NUCLEAR RECEPTORS MODULATE IMMUNE FUNCTIONS IN DENDRITIC CELLS

THE CASE OF VITAMIN D RECEPTOR AND THE REGULATION OF IMMUNE FUNCTION-RELATED GENES

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Nuclear receptors modulate immune functions in dendritic cells

The case of vitamin D receptor and the regulation of immune function-related genes

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Supplement 1.

1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype.

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Supplement 2.

PPARgamma in immunity and inflammation: cell types and diseases.

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1. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A dendritikus sejtek nélkülözhetetlenek az adaptiv immunválaszok és a tolerancia kiváltásában, de a természetes immunitáshoz is hozzájárulnak. Az immunválaszt kiváltó folyamatokban elsősorban immunogén, míg az immunválaszok csendesítésében tolerogén dendritikus sejtek vesznek részt. Az utóbbi időben egyértelművé vált, hogy a dendritikus sejtek fejlődésének és számos funkciójának szabályozásában részt vesznek a magreceptorok is. A jelen értekezésben tárgyalt kísérletek során egy ilyen magreceptor, a D-vitamin receptor (VDR), által indukált változásokat vizsgáltuk két másik program, a differenciálódás és a retinsav receptor α (RARα)-indukálta transzkripciós program viszonyában.

(1) Korábbi tanulmányok igazolták, hogy a kalcitriol gátol számos olyan változást, amely a dendritikus sejtek előalakból (elsősorban monocitából) történő differenciálódása és érése során következik be. Emellett az is világossá vát, hogy a kalcitriol kezelt dendritikus sejtek mind fenotipusukban mind funciójuk szempontjából tolerogén dendritikus sejteknek tekinthetőek. Kísérleteinkben azt vizsgáltuk, hogy hogyan függ össze a differenciálódás és a kalcitriol által szabályozott traszkripciós program. Hogy erre a kérdésre választ kapjunk, microarray és qPCR vizsgálatokat végeztünk el humán monocitából differenciálódó dendritikus sejteken és vérből izolált mieloid dendritikus sejteken. Eredményeink alapján megállapitottuk, hogy a differenciálódás és a kalcitriol által szabályozott gének átfednek ugyan, de nagyszámú olyan gén is van, amit vagy csak az egyik, vagy csak a másik program szabályoz. További vizsgálatok azt is megmutatták, hogy sok esetben akkor is függetlennek tekinthető a két program egymástól, amikor mindkettő képes szabályozni egy adott gént. A kísérleteink alapján arra a következtetésre jutottunk, hogy az exogén (vagy a dendritikus sejtek által átalakitott) kalcitriol olyan programot szabályoz, amely nagymértékben független a dendritikus sejtek differenciálódási folyamatától. Ez arra is utalt, hogy a kalcitriol nem kizárlólag immunogén programok gátlása által, hanem jelentős részben aktív folyamat révén szabályozza a dendritikus sejtek tolerogén fenotípusát.

(2) Korábbi eredményeink azt mutatták, hogy a RARα és a VDR sok esetben hasonló változásokat idéz elő a dendritikus sejtek fenotípusában, ráadásul a két magreceptor több funkciót is hasonlóképpen befolyásol. A jelenleg folyó kísérletinkben azt vizsgáltuk, hogy mennyire tekinthető általánosnak a közös szabályozás célgének szintjén. Microarray kísérltek során jelentős átfedést tapasztaltunk az AM580 (RARα agonista) és a kalcitriol kezelés nyomán. Eredményeink azt mutatták, hogy a kalcitriol által szabályozott gének mintegy 50%-át az AM580 is szabályozta a dendritikus sejtek differenciálódásának 5 napja alatt. A továbbiakban ennek a megfigyelésnek szeretnénk lehetséges molekuláris mechanizmusait vizsgálni. Elsősorban arra vagyunk kíváncsiak, hogy vannak-e olyan, mindkét receptor által szabályozott célgének, amelyeket a VDR és a RARα úgy szabályoz, hogy ugyanahhoz a válaszadó elemhez kötődnek az adott gén szabályozó szekvenciájában.

LISTS OF ABBREVIATIONS

Cell types

DC: dendritic cell

IDC: immature dendritic cell

MDC: mature dendritic cell

iNKT: invariant natural killer T cell

Treg: regulatory T-cell

Th1: T helper 1 cell

Th2: T helper 2 cell

Th17: T helper 17 cell

Nuclear receptors

VDR: vitamin D receptor

PPAR: peroxisome proliferator-activated receptor

RXR: retinoid X receptor

RAR: retinoic acid receptor

LXR: liver X receptor

GR: glucocorticoid receptor

FXR: farnesoid X receptor

TR: thyroid hormone receptor

VDR and other nuclear receptor ligands

1,25-vitD: 1,25-dyhidroxyvitamin D_3

25-vitD: 25-hidroxyvitamin D_3

vitD: vitamin D_3

ATRA: all-trans retinoic acid

9cis-RA: 9-cis retinoic acid

2. INTRODUCTION

2. 1. Nuclear receptors in the control of diverse biological processes

Nuclear receptors, such as estrogen receptor, androgen receptor, glucocorticoid receptor, vitamin D receptor, retinoic acid receptor are ligand-activated transcription factors that have diverse roles in regulating developmental, homeostatic, metabolic, inflammatory and immune processes (1-5). The human genome contains 48 members (6) of this family and many of them are indispensable for life. The ligands for these receptors are steroids, cholesterol, fatty acids, and fat-soluble vitamins (3-5). Some nuclear receptor ligands (e.g. classic steroids, such as glucocorticoid and estrogen) had been known decades before their receptors were cloned and identified. In contrast, in the case of adopted orphan receptors the investigation followed a different order: first the receptor was cloned and later ligands were identified. This "reverse endocrinology" strategy has resulted in the discovery of unanticipated nuclear signaling pathways for retinoids, fatty acids, eicosanoids, and steroids (7). Some nuclear receptors remained “orphan” partly because ligands of these receptors have not been identified, and partly because some of them do not have a pocket for ligand binding. Nuclear receptors can regulate the transcriptional activity of their target genes by various mechanisms (Figure 1). Generally, in the absence of the ligand they bind co-repressor molecules that recruit complexes that contain protein with histone deacetylase activity (Figure 1A). Upon ligand binding nuclear receptors undergo a conformational change that dissociates co-repressors and facilitates recruitment of co-activators to enable transcriptional activation (Figure 1B) (8, 9). Other direct and indirect mechanisms were also documented by which nuclear receptors regulate gene expression. The consensus sequence of the hexamer is the same (AGGTCA) for most nuclear receptors and the hormone response elements contain usually degenerate variants of this consensus sequence (2, 4, 10). However, in the case of nuclear receptors functioning as heterodimers, response elements contain two hexamers separated by 1, 2, 3 or more base pairs. The orientation of the repeats can also differ (everted repeats and direct repeats) and complex response elements also occur. These differences make it possible

that heterodimers may bind to receptor-specific response elements (e.g. PPAR-RXR for DR1, VDR-RXR for DR3, RAR-RXR for DR5) (4, 10).

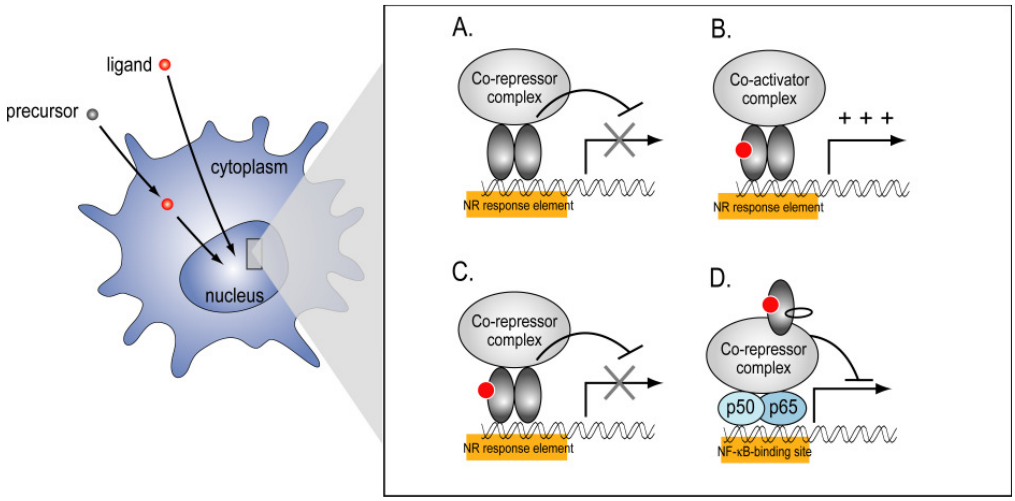

Figure 1: *Nuclear receptors are activated by lipophilic molecules and regulate the transcription of their direct or indirect target genes. The biological active ligand can be extracellular or can be synthesized in the cell from its precursor(s). Several mechanisms are proved to be involved in lipid-dependent regulation of transcription. (A) A couple of nuclear receptors are known to repress their targets in the absence of their cognate ligand. (B) Ligand-binding initiates a conformational change that results in the degradation of co-repressor complexes and the recruitment of co-activator complexes, which results in induction of the transcriptional activity of target genes. (C) In the case of ligand-dependent direct repression, target genes contain a negative nuclear receptor response element. Nuclear receptors recruit co-repressors in the presence of the receptor agonist and inhibit the expression of the regulated gene. (D) Nuclear receptors can also inhibit the activity of other transcription factors such as NF-κB via transrepression.*

The in vivo significance of a certain nuclear receptor in any tissues or cell types is obviously dependent on two factors: (1.) the expression of the receptor itself, and (2.) the availability of the endogenous ligands. However, pharmacological (exogenous: natural or synthetic) ligands activating various receptors also have clinical implications (e.g. thiazolidinediones in the treatment of diabetes type 2, dexamethasone in the context of congenital adrenal hyperplasia, etc.). Remarkably, some nuclear receptors are considered as master regulators of different processes. Sex hormones promote the development of sex characteristics, testosterone has also profound anabolic effects and female sex hormones are essential for regulating the menstrual cycle. VDR regulates the calcium and phosphorus levels, while PPARγ is essential for adipogenesis (11, 12)

and thyroid receptor for energy metabolism. Numerous studies demonstrated that besides these "classical" functions several nuclear receptors have role in regulating immune responses in various cell types. Our research group is interested in investigating the roles of a few of nuclear receptors (VDR, RAR, LXR, PPAR) in DCs and macrophages, and the rest of the introduction will cover some aspects of DC, and partly macrophage, biology and the role of nuclear receptors in regulation of phenotype and function of these cell types.

2.2. Dendritic cells (DCs) and the control of immunity and tolerance

Main features of DCs

DCs are professional antigen presenting cells which have the superior ability to stimulate naive T-cells and regulate their functions (13-15). However, dendritic cells are heterogeneous and subtypes differ in location, migratory pathways and immunological function (13, 15, 16). Moreover, the same DC subtype can be activated in distinct ways in response to a spectrum of environmental and endogenous stimuli and can initiate different ways of immunity or tolerance (13, 15). As introduction to DC biology we first discuss the functions of migratory DCs (16) (Figure 2). The migratory DCs in their immature state can be found at body surfaces like in the skin, pharynx, upper esophagus, vagina, ectocervix and anus, and at mucosal surfaces, such as the respiratory and gastrointestinal system (15). They sense and translate environmental cues by sampling and processing antigens of dying cells and various pathogens. DCs are able to pick up antigens via various mechanisms, such as phagocytosis, macropinocytosis and endocytosis (13, 15, 17, 18). Antigen uptake and maturation signals (pro-inflammatory cytokines, exogenous microbial products that bind to TLRs) trigger maturation of DCs. Maturation is a complex process, which includes phenotypic and functional changes (for details see below). During maturation, DCs loose their capacity to take up antigens, and migrate to draining lymph nodes, where they present antigens and activate naive T-cells and other lymphocytes (13, 15). The mobilization of

DCs from the periphery to the lymph nodes is regulated by the chemokine receptor CCR7 (19).

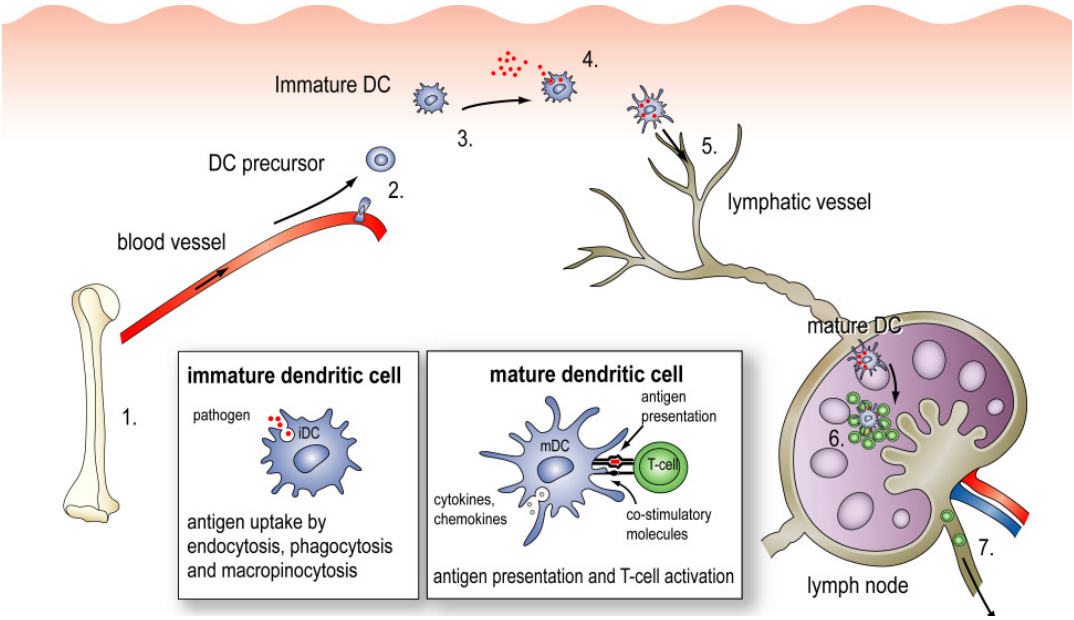

Figure 2. *Differentiation and migration of tissue resident DCs. DCs precursors are bone marrow derived cells (1) that leave the blood vessels (2) and reside in various tissues (3) in the periphery, e.g. in the skin or mucosal surface of gastrointestinal system, as immature DCs (IDC). IDCs cells are well armed for sampling antigens and receive various cues from the surrounding tissues. Antigen uptake (4) associated with other signals leads to DC maturation and migration to draining lymph nodes (5). In the lymph nodes, mature DCs present antigens to naive T-cells (6) or other lymphocytes and activate them (7).*

CCR7 expression is induced together with the maturation, while the expression of other chemokine receptors is down-regulated. Interstingly, signals that are expected to be found at sites of inflammation are required to sensitize CCR7 to its ligands CCL19 and CCL21 (19). T-cell stimulation and Th1/Th2 polarization are thought to be dependent mainly on three types of DC-derived signals (20). “Signal 1” is the antigen-specific signal that mediated by T cell receptor triggering by MHC-II associated peptides. “Signal 2” is the co-stimulatory signal, mainly mediated by triggering CD28 of T-cells by CD80 and CD86 that are expressed by DCs after ligation of pattern recognition receptors. “Signal 3” is a polarizing signal that is mediated by various soluble or membrane bound factors, such as IL-12 and CCL2, which promote the development of Th1 and Th2 cells, respectively. The antigen-presentation of DCs function is not restricted to the presentation of peptides by MHC-II molecules; they also present

glycolipids in complexes with CD1 molecules and "cross-present" endo- or exogenous antigens on MHC-I to elicit $CD8^+$ killer T cells (15, 21, 22). In-vivo mouse experiments demonstrated that skin-derived mature DCs can stay in lymph nodes up to two weeks before being eliminated (23). Besides eliciting immune response, DCs could also provoke immunological tolerance in thymus and in the periphery (24, 25).

DCs have important medical implications: on the one hand, DCs can induce unwanted responses during allergy, autoimmunity and transplant rejection. On the other hand, DCs have become an attractive cell type for therapeutic manipulation in inducing (in cancer and infections) and silencing (in autoimmunity, allergy and transplantation, etc.) immune responses (25).

Subsets, localization and development of DCs

The most common division of dendritic cells is conventional (cDC) and plasmacytoid DCs (pDC). pDC are the main type-I interferon-producing cells, in the immune system and circulate as precursor cells in the blood or reside as immature cells in primary and secondary lymphoid tissues (26, 27). cDCs can be divided into subsets according their tissue localization (28), such as skin DCs including Langerhans cells in epidermis and dermal DCs in dermal area; mucosal tissue associated DCs; lymphoid tissue-associated DCs; and interstitial tissue DCs including liver DCs and lung DCs. In addition, DCs that are not found in the steady state but develop after infection or inflammation include monocyte-derived DCs and TNFα-producing and iNOS-expressing DCs (28). The combined treatment of $CD14^+$ monocytes with GM-CSF and IL-4 in vitro is a commonly used model system which very likely recapitulates inflammatory DC development (16, 29, 30). Many cytokines and transcription factors are required for the overall process of DC development, but only few "master regulators" have been identified so far (16, 31).

Maturation of DCs

After maturation, antigen capturing DCs become professional antigen presenting cells. Maturation is triggered by numerous stimuli, including endogenous ligands, microbial products, lymphocytes and neutrophils (13, 15). Maturation processes alter the

expression of hundreds of genes (15) such as MHC molecules, T-cell costimulatory molecules and cytokines including IL-12 and type I interferons and affects several functions of DCs (e.g. endocytic capacity, antigen processing, migration to lymph nodes, T-cell priming ability). Expression of cell surface levels of MHC molecules, CD40, CD80, CD83 and CD86 often correlates with T-cell-priming ability, so it has been assumed that DCs that are mature by phenotypic criteria (expressing those maturation markers) are also functionally mature, that is immunogenic (14). In other words, DCs were originally thought to exist in two states, „off" (immature) or „on" (mature) (14), with only mature DCs able to drive T-cell clonal expansion and prime immune responses. Because of this linear model (immature/off and mature/on), the term „mature" has been used often not only functionally (mature DC = immunogenic DC) but also as phenotypic description (mature DC= DC which express high levels of MHC molecules, adhesion and co-stimulatory molecules). However, the functional description has been challenged by several reports (27) showing that phenotypically mature DCs are not immunogenic, e.g. myeloid DCs and CD40L-matured plasmacytoid DCs can induce regulatory T-cell responses. In fact, the expression of co-stimulatory molecules is often necessary for tolerance induction (14). Because of this reason, as reviewed by Reis e Sousa (14), there is a trend in the field to complement „maturation" nomenclature with one that defines DCs by their „effector" function. The terms immunogenic and tolerogenic DCs refer to this effector function. However, the "maturation markers" term in phenotypic sense is acceptable and in use (maturation markers as markers that are highly expressed by cultured LCs or in vitro culture DCs exposed to maturation stimuli).

Tolerogenic / regulatory DCs

As mentioned above, DCs are involved not only in inducing and regulating strong innate and adaptive immunity to infections and other antigens but also in the induction of tolerance (25). Tolerogenic DC and T-cell interactions can be manifested in T-cell death, T-cell anergy (T-cell unresponsiveness to stimulation with antigen) or regulatory T-cell expansion or generation (24). These mechanisms are essential to avoid excessive inflammation or the development of autoimmunity and allergy (25, 32).

Tolerogenicy is not specific to a DC subset or restricted to the immature state of DCs. Several biological and pharmacological agents, such as dexamethasone, 1.25-vitD, rapamycin, cyclosporine A, can also alter DCs to become tolerogenic (24, 27). These agents can interfere with the differentiation and maturation of DCs, and affect various functions of DCs such as antigen uptake and presentation, migration, cytokine production and DC survival (24, 27). Interestingly, particular pathogens also can induce tolerogenic DCs that drive the generation of Tregs (32). As we discussed previously, the down-modulation of molecules required for antigen-specific stimulation and co-stimulation (MHC molecules, CD40, and CD80/CD86) is not sufficient and nor necessary to induce tolerogenic DCs. Indeed, the tolerogenic phenotype is achieved by modulation/altered expression of a series of molecules (24, 27). However, the down-modulation of MHC molecules, CD40, and CD80/CD86 is not sufficient alone but can contribute to development of tolerogenic DCs. Other factors have also been identified, such as low production of IL-12p70 and prevention of the nuclear translocation of NF-κB (24, 27) as well as resistance to maturation signals in response to danger signals. Interestingly, not only inhibition of "immunogenic molecules" but also induction/high expression of "immunosuppressive molecules" (24, 27) is an important component of tolerogenic DC phenotype. High expression of anti-inflammatory cytokine IL-10, and immunomodulatory enzyme Indoleamine 2,3-dioxygenase (IDO) can suppress T-cell responses and promote tolerance (33). Released TGFβ1 is involved in the development of Tregs, while inhibitory receptor ILT3 is involved in the control of regulatory T-cell activation (24, 27, 34).

2. 3. The role of nuclear receptors in DC biology

In this section we discuss the common and receptor-specific roles of nuclear receptors in regulating DC phenotype and function. Microarray experiments provide a valuable tool to identify the expression of nuclear receptors in DCs. Our global gene expression profiling showed that 20 out of 48 human nuclear receptors are present in developing human monocyte derived DCs (35). The expression of a subset of nuclear receptors does not change significantly during the differentiation process (e.g. RXRα and GR).

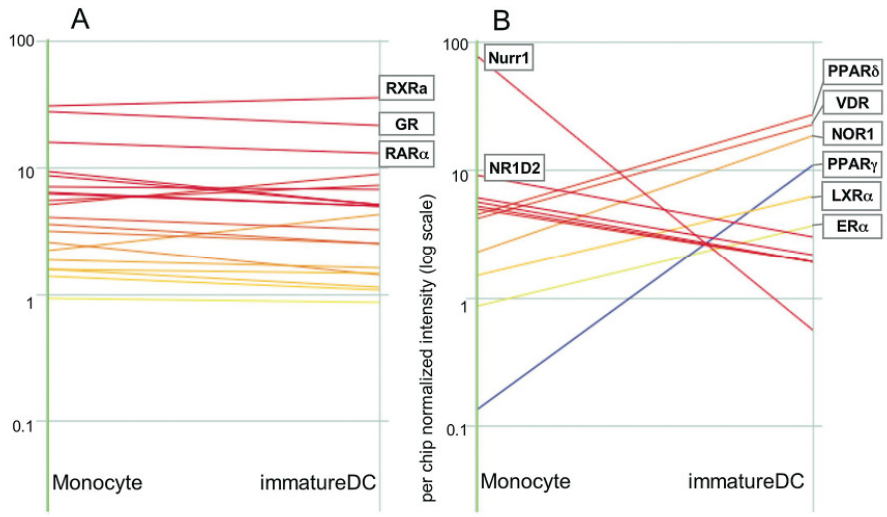

Figure 3. *Expression of different nuclear receptors in dendritic cells as determined by microarray analysis is shown. Several of the 48 members of the nuclear receptor superfamily are expressed in monocyte and dendritic cells, (A) at constant or (B) changing levels.*

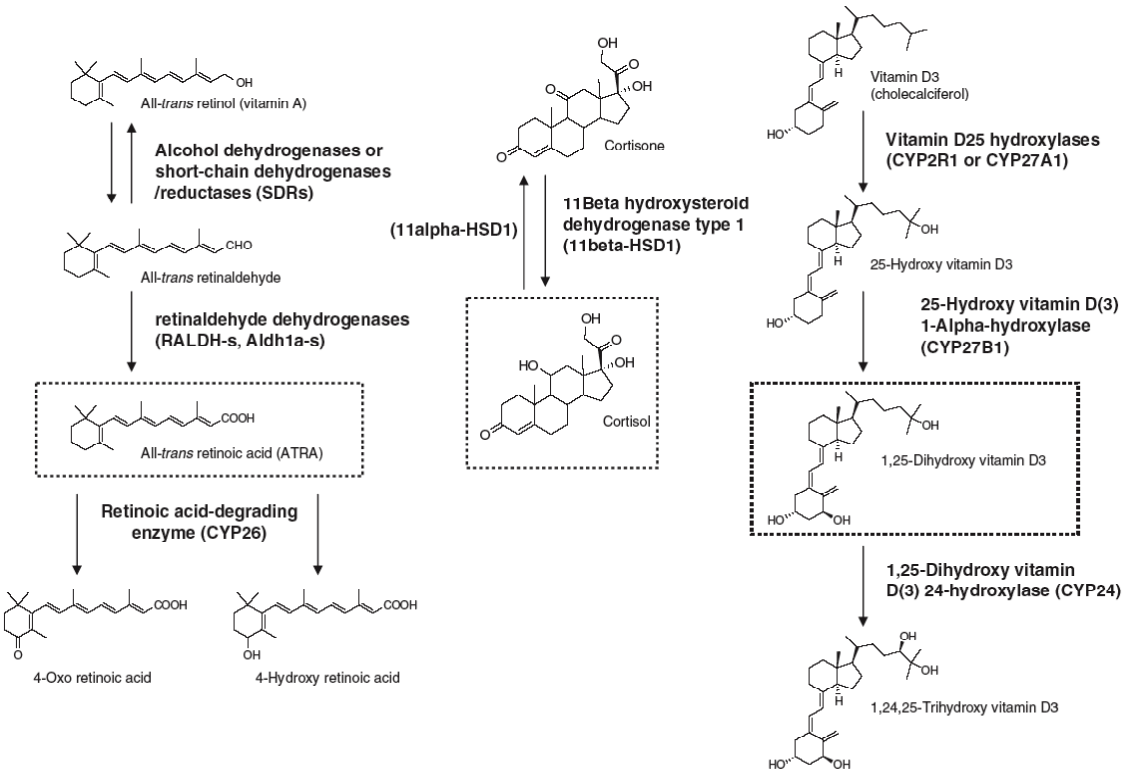

Figure 4. *The conversion steps of the three nuclear receptor ligand precursors (vitamin A, cortisone and vitamin D) to the active compounds are shown. The figure is adopted from Szatmari and Nagy (2008) EMBO J. Sep 17;27(18):2353-62 with the permission of the authors.*

Others are induced (e.g. LXRα, PPARγ and VDR) or down-modulated (e.g. Nurr1) constantly or transiently during differentiation (Figure 3).

A growing body of evidence suggests that nuclear receptors are not only expressed but play an important role in regulation of DC development and functions. GR, VDR and RAR were among the first nuclear receptors which effects on DCs were documented. We and others also investigated and identified important aspects of other receptors, PPARs, LXRs and RXR. Various DC types treated with high affinity, natural and synthetic agonists were effective in regulating cell surface markers, cytokine profile, antigen uptake, and presentation by DCs, and their T-cell-activating capacity (for details see below). The importance and biological relevance of nuclear receptor signaling in DCs were supported by the findings proving that DCs are actively participating in the production of natural ligands for GR, RAR, VDR and PPAR (35-42) (Figure 4). These active metabolites may be involved in autocrine regulation of DCs under certain circumstances. Remarkably, all-trans retinoic acid (ATRA) and 1,25-vitD released by DCs contribute to T-cell tropism by regulating integrins and chemokine receptors expressed by T-cells (39, 40).

Interestingly, several nuclear receptors can affect various pathways similarly: differentiation markers (e.g. down-modulation of CD1A and up-regulation of CD14 by agonists of GR, PPAR, VDR, RAR), antigen uptake capacity (enhanced by agonists of PPAR, VDR, RAR and GR), and T-cell activation capacity (down-modulated by agonists of VDR, GR, LXR). Moreover, our microarray studies also indicated that there is a significant overlap between the transcriptional targets of these nuclear receptors (PPAR vs. RAR and VDR vs. RAR). Our research group elucidated some aspects of PPAR and RAR crosstalk in DCs and PPAR, RAR and LXR crosstalk in macrophages (Figure 5). We also make an effort in understanding why activation of VDR and RAR can regulate significantly overlapping sets of genes (see below).

The role of glucocorticoid receptor in DC and macrophage biology

Corticosteroids were among the first immunosuppressive drugs used in clinical application (27). Agonists of glucocorticoid receptor have profound effects not only on

lymphocytes, but also on other immune cells such as macrophages and DCs. Human studies on monocyte-derived DCs treated with dexamethasone demonstrated that these DCs were not fully differentiated (43). They expressed low levels of CD1A und unlike untreated cells, high levels of CD14 and CD16.

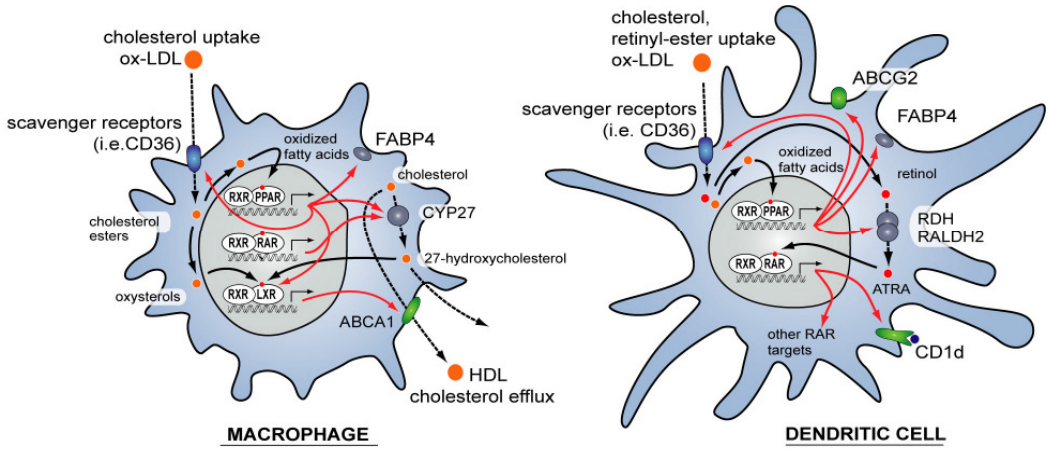

Figure 5. *Various mechanisms involved in cross-talks between nuclear receptors have been identified in macrophages and DCs. In macrophages CD36-mediated uptake of oxidized LDL (ox-LDL) leads to the accumulation of intracellular cholesterol esters, from which oxidized fatty acids and sterols are derived. Oxidized fatty acids and oxysterols can activate PPAR and LXR receptors, respectively. Activation is resulted in the induction of target genes, among them CD36, initiating a positive feedback. LXRα expression is also induced by PPARγ, while ABCA1 is regulated by LXR. The lipid accumulation is counterbalanced by ABCA1-mediated cholesterol efflux to HDL. Production of endogenous LXR activator 27-hydroxycholesterol is enhanced by induction of CYP27. In DCs activated PPARγ not only regulates genes directly, but it turns on retinoic acid synthesis by inducing the expression of retinol and retinal metabolizing enzymes such as retinol dehydrogenase 10 (RDH) and retinaldehyde dehydrogenase type 2 (RALDH2). PPARγ-regulated expression of these enzymes leads to an increase in the intracellular generation of all-trans retinoic acid (ATRA) from retinol. ATRA regulates gene expression via RARα, and RARα acutely induces the expression of CD1D and other target genes. (In dendritic cells CD36 is also regulated by activated PPARγ.) Red arrows indicate transcriptional regulation by nuclear receptors, black arrows represent influx, efflux and metabolism of lipids and nuclear receptor ligands.*

Molecules involved in antigen presentation (CD40, CD86, and CD54) were also impaired. Interestingly, endocytic activity of DCs was enhanced in the presence of dexamethasone (43). This phenomenon was consistent with the elevated level of potential antigen uptake receptors (e.g. mannose receptor, CD32). After exposure to

TNFα or CD40 ligand, DCs treated with dexamethasone expressed lower level of CD83 and CD86 than untreated cells. Cytokine production was also affected by GR, reduction of IL-12p70, IL-1β, and TNFα was detected in the supernatant of dexamethasone-treated DCs (43). MLR experiments demonstrated that dexamethasone down-regulated the immunostimulatory capacity of T-cells. However, dexamethasone and prednisolone did not affect Langerhans cell development but they blocked the generation of dermal/interstitial type CD34+ derived DC (44).

Remarkably, differentiation of human immature DCs from monocyte precursors is associated with induction of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) activity that permits conversion of inactive cortisone to active cortisol (28) (Figure 4). Synthesis of cortisol from cortisone added at physiologic concentrations leads to autocrine-negative regulation of DC differentiation from monocyte precursors (36). Global transcriptome studies have also been conducted to investigate the effect of activation of GR in human and mice DCs and macrophages (45-47). When ligands of VDR and GR were added together, the combined treatment resulted in significant additive inhibition of pro-inflammatory cytokines, T-cell stimulation, chemokines, chemokine receptors, and NF-κB components (48).

Microarray studies on human monocytes/macrophages treated with GR activator fluticasone propionate, revealed other important aspects of GR activation (45). Ehrchen et al. in their study performed *in silico* analysis of regulated genes followed by functional analysis of glucocorticoid-treated monocytes. Glucocorticoid treatment resulted in increased migratory, chemotactic and phagocytic activity and decreased oxidative burst. They concluded that GR activation not only suppresses proinflammatory properties but rather induces a distinct functional phenotype, which seems to be actively involved in resolution of inflammatory reactions. This conclusion is especially interesting in our point of view. We also found that up-regulation of genes is more important in the case of 1,25-vitD action than previously thought and tolerance is a consequence of both inhibition of immunogenic effect and enhancement of active tolerogenic mechanisms (see below).

The role of PPARs in DC biology

The three PPAR isoforms (α, γ and δ) have different tissue distributions and seem to have distinct but overlapping biological functions (1, 49, 50). PPARγ is expressed in various murine and human DCs and its role has been intensively investigated by our research group (see below). PPARδ is also expressed in DCs but the potential function of this receptor is poorly understood. PPARγ was first detected in murine immature and mature spleen-derived DCs (51). In human, microarray analyses revealed that PPARγ is highly up-regulated in monocyte-derived DCs during differentiation triggered by cytokines (52). This result was confirmed by several groups (53-55), and the protein product of the PPARγ gene was also detected. The presence of PPARγ was also demonstrated in other DC subtypes such as blood-derived myeloid DC, S100-positive antigen presenting cells in human tonsils (55), murine Langerhans cells and bone marrow derived murine DCs (56). As our research group has observed in macrophages, PPARγ has an impact on both lipid homeostasis and immunoregulation. In DCs, we and others detected up-regulation of lipid metabolism and transport related genes (CD36, FABP4, LXRα, and PGAR) upon PPARγ ligand treatment. We found that up-regulation of these genes could be blocked by PPARγ-specific antagonist suggesting a PPARγ-dependent regulation (55). To reveal the role of PPARγ ligands as immunoregulators, in several laboratories different processes and immune functions were tested such as antigen uptake, DC migration, modulation of Th1/Th2 balance, cytokine production and antigen presentation. We found that PPARγ, similarly to glucocorticoids and 1,25-vitD, (57, 58), enhanced endocytosis in immature DCs (55). Antigen uptake and presentation is under tight developmental control: monocytes and immature DCs have the highest capacity to take up antigens. Nuclear receptor ligands can control differentiation and maturation processes of DCs, so it is possible, that PPARγ ligands similarly to the two other nuclear receptor ligands (27) act developmentally, and interfere with endocytosis indirectly. Nencioni et al. reported (54) that activation of PPARγ in DC inhibited the expression of CCR7. Using an experimental murine DC model of Langerhans cell migration induced by TNFα, Angeli et al. found (59) that PPARγ agonist rosiglitazone specifically impairs the departure of

Langerhans cells from the epidermis. This phenomenon may be explained by the reduced level of CCR7. The first papers on PPARγ in murine and human DCs reported that 15d-PGJ_2 and/or rosiglitazone down-regulate the CD40-induced secretion of IL-12 (51, 53). These and other papers (54, 55) also reported a series of observations suggesting that activation of PPARγ in DCs might have an impact in the orientation of immune responses by favoring Th2 responses. Observations included decreased IL-12 production, decreased CD80 and induced CD86 level, down-regulation of chemokines involved in the recruitment of Th1 lymphocytes, namely CXCL10 and RANTES. We and others found (54, 55) that activation of PPARγ changed the expression level of CD1A. CD1 molecules bind and present glycolipids and are important in lipid-antigen presentation of DCs. Unexpectedly, we found that PPARγ ligand treatment induced CD1D in human monocyte-derived DCs. Since CD1D mediated lipid presentation is indispensable for the activation and expansion of invariant Natural Killer T-cells (iNKT) cells (60), we hypothesized that increased CD1D protein levels should translate into increased activation of iNKT-cells. Further studies showed that DCs treated with PPARγ ligands, induced iNKT-cell proliferation, causing a significant increase in the number of iNKT-cells in the lymphocyte population (55). These findings proved that increased CD1D levels in DCs regulated by PPARγ are functional and can play a central role in the immunomodulatory effects of the activated cells. Our further studies (41) revealed that PPARγ regulates CD1D indirectly, by turning on retinoic acid synthesis via the induction of the expression of retinol and retinal metabolizing enzymes such as retinol dehydrogenase 10 and retinaldehyde dehydrogenase type 2 (RALDH2). PPARγ-regulated expression of these enzymes leads to an increase in the intracellular generation of all-trans retinoic acid from retinol. All-trans retinoic acid regulates gene expression via the activation of the RARα in human DCs, and RARα acutely regulates CD1D and other RARα target genes. These studies established dendritic cells as a very relevant target in PPARγ mediated immune regulation. It remains to be seen what portion of PPARγ's activation in vivo is mediated by receptors residing in dendritic cells. Dendritic cell specific knockouts may prove to be

particularly useful in defining the role of the receptor in innate immune responses and autoimmunity.

Remarkably, out research group also demonstrated that PPARγ is not only involved in turning on retinoid signaling but PPARγ itself can be ligand-activated during DC development (42). When DCs were cultured in human serum instead of FBS, PPARγ-regulated FABP4 gene was up-regulated. Moreover, most genes whose expression was up-regulated (42) on synthetic PPARγ-ligand-treatment also showed an elevated expression if DCs were cultured in human serum-containing medium. These results suggested that in the presence of human serum, PPARγ ligands are generated/accumulated in DCs.

The role of LXRs in macrophage and DC biology

LXRs (α and β isoforms) are cholesterol sensors that regulate both cellular and systemic cholesterol homeostasis (1, 61-63). Similarly to PPARs, LXRs form permissive heterodimers with RXR, where the complex can be activated through either partner. The two isoforms show different tissue distribution but seem to have almost identical target genes. Natural ligands which can bind to and activate LXRs at physiologic concentrations are 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol and 27-hydrocholesterol (1, 2, 64). The role of LXR in macrophages is intensively investigated, especially in mice. We have far less information about the role and regulated targets of LXRs in DCs. Because very likely there are parallels between DCs and macrophages in function and mechanism how PPARs regulate their targets, briefly the role of LXRs in macrophage biology has been discussed.

Recent papers (1, 65, 66) documented that LXRs and their ligands are negative regulators of macrophage inflammatory gene expression in mice. Joseph et al. (65) demonstrated a reciprocal LXR-dependent regulation of genes involved in lipid metabolism (up-regulation by LXR agonist: ABCA1, SCD2) and the innate immune response (inhibited expression of iNOS, COX2, IL6 and MMP9) in the presence of LPS. *In vivo*, LXR agonists reduce inflammation in a model of contact dermatitis and inhibit inflammatory gene expression in the aortas of atherosclerotic mice (1).

Activation of LXRs antagonizes downstream not only TLR4 signaling but also IL-1β-mediated and TNFα-mediated signaling (65, 66). The mechanism of LXR-mediated transrepression, similarly to PPAR, depends on protein-protein interactions that preserve the integrity of the co-repressor complex in the presence of LXR ligands after SUMOylation (Figure 1) (1). Although a microarray study by Le Naour et al. documented the elevated expression of LXRα during monocyte to DC differentiation in 2001 (52), we still have limited knowledge about the role of LXRs and genes regulated by these receptors in DCs. However, Geyeregger et al. (67) found that LXRα is expressed in human myeloid DCs and induced during differentiation of monocyte-derived DCs, whereas LXRβ is expressed constitutively at a very low level. LXR activation by LXR agonists strongly interfered with LPS-induced but not with CD40L-induced DC maturation by altering DC morphology and suppressing IL-12-secretion, but enhancing IL-10-secretion. LXR activation in DCs largely blocked their T-cell stimulatory ability despite essentially unaltered expression of various antigen-presenting and co-stimulatory molecules. Immunologic synapse formation was significantly inhibited by LXR activation along with a complete block in LPS- but not CD40L-induced expression of the actin-bundling protein fascin (67).

Regulation of DCs by retinoids and other RAR and RXR agonists

Retinoids are vitamin A derivatives and exert a modulatory effect on the immune system, regulating functions, activity and survival of various immune cells among them DCs (68-70). Retinoic acid receptor (RAR) and retinoid X receptor (RXR), both having 3 isoforms, are responsible for mediating the effect of various retinoids. All-trans retinoic acid (ATRA) is an agonist of RAR, however, an isomeric form, 9-cis-retinoic acid (9cis-RA), can activate both RARs and RXRs (71-73). The essential and central role of RXRs is due the fact that they serve as heterodimerization partners for a series of nuclear receptors, such as PPARs, LXRs, RARs, VDR, FXR and TRs (2, 4, 74). Some of these heterodimers (e.g. LXR-RXR and PPAR-RXR) are called “permissive”, as they become transcriptionally active in the sole presence of an RXR-selective ligand (rexinoid) (75). Other (non-permissive and conditional) heterodimers do not respond to rexinoids alone. However, combining RXR and RAR agonists has a greater effect than

RAR agonist alone so RAR-RXR heterodimer is considered as “conditional”. Additive effect of RXR agonist cannot be detected in the case of “non-permissive” heterodimers (e.g. TR-RXR and VDR-RXR) in transfection assays.

In this way, addition of 9cis-RA to a certain cell type may result in the activation of different dimers: RAR-RXR (from RAR side), LXR-RXR and PPAR-RXR (from RXR side) or probably RXR-RXR homodimers, while RXR specific rexinoids very likely cannot activate RAR-RXR in the absence of RAR agonist (75). Synthetic RAR specific agonists regulate only RAR-RXR, while ATRA may activate RAR-RXR and/or PPARδ-RXR heterodimers, depending on CRABPs and FABP5 ratios (69).

There are conflicting results on whether RAR and RXR activators enhance or repress the immunogenicity of DCs (35). Data documenting suppressive/inhibitory effects leading to less stimulatory DC phenotype are:

(1.) Mouse splenic DCs are less stimulatory on retinoic acid-treatment (76).

(2.) Retinol, ATRA and 9cis-RA induced apoptosis of human LC-type DCs (68). This effect was restricted to DCs their immature state, because neither MDC nor monocytes died after exposure to retinoids. Using selective agonist it was concluded that apoptosis was mediated through RARα-RXR.

(3.) 9cis-RA and synthetic RXR agonists exert immunosuppressive effects (e.g. down-regulated CD80, CD83, CD86 and IL-12; decreased lymphoproliferative stimulation capacity of 9cis-RA treated DCs) on human monocyte derived DCs, through the activation of PPARγ-RXR heterodimer (70).

However, there are findings suggesting that DCs on retinoic acid-treatment become more potent or effective in some aspects:

(1.) Langerhans cells have an enhanced T-cell activation capacity (77).

(2.) Retinoids promote the differentiation and maturation of monocytes to DC-like cells (78).

(3.) Retinoid pretreated DCs, if injected into tumors in mice, showed and increased accumulation in draining lymph nodes. It was concluded that enhanced DC migration was due to the elevated matrix metalloproteinase (MMP) production (79).

(4.) Via a cross talk with inflammatory cytokines, retinoids increase DNA-binding activity of NF-κB, trigger MHC-II and co-stimulatory molecule expression, induce the differentiation of IDCs into MDCs, and enhance antigen-specific T-cell response (68).
Our laboratory also investigated the effects of activation of RAR using synthetic agonist on monocyte-derived DCs and found that these cells have an enhanced iNKT-cell activation capacity as a consequence of the induction of CD1D (41). In a breakthrough study, Iwata et al. discovered (39) that retinoic acid produced by gut-associated lymphoid tissue (GALT)-resident DCs and probably by other cells, potently induces the expression of the gut-homing receptors ($\alpha_4\beta_7$-integrin and CCR9) by activated $CD4^+$ and $CD8^+$ T-cells. This study established that a DC-derived nuclear receptor ligand is able to reprogram the local T-cells and imprint T-cell tropism. Moreover, GALT-DC–derived retinoic acid conferred gut tropism of B cells and potently synergized with GALT-DC-derived IL-6 or IL-5 to induce IgA secretion (69). Recent papers also documented that retinoic acid, in combination with other immunological mediators, also modulates the differentiation of various lymphocyte populations. Retinoic acid blocks the differentiation of Th17 cells and induces FoxP3+ Tregs in the presence of TGFβ and also imprints Tregs with gut tropism (69, 80-82).

Vitamin D metabolism, the role of 1,25-vitD and its receptor in classical target organs and in the immune system

Vitamin D is a fat-soluble secosteroid that is naturally present in very few foods (e.g. in flesh of fish, fish liver oils, egg yolks) (83). It is also produced endogenously from 7-dehydrocholesterol when ultraviolet rays from sunlight strike the skin and trigger vitamin D synthesis. There are several nutritional forms of vitamin D; the best-known examples are vitamin D_3, which can be produced in the skin and vitamin D_2, which is derived from plant tissues. (Vitamin D_2 has been shown to contribute to the overall vitamin D status and can be metabolized in a similar fashion to produce several metabolite analogues to the vitamin D_3 system, including the active 1,25-dihydroxyvitamin D_2). Vitamin D_3 obtained from sun exposure, food, and supplements is biologically inert and must undergo two hydroxylation steps for activation. The first occurs mainly in the liver and converts vitamin D_3 to 25-hydroxyvitamin D_3 (25-vitD).

This is the major circulating form of vitamin D that is used by clinicians to determine the vitamin D status (83). The second step occurs primarily in the kidney and forms the physiologically active 1,25-dihydroxyvitamin D_3 (1,25-vitD), also known as calcitriol. Vitamin D_3 is essential for promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations to enable normal mineralization of bone. It is also needed for bone growth and bone remodeling by osteoblasts and osteoclasts. The classical manifestations of vitamin D deficiency are rickets (in children), osteomalacia and osteoporosis (in adults). However, vitamin D deficiency may also be linked to other problems such as increased susceptibility to several chronic diseases and muscle weakness (83).

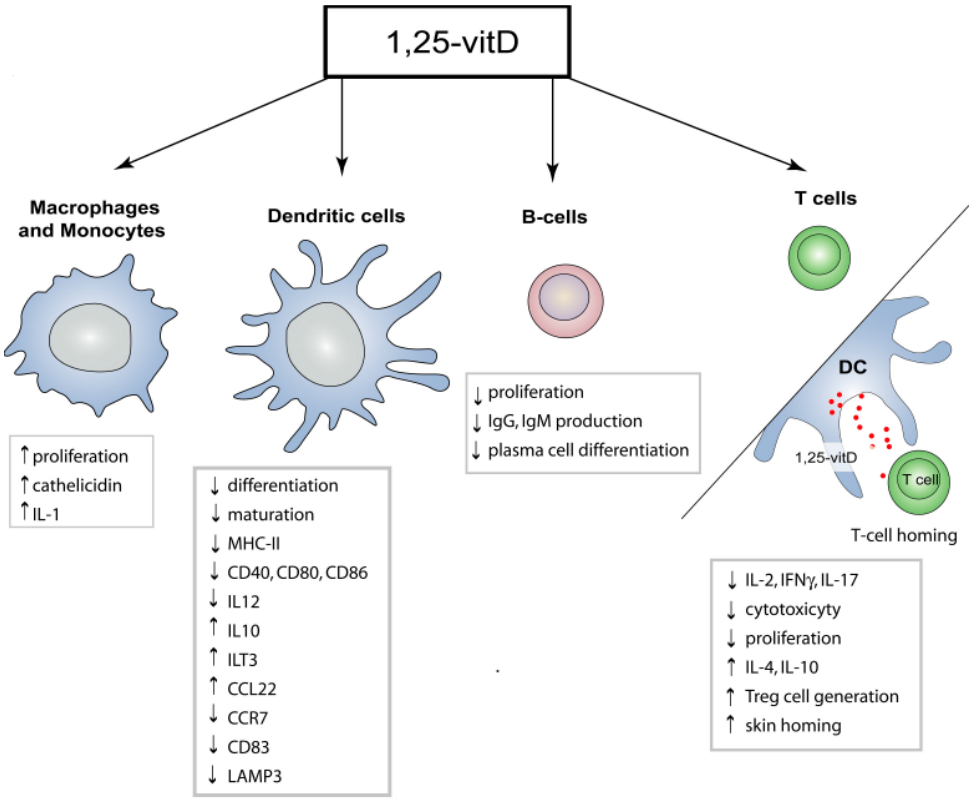

Figure 6. *1,25-vitD exerts its effects on several immune cell types, including macrophages, DCs, T-cells and B-cells. Most important affected functions and regulated genes are indicated.*

The influence of 1,25-vitD in the immune system has been known for 20 years (69, 84, 85). 1,25-vitD inhibits T-cell proliferation, down-modulate the expression of IL-2, IFNγ, and CD8+ T-cell mediated cytotoxicity while IL-4 production is enhanced (27,

69, 86) (Figure 6). By inhibiting IFNγ, which is a positive feedback signal to DCs, 1,25-vitD prevents further antigen presentation to and recruitment of T cells (86). IL-2 is an autocrine growth factor for T cells and its inhibition prevents their further activation and proliferation (86). The net result of 1,25-vitD action on T-cells is to block the induction of Th1 cell and Th17 responses, while promoting Th2 cell and Treg responses (69). In addition to its inhibitory effects on T-cells, 1,25-vitD decreases B-cell proliferation plasma cell differentiation and IgG secretion (69). It should be noted that it is not clarified that 1,25-vitD acts directly on B-cells, or rather via antigen presenting cells and/or T-cell help (69, 87-90). Interestingly, some of 1,25-vitD effects on monocytes/macrophages are stimulatory: 1,25-vitD can induce the proliferation of human monocytes in vitro (69), and increase the production of IL-1 (69). TLR activation of human macrophages up-regulated expression of VDR and the vitamin D-1α hydroxylase genes, leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular Mycobacterium tuberculosis (91).

A number of studies (58, 92-97) provided evidence that addition of 1,25-vitD, has impact on differentiation, function and maturation of human and mouse DCs resulting in T-cell hyporesponsiveness. Importantly, DCs differentiated in the presence of 1,25-vitD share several features with tolerogenic DCs (24, 98). These include low surface expression of MHC-II and co-stimulatory molecules (CD40, CD80, CD86); up-regulation of inhibitory molecules (ILT3), decreased production of IL-12, and enhanced secretion of CCL22 and IL-10 (34, 58, 93-97, 99). The effect of 1,25-vitD on inhibiting the maturation of DCs was dependent on VDR (94). Furthermore, $VDR^{-/-}$ mice have enlarged lymph nodes with an increased proportion of mature DCs implicating 1,25-vitD in the differentiation and/or maturation of DCs *in vivo* (94). In addition animal studies demonstrated that treatment with 1,25-vitD arrests the development of autoimmune diabetes (100) and mediates tolerance to transplants (101), supporting a potential pharmacological application for this hormone or its analogs (102).

Remarkably, DCs are not only sensitive to 1,25-vitD but they can produce it from its precursors. The inactive 25-vitD is converted to 1,25-vitD by 25-hydroxyvitamin D_3 1-α-hydroxylase (CYP27B1). The main site of this conversion is the kidney, but

CYP27B1 expression has been shown in several extrarenal sites including DCs (37, 38, 40). DCs also express sterol 27-hydroxylase (CYP27A1) the main (40) 25-hydroxylase of vitamin D in the liver. Due to the expression of these two enzymes DCs can convert both vitamin D_3 and 25-vitD into 1,25-vitD (40, 103) (Figure 4). In this way, endogenously generated 1,25-vitD circuit enables DCs to respond to pro-inflammatory stimuli with the generation of 1,25-vitD that limits the extent of DC activation and might function as a negative-feedback mechanism (27). The other consequence was documented by Sigmundsottir et al. (40). 1,25-vitD produced by DCs in the skin instructs the local T-cells to express CCR10, thus enabling them to migrate to the epidermis. These results suggested that similar to retinoic acid, which elicits gut-homing specificity of T-cells, the active form of vitamin D is also involved in T-cell homing by imprinting skin-homing specificity of T-cells.

2.4. Aims of the studies

We investigated the program initiated by the activation of VDR using microarray analyses. The list of genes regulated by 1,25-vitD was compared to two other gene lists: the list of differentiation-affected and the list of RARα-regulated genes. These comparisons served as the basis of two studies discussed in this thesis.

(1.) The ways how immunogenic and tolerogenic DCs develop and the mechanisms immunosuppressive drugs can modify the function, differentiation and maturation of DCs are central questions in DC biology. 1,25-vitD induces the tolerogenic DC phenotype by various independent pathways or via a combination of those pathways such as inhibition of differentiation and/or maturation, interference with NF-κB signaling, or by direct transcriptional evens. It is an important issue to determine if 1,25-vitD acts via inhibition of immunogenic mechanisms or it acts autonomously. In the first study we aimed at **investigating the role of 1,25-vitD on the DCs in a developmental context** to clarify how 1,25-vitD-regulated genes are connected to the differentiation-affected changes.

(2.) Previously we demonstrated that PPARγ-specific rosiglitazone and RARα-specific AM580 regulate overlapping gene sets in monocyte-derived DCs. Interestingly, we found that several genes, regulated by 1,25-vitD, were also affected by AM580. This raised the question **whether and how the programs initiated by RARα and VDR overlap.** In our second study we investigated this issue.

3. MATERIALS AND METHODS

3.1. Isolation and differentiation of DCs

$CD14^+$ monocytes and blood myeloid DCs were obtained from platelet-free buffy coats from healthy donors by Ficoll gradient centrifugation followed by immunomagnetic cell separation with anti-CD14-conjugated or CD1c-conjugated microbeads, respectively (VarioMACS Separation System; Miltenyi Biotec). Blood myeloid DCs were cultured at a density of 3.5 x 10^5 cells/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (Sigma-Aldrich). Monocytes were cultured in multiwell culture plates or tissue flasks at a density of 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 800 U/ml GM-CSF (Leucomax; Gentaur Molecular Products), 500 U/ml IL-4 (PeproTech), and penicillin/streptomycin. IL-4 and GM-CSF were replenished on day 3. To obtain MDCs, the medium was supplemented with mixture of proinflammatory cytokines containing 10 ng/ml TNF-α, 10 ng/ml IL-1β, 1,000 U/ml IL-6 (PeproTech), and 1 μg/ml prostaglandin- E_2 (Sigma-Aldrich). Immature DCs were also challenged with various TLR ligands: 100 ng/ml LPS (TLR4 ligand) (Sigma- Aldrich), 2 μg/ml CL075 (TLR8/7 ligand) (InvivoGen), and 20 μg/ml polyinosinic:polycytidylic acid (TLR3 ligand) (Sigma-Aldrich) for 18 h.

3.2. Ligand treatment of DCs

1,25-VitD (Biomol) was used at 10 nM and at 1 pM to 100 nM for dose response experiments, 25-hydroxyvitamin D_3 (25-vitD) (Biomol) was used at 100 nM, and ZK159222 (Bayer Schering Pharma) was used at 1 μM. The vehicle (ethanol:DMSO at 1:1) had no detectable effect on the differentiation (data not shown). For activation of RARα receptor, synthetic agonist, AM580 was used at 100 nM.

3.3. Microarray analysis: sample preparation, labeling, and hybridization

Monocytes differentiating into DCs were treated with 10 nM 1,25-vitD, 100 nM AM580 or vehicle 14 h after plating. Cells were harvested 12 h or 5 days thereafter. Total RNA from 6 x 10^6 cells was isolated using the RNeasy kit (Qiagen). Experiments

were performed in biological triplicates representing samples from different donors. Further processing and labeling, hybridization to GeneChip human genome U133 Plus 2.0 arrays (Affymetrix), and scanning were conducted at the Microarray Core Facility of EMBL (Heidelberg, Germany). Microarray data have been deposited into the Gene Expression Omnibus database under accession number GSE13762.

3.4. Microarray data analysis

Image files were imported to GeneSpring 7.3 (Agilent). Raw signal intensities were normalized per chip (to the 50th percentile) and per gene (to the median). We removed probe sets that failed to reach a signal intensity of at least 200 in three of 15 samples. To identify significantly regulated genes between two compared samples, we then identified probe sets that showed at least 2-fold up or down-regulation by eliminating probe sets with a ratio of signal intensity between 0.5 and 2. Finally, we performed a t-test for each pair of probe sets and filtered for values of $p \leq 0.05$ (samples were normalized to median or to control samples). For comparison of RARα and VDR affected genes ANOVA t-test were performed. For heat map and scatter plot visualization of signal intensities, each probe set was normalized to the signal intensities of vehicle controls (fold change). The PANTHER (Protein Analysis through Evolutionary Relationships) classification system was used for the functional classification of genes (www.pantherdb.org/tools/genexAnalysis.jsp).

3.5. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qPCR) was conducted as described earlier (42, 55) using TaqMan probes (Applied Biosystems). For details see Table 1. Gene expression was quantified by the comparative cycle threshold (CT) method and normalized to cyclophilin A expression. All experiments were conducted as biological triplicates. Values are expressed as mean ± SD of the mean.

Gene symbol	Assay code or probe and primer sequences
ALOX5	Hs00386528_m1
CCL22	Hs00171080_m1
CD14	(879+) CGCTCCGAGATGCATGTG (938 -) AGCCCAGCGAACGACAGA (898 +) FAM-TCCAGCGCCCTGAACTCCCTCA-TAMRA
CD1A	(1357+) ACCTGTCCTGTCGGGTGAA (1435-) CCCACGGAACTGTGATGCT (1382+) FAM-CAGTCTAGAGGGCCAGGACATCGTCCT-TAMRA
CD1C	Hs00233509_m1
CD1E	Hs00229421_m1
CD300LF (IREM-1)	Hs00371178_m1
CD36	(969+) TGGGAAAGTCACTGCGACAT (1048-) TGCAATACCTGGCTTTTCTCA (990+) FAM-ATTAATGGTACAGATGCAGCCTCATTTCCAC-TAMRA
CD80	Hs00175478_m1
CYP24A1	Hs00167999_m1
CYP27B1	Hs00168017_m1
F13A1	Hs00173388_m1
IER3	Hs00174674_m1
IRF4	Hs00180031_m1
IRF8	Hs00175238_m1
LGALS9	Hs00371321_m1
PPIA (Cyclophilin A)	(52+) ACGGCGAGCCCTTGG (117-) TTTCTGCTGTCTTTGGGACCT (69+) FAM-CGCGTCTCCTTTGAGCTGTTTGCA-TAMRA
THBD	Hs00264920_s1
VDR	Hs01045840_m1

Table 1. *Real-time quantitative RT-PCR primers and probes.*

3.6. 1,25-VitD ELISA

Monocytes were plated at a density of 10^6 cell/ml (total 6.5 x 10^6 cells/ sample), cultured as described, and treated with 100 nM 25-vitD. Cells were harvested at days 1–6, washed, and stored at ⁻20°C. Pellets were resuspended in saline and sonicated (Bioruptor; Diagenode) for 10 min to achieve complete lysis. Lysates were cleared by centrifugation and the 1,25-vitD content of supernatant was concentrated by column chromatography and measured by ELISA (1,25-(OH)2 vitamin D ELISA kit; Immundiagnostik) as recommended by the manufacturer.

3.7. CCL22 ELISA

Monocytes were cultured in 12-well dishes in the presence of 1,25-vitD, 25-vitD, or vehicle as described earlier. CCL22 content of supernatants was measured by sandwich ELISA specific for CCL22 (human MDC immunoassay; R&D Systems) as recommended by the manufacturer.

3.8. Flow cytometry

Surface expression of CD300LF (also known as IREM-1) was detected with anti-IREM-1 UP-D2 mAb (a gift from M. López-Botet, Universitat Pompeu Fabra, Barcelona, Spain). Isotype control anti-IgG1 mAb (R&D Systems) was used as a negative control. FITC-conjugated polyclonal goat anti-mouse Ig (Dako) was used as a secondary Ab. Flow cytometric analysis of differentiation and maturation markers was performed using the following antibodies: anti-CD1A PE, anti-HLA-DR PE, anti-CD83 PE (BD Pharmingen), anti-CD14 PE and anti-CD40 PE (R&D Systems). Cell surface staining was measured with a FACSCalibur cytometer and analyzed with the CellQuest software package (BD Pharmingen).

3.9. Western blot analysis

Cell lysates (25 μg protein) were separated on an 8% SDS-polyacrylamide gel and electroblotted onto a PVDF (Millipore) membrane. The membrane was probed with a polyclonal Ab against CYP27B1 (1/5000; article no. HYD001 from Biologo), stripped, reprobed with an Ab to VDR (1/7000; C-20, sc1008from Santa Cruz Biotechnology), stripped again, and reprobed with mouse anti-GAP3DH (clone 6C5) (1/5000; catalog

no. ab8245 from Abcam). The Ag-Ab complexes were labeled with appropriate HRP-conjugated secondary Abs (Sigma-Aldrich) and visualized by Immobilon Western HRP substrate kit (Millipore).

3.10. Immunohistochemistry

Cells were collected by centrifugation, fixed in buffered formalin, and embedded into paraffin. Sections (5 μm) were immunostained with antihuman VDR mAb (1/2000; clone H4537 from Perseus Proteomics) after wet heat-induced Ag retrieval. The EnVision -HRP system (Dako) was used to visualize the labeling according to manufacturer's instructions.

4. RESULTS

4.1. VDR is expressed early in developing monocyte-derived DCs

We isolated $CD14^+$ monocytes from the peripheral blood of healthy donors and cultured them in the presence of GM-CSF and IL-4. This represents a well-established model (30) in which we and others have previously mapped nuclear hormone receptor mediated transcriptional events (42, 43, 55, 58, 96).

First we determined the kinetics of VDR expression. Monocytes expressed VDR at low, but detectable levels. Upon culturing monocytes in GM-CSF and IL-4 transcription of the VDR gene increased rapidly. The amount of VDR transcript detected peaked after 18-24h. Interestingly, the VDR transcription rate decreased to lower levels in later phases of the differentiation process (Figure 7A). Western blotting experiments showed that the VDR protein level rapidly increased in the first few days and remained at a high level in later phases of the differentiation (Figure 7B). The VDR protein was located in the nuclei of IDCs as determined by immunohistochemistry (Figure 7A, insert). We thus demonstrated that VDR is expressed rapidly and at high levels in differentiating DCs.

4.2. 1,25-vitD directs the transcription of a large set of genes independent of the differentiation program

The receptor expression peaks around 18h after induction of differentiation therefore we added 1,25-vitD or vehicle 14h after plating. Our goal was to assess both the early and late transcriptional changes caused by 1,25-vitD; we therefore harvested differentiating DCs at 12h or IDC at 5d after addition of ligand. $CD14^+$ monocytes served as reference. The transcriptomes of these cells were studied using Affymetrix GeneChip Arrays. This experimental setup (Figure 7D) allowed us to compare the transcriptomes of monocytes, differentiating DCs and IDCs to define genes that are differentially expressed during differentiation and upon 1,25-vitD-treatment at early (12h) and late (5d) time points. We and others have already shown (42) that the differentiation of myeloid cells from precursors will lead to differential expression of several thousand genes.

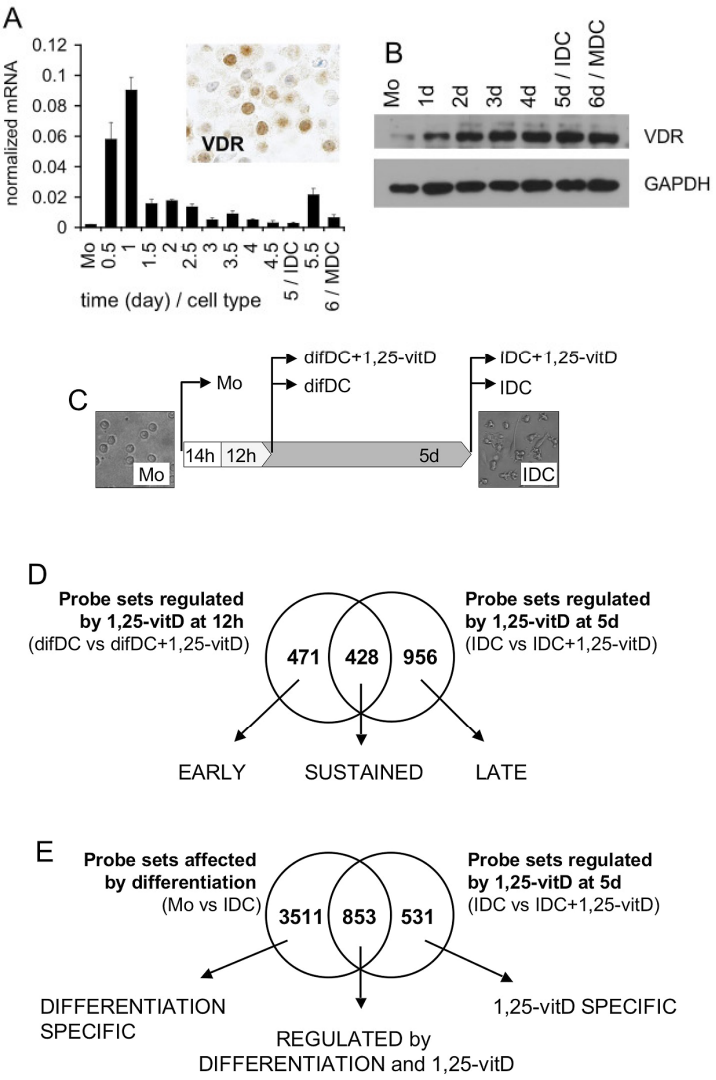

Figure 7. *VDR is expressed early in developing monocyte-derived DCs and directs the transcription of a large set of genes independent of the differentiation program. (A) Expression of VDR in differentiating and maturing human monocyte-derived DCs as determined by qPCR (Mo - monocyte, IDC - immature DC, MDC - mature DC). To obtain MDCs, cells were treated with cocktail of proinflammatory cytokines. VDR is strongly expressed and is localized to the nucleus in IDCs as demonstrated by immunohistochemical staining (panel A insert). (B) VDR protein is accumulated in an early phase during differentiation as shown by Western blotting analysis. (C) $CD14^+$ monocytes were isolated from peripheral blood and were cultured in the presence of IL-4 and GM-CSF. Fourteen hours after setting up the culture the differentiating cells were treated with 10 nM 1,25-vitD or vehicle for twelve hours or 5 days. The transcriptomes of monocytes, differentiating DCs (difDC) or 5-day-immature DCs (IDC) treated with 10 nM 1,25-vitD or vehicle were analyzed by Affymetrix microarrays. (D) Probe sets regulated by 1,25-vitD-treatment in differentiating DCs (12h) and IDCs (5d) were identified and compared. The results are visualized as a Venn-diagram. The regulated probe sets were categorized as EARLY (regulated at 12h only), SUSTAINED (regulated both at 12h and 5d) and LATE (regulated at 5d only). Note the large number of genes that are regulated already at 12h. (E) Probe sets differentially expressed during the Mo to IDC differentiation as well as probe sets regulated by the 5-day 1,25-vitD treatment were identified and compared. The results are visualized as a Venn-diagram. Note the significant number of probe sets regulated by 1,25-vitD, but not the differentiation process itself.*

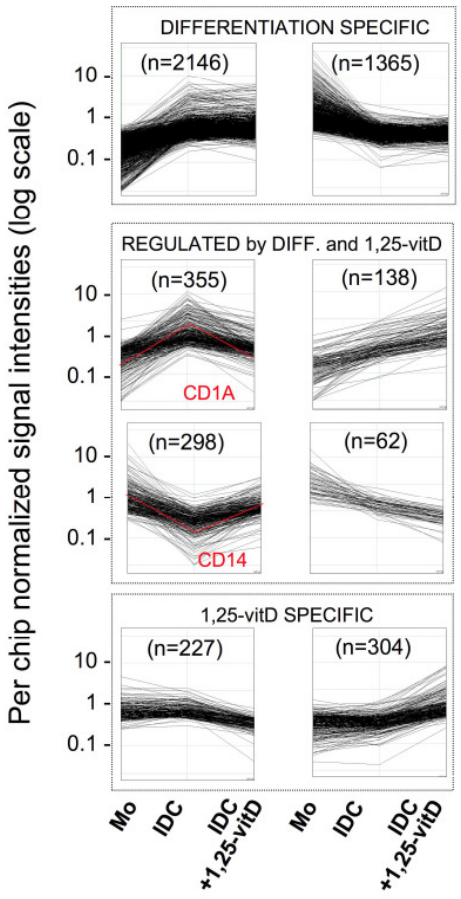

Figure 8. *Probe sets regulated by 1,25-vitD and affected by differentiation only partially overlap. Probe sets regulated by 1,25-vitD and/or affected by differentiation were grouped based on their expression patterns. Line charts and number of probe sets belonging to a certain group are shown. (Mo – monocyte, IDC – 5-day-immature DC, IDC+1,25-vitD – immature DC treated with 1,25-vitD for 5d.)*

Consistent with this, we found 4364 differentially expressed probe sets (representing 2766 genes) between the transcriptomes of monocytes and IDCs. Comparing the transcriptomes of 1,25-vitD-treated and vehicle-treated samples we detected 899 and 1384 differentially expressed probe sets (representing 578 and 918 genes) at 12 hours and 5 days, respectively (Figure 7D-E). If VDR ligands exert their effect mainly by inhibiting the differentiation and maturation program this would imply that 1,25-vitD transcriptionally regulates part of the gene set that is also developmentally regulated. Remarkably, 3511 probe sets affected by differentiation were not regulated by 1,25-vitD (Figure 7E). Furthermore, only 853 of the 1384 probe sets that were found to be regulated by 1,25-vitD were also among the probe sets that were differentially expressed in monocytes and IDCs (Figure 7E and Figure 8). These results thus strongly suggest that 1,25-vitD should not be simply viewed as a general and global inhibitor of differentiation.

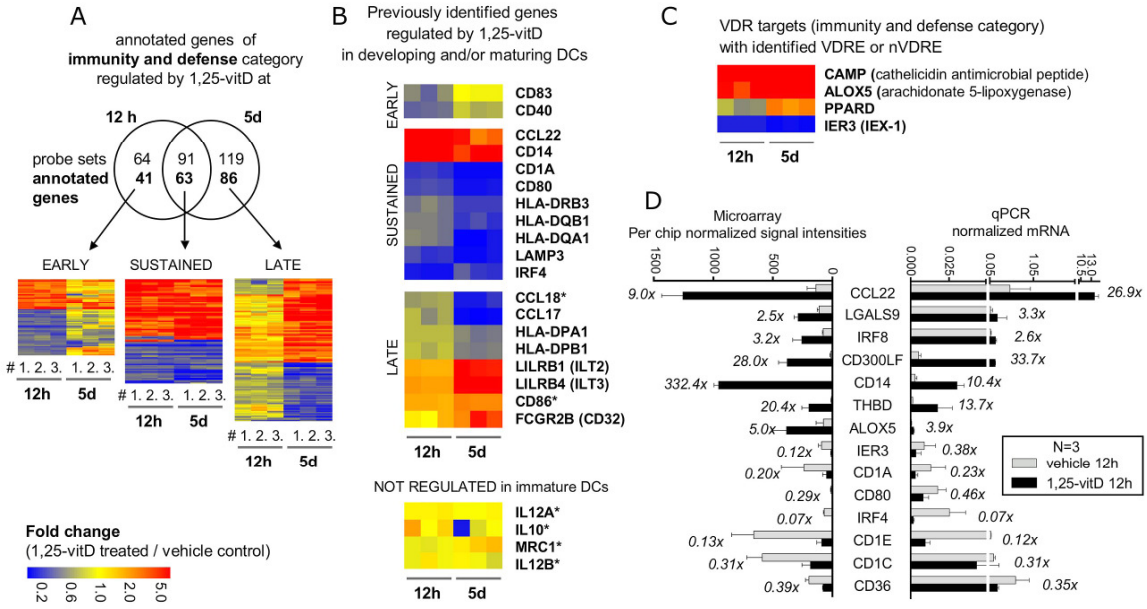

Figure 9. *1,25-vitD treatment leads to the regulation of many "immunity and defense" genes in differentiating and immature DCs. (A) Genes belonging to the EARLY, SUSTAINED and LATE groups were functionally categorized using the Panther Classification System. Genes falling to the functional category "immunity and defense" are shown as a Venn-diagram and heat maps. In heat maps columns represent expression profiles of independent donors for the two studied time points. Color intensities reflect the ratios of signal intensities as shown. (B) Known 1,25-vitD-regulated "immunity and defense" genes in DCs as observed by our microarray analysis shown as a heat map. Note that in the vast majority of cases our results were concordant with the previously published literature. Exceptions are shown with asterisks. (C) The effect of 12-hour and 5-day 1,25-vitD treatment on the expression of "immunity and defense" direct VDR target genes regulated by well-characterized positive and negative vitamin D response elements shown as a heat map. (D) Validation of the microarray results (on the left) by qPCR (on the right) on a list of genes connected to "immunity and defense" properties of DC. Bars show the signal intensities of the mean of biological triplicates for vehicle-treated and 1,25-vitD-treated samples. Note that the axis showing the qPCR signal intensities is twice broken. Fold changes (1,25-vitD-treated / vehicle-treated) are indicated by numbers next to the bars.*

4.3. 1,25-vitD-treatment leads to the regulation of many “immunity and defense” genes in differentiating and IDCs

The tolerogenic phenotype is likely to be brought about by transcriptional modulation of immune function related genes. We used the PANTHER Classification System that utilizes an unbiased gene ontology classification to assign function to the affected genes (Table 2.). We found 41+63=104 and 63+86=149 genes that fell into the functional category "immunity and defense" regulated at the 12 hour and 5 day time point, respectively (Figure 9).

	EARLY			SUSTAINED			LATE		
Biological Process	# of genes	# of expected	P-value	# of genes	# of expected	*P-value*	# of genes	# of expected	P-value
Immunity and defense	41	16.07	<0.00001	63	14.82	<0.00001	86	33.79	<0.00001
Signal transduction	86	41.52	<0.00001	71	38.3	<0.00001	165	87.32	<0.00001
T-cell mediated immunity	10	2.36	<0.05	12	2.18	<0.001	10	4.97	NS
MHCII-mediated immunity	0	0.41	NS	6	0.38	<0.001	4	0.87	NS
Other carbon metabolism	2	1	NS	7	0.92	<0.01	1	2.1	NS
Other immune and defense	8	1.87	NS	9	1.72	<0.05	6	3.92	NS
Induction of apoptosis	5	2.01	NS	9	1.86	<0.05	13	4.23	NS
Ligand-mediated signaling	10	5.13	NS	15	4.73	<0.05	31	10.79	<0.0001
Other metabolism	7	6.81	NS	16	6.29	<0.05	18	14.33	NS
Apoptosis	15	6.47	NS	15	5.97	<0.05	29	13.61	<0.01
Carbohydrate metabolism	13	7.22	NS	16	6.66	<0.05	25	15.18	NS
Lipid, fatty acid and steroid metabolism	24	9.39	<0.01	19	8.66	<0.05	39	19.74	<0.01
Amino acid metabolism	5	2.8	NS	9	2.59	<0.05	8	5.9	NS
Cell proliferation and differentiation	27	12.53	<0.01	22	11.56	NS	47	26.36	<0.01
Transport	30	15.92	<0.05	26	14.69	NS	46	33.48	NS
Cell communication	29	14.79	NS	26	13.64	NS	63	31.1	<0.0001
Cell surface receptor mediated signal transduction	34	19.97	NS	32	18.42	NS	75	42	<0.001
Intracellular signaling cascade	31	10.62	<0.0001	20	9.8	NS	53	22.33	<0.00001
B-cell- and antibody-mediated immunity	8	1.18	<0.01	5	1.09	NS	6	2.49	NS
Macrophage-mediated immunity	9	1.71	<0.01	6	1.57	NS	10	3.59	NS
Biological process unclassified	87	138	UR <0.00001	64	127.32	UR <0.00001	188	290.25	UR <0.00001

Table 2. Biological processes affected by 1,25-vitD in DCs. *Using Panther Classification system genes of EARLY (regulated at 12h only), SUSTAINED (regulated both at 12h and 5d) and LATE (regulated at 5d only) groups were categorized. "Immunity and defense" category was amongst the most overrepresented processes in all groups. Number of expected genes and p-values of categories were calculated by comparing uploaded gene lists to NCBI Homo sapiens reference list. Biological processes over-represented significantly (p-values ≤ 0.05) are listed in the table. (NS – not significant, UR – under-represented.)*

Importantly, this category was significantly overrepresented among the functional classes (Table 2.) at both time points. We chose two approaches to validate these findings. First, we selected a set of genes previously reported (34, 58, 92, 95, 96, 99, 104, 105) to be 1,25-vitD-regulated in DCs with the caveat that in most studies the effect of 1,25-vitD was not tested at the transcriptional level. We investigated if these genes were regulated by 1,25-vitD in our experimental model at the mRNA level. The majority was indeed regulated as expected (Figure 9B) with the notable exceptions of IL-10, IL-12 and mannose receptor, C type 1 (no regulation); and CD86 and CCL-18 (opposite regulation). It is likely that 1,25-vitD can modulate the expression of IL-10 and IL-12 only during maturation. We also looked at the direct VDR target genes (106-109) falling into the "immunity and defense" category. As anticipated all direct VDR targets, expressed in this cell type, were regulated, most of them at both time points (Figure 9C). Second, we also validated our microarray results on selected targets (choosing genes expressed at low and at high levels) using qPCR. We found that the results of the two independent methods for measuring gene expression at the transcriptional level showed a good agreement (Figure 9D). The validation of our expression data thus gave us confidence to further investigate the relationship of differentiation and 1,25-vitD-treatment at the transcriptional level.

4.4. 1,25-vitD and differentiation regulated "immunity and defense" gene sets only partially overlap

The suggestion that VDR ligands inhibit the differentiation and maturation program comes from the investigation of individual immunity and defense genes (CD1A, CD14 etc.). No system-level analysis involving hundreds of genes was ever carried out to investigate this issue. To reveal the relationship of the effects of 1,25-vitD and differentiation on "immunity and defense" genes we first derived and compared the gene sets regulated by 1,25-vitD and/or differentiation belonging to this category (Figure 10A). This comparison proved that 1,25-vitD and differentiation regulated "immunity and defense" gene sets only partially overlap, similarly to the entire gene sets (Figure 7E). To get a more complex view we also determined the ratios of gene expression in DC vs. monocyte (differentiation effect) and 1,25-vitD-treated vs. vehicle-treated DC (1,25-vitD effect) and plotted these for both the early and late (Figure 10B) time points.

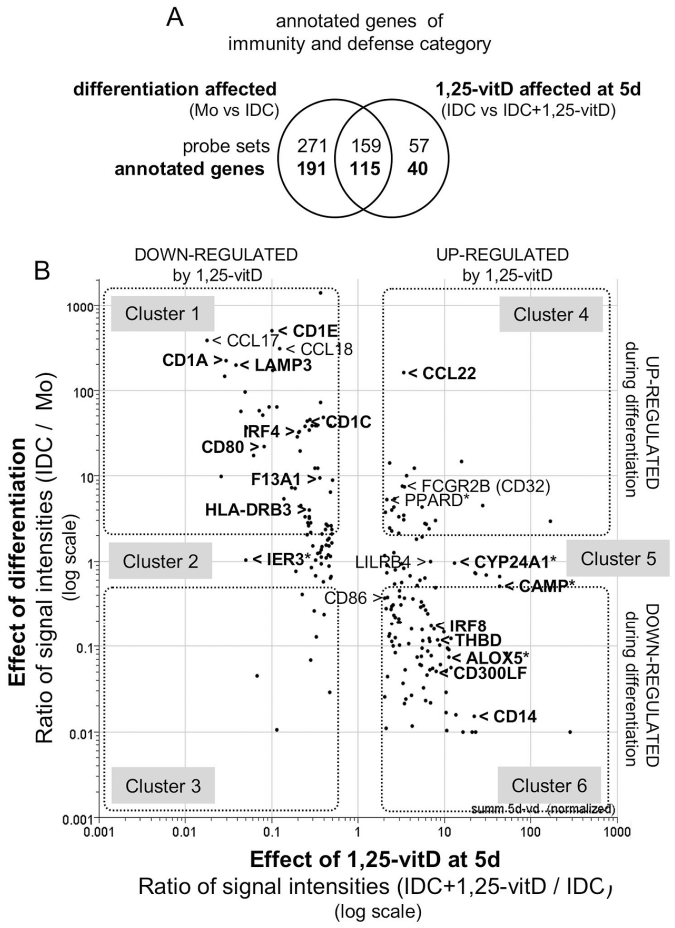

Figure 10. *1,25-vitD and differentiation regulated "immunity and defense" gene-sets only partially overlap. (A) Annotated genes of "immunity and defense" category differentially expressed during the Mo to IDC differentiation as well as probe sets regulated by the 5-day 1,25-vitD-treatment were identified and compared. The results are visualized as a Venn-diagram. (B) For each probe set representing an "immunity and defense" gene, the ratios of transcript levels in 5d IDC vs. monocyte (effect of 5d differentiation) were determined. Similarly, the ratios of transcript levels of 5d 1,25-vitD-treated IDC vs. vehicle-treated DC (effect of 5d 1,25-vitD) were calculated. The two ratios of transcript levels were plotted against each other resulting in a scatter plot that shows the relationship of transcriptional changes caused by the differentiation process and the 1,25-vitD treatment. Genes relevant for this study are marked. Genes already regulated at the 12h time point are marked in bold. Known direct VDR targets are shown with an asterisk. For clarity only one HLA gene is indicated and probe sets not affected by the 1,25-vitD-treatment are omitted from the scatter plot.*

The majority of genes (Cluster 1 and 6) showed opposite regulation during differentiation and upon 1,25-vitD-treatment as one could have predicted. Importantly, we also found a smaller number of genes where the effect of differentiation and 1,25-vitD-treatment pointed to the same direction (Cluster 3 and 4). In addition, many 1,25-vitD-regulated genes were not affected by differentiation at all (Cluster 2 and 5) providing evidence to suggest that the differentiation program and the 1,25-vitD-induced program are indeed, non-overlapping.

4.5. Many early "immunity and defense" genes are autonomously regulated in differentiating DCs and blood myeloid DCs

Interestingly, the expression profile of ALOX5 (in Cluster 6 on Figure 10) suggested that genes showing opposite regulation during differentiation and upon 1,25-vitD-treatment might also be regulated independent of differentiation. The fact that ALOX5 (arachidonate 5-lipoxygenase) is a direct VDR target (109) made it likely that the 1,25-vitD-treatment altered its expression directly. This suggests that 1,25-vitD and differentiation can regulate the expression of certain genes, independently of one another. Autonomous regulation by 1,25-vitD would thus imply that 1,25-vitD changes the rate of transcription of its target genes independent of the differentiation state of DCs. In order to test this hypothesis we chose a number of genes that were up-regulated by 1,25-vitD at 12h and inhibited during differentiation (Cluster 6, Figure 10) and tested whether they can be up-regulated in a later phase of DC differentiation by 1,25-vitD.

We treated monocytes with GM-CSF and IL-4 for four days, and then added 10 nM 1,25-vitD or vehicle for an additional 24h and determined gene expression by qPCR. Our results showed that 1,25-vitD-treatment led to the induction of CD14, THBD, CD300LF, ALOX5 but not IRF8 (Figure 11A), suggesting that a large fraction of genes is regulated indeed autonomously by 1,25-vitD. To underscore the biological significance of our finding and also to test whether the genes regulated seemingly independent of differentiation (Cluster 1 and 6, Figure 10) can also be regulated autonomously by 1,25-vitD, we aimed to validate our results in a distinct *ex vivo* DC type. We isolated myeloid blood DCs from peripheral blood of healthy human donors and cultured them in the absence of cytokines and in the presence of 1,25-vitD for 24 hours. Our qPCR experiments showed that 11 of the 12 studied genes showed similar regulation in blood DCs and *ex vivo* differentiating DCs (Figure 11B), providing further evidence that many genes are regulated by 1,25-vitD-treatment independent of differentiation signals.

4.6. Characterization of 1,25-vitD-dependent regulation of CYP24A1, CCL22 and CD300LF

We selected three genes induced by 1,25-vitD, but differentially regulated by differentiation for further characterization: 24-hydroxylase (CYP24A1, a direct target

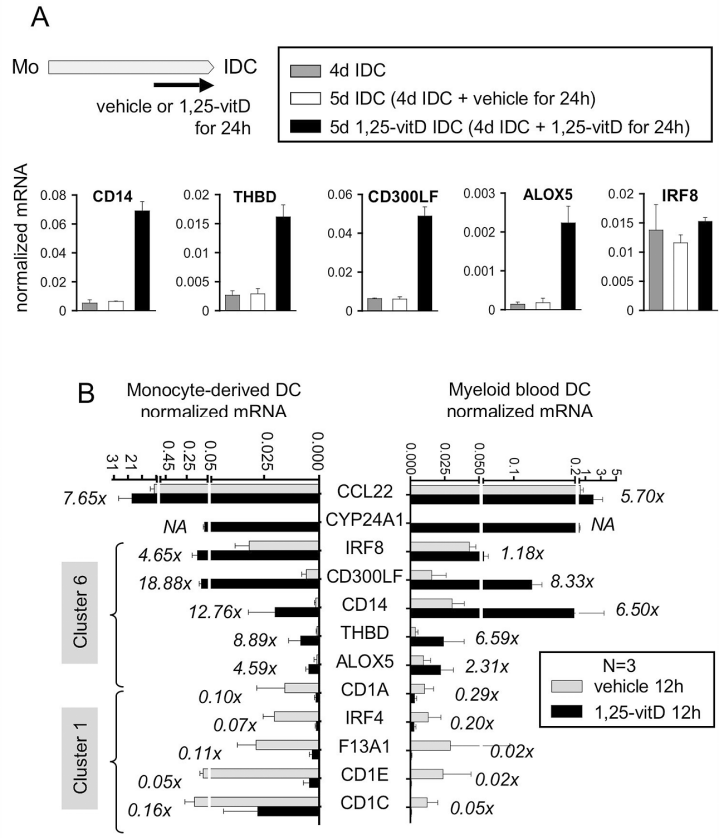

Figure 11. *Many early "immunity and defense" genes are autonomously regulated in differentiating DCs and blood myeloid DCs. (A) 1,25-vitD changes the expression levels of its target genes independent of the differentiation state of DCs. Monocytes were cultured with GM-CSF and IL-4 for four days, and then differentiating DCs were treated with 10 nM 1,25-vitD or vehicle for 24h. The expression of selected "immunity and defense" genes, which's expression is oppositely regulated by differentiation and 1,25-vitD treatment, was measured by qPCR. (B) The 1,25-vitD sensitivity of selected "immunity and defense" genes was verified in monocyte-derived DCs and blood myeloid DCs by qPCR. Genes tested included a selected panel of "immunity and defense" genes, which's expression is oppositely regulated by differentiation and 1,25-vitD treatment, and CCL22 and CYP24A1 (belonging to Clusters 4 and 5, respectively). Gene expression levels were measured by qPCR. Bars show the mRNA levels of the indicated genes for vehicle-treated and 1,25-vitD-treated samples. Note that the axis showing the qPCR signal intensities is twice broken in order to accommodate the large range of expression levels. If applicable, fold changes (1,25-vitD-treated / vehicle-treated) are indicated by numbers next to the bars (NA - no applicable).*

of VDR (110)), chemokine ligand 22 (CCL22, a chemokine attracting regulatory T-cells (99, 111)) and CD300LF (an inhibitory receptor (112), also known as IREM-1). These three genes were all shown to be regulated by 1,25-vitD-treatment by our expression study, but were either up-regulated (CCL22), not regulated (CYP24A1) or

down-regulated (CD300LF) during differentiation (Figure 10), therefore represented different Clusters in our analysis.

We first determined the time course of the expression of the three genes using qPCR. CYP24A1, CCL22 and CD300LF were induced as early as three hours of 1,25-vitD-treatment, and the expression of all three genes remained up-regulated after 6h, 12h and 24h (Figure 12A). The early and sustained up-regulation of CYP24A1, CCL22 and CD300LF suggested a direct regulation by ligand-bound VDR. To show that the effect of 1,25-vitD is indeed mediated through VDR, we used ZK159222, a partial VDR antagonist (113), and could show that treatment with ZK159222 has significantly repressed the 1,25-vitD-elicited transcriptional response of all three genes (Figure 12B). Next, we determined dose response curves of CYP24A1, CCL22 and CD300LF gene expression upon 1,25-vitD treatment and calculated the EC_{50} values for 1,25-vitD. The EC_{50} value for 1,25-vitD for CD300LF was very similar to that of CYP24A1 (2-3 nM), while the EC_{50} of CCL22 was an order of magnitude lower suggesting that this gene is more sensitive to VDR activation (Figure 12C).

We next reasoned that if the effect of 1,25-vitD is really independent of differentiation, it should induce these three genes at different time points during the monocyte to IDC differentiation program and also during maturation of IDC to MDC provoked by cocktail of proinflammatory cytokines. The obtained data supported our hypothesis as shown on Figure 12D. However, CCL22 was not induced significantly in MDCs probably due to its already high expression level.

4.7. Endogenously produced 1,25-vitD regulates the expression of CYP24A1, CCL22 and CD300LF

The physiological serum levels of 1,25-vitD (~ 40-130 pM) (114) are unlikely to be sufficient to turn on 1,25-vitD signaling in DCs (Figure 12C). However, previous studies revealed that 1,25-vitD can be generated endogenously (37, 38, 40, 103). We therefore sought to determine whether and when the >1,000 times more abundant (114-116) precursor, 25-hydroxyvitamin D_3 (25-vitD) is actively converted to 1,25-vitD in DCs. The hydroxylation step of the conversion process of inactive 25-vitD to 1,25-vitD is catalyzed by CYP27B1, a cytochrome p450 hydroxylase (117, 118).

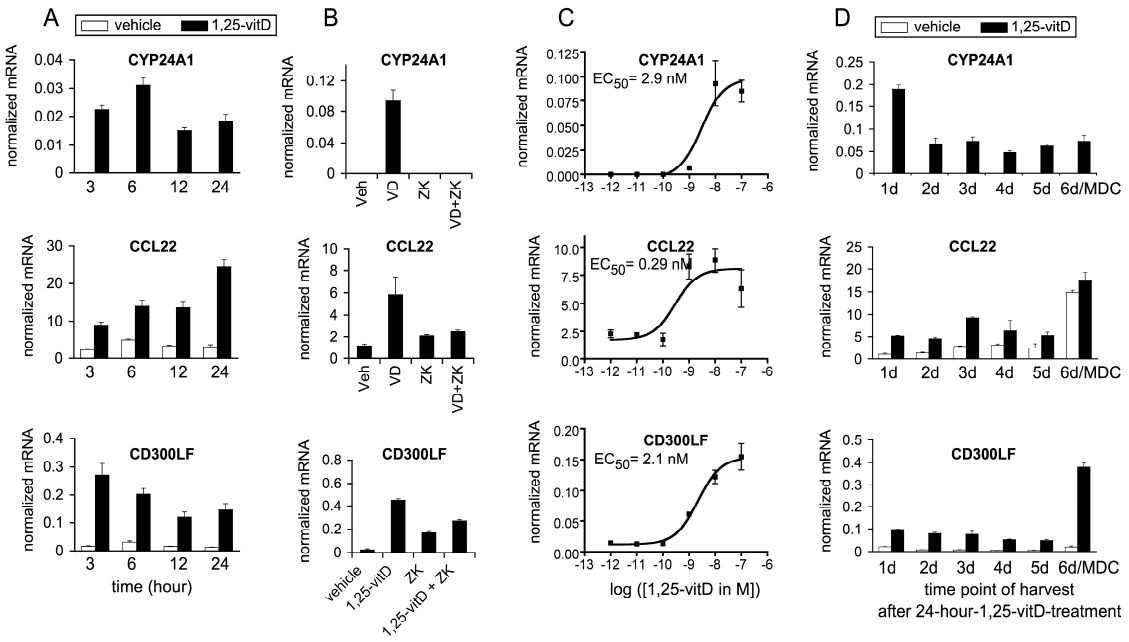

Figure 12. *Characterization of 1,25-vitD-dependent regulation of CYP24A1, CCL22 and CD300LF. CD14$^+$ monocytes were isolated from peripheral blood and were cultured in the presence of IL-4 and GM-CSF. Differentiating cells were treated with 1,25-vitD, VDR antagonist ZK159222 or vehicle 14 hours after setting up the culture (A-C) or as shown (D). Expression of CYP24A1, CCL22 and CD300LF was determined by qPCR. (A) CYP24A1, CCL22 and CD300LF expression of differentiating DCs treated with 10 nM 1,25-vitD for various times. (B) The effect of 10 nM 1,25-vitD, 1 μM ZK159222 or the combination of them on the transcription of CYP24A1, CCL22 and CD300LF after 12 hours. Vehicle was used as a negative control. (C) CYP24A1, CCL22 and CD300LF expression after a 12-hour treatment with varying concentrations of 1,25-vitD. EC_{50} values for 1,25-vitD are indicated. (D) 1,25-vitD-sensitivity is retained during differentiation and partly during maturation. Effects of 24-hour-ligand treatment were tested by adding 1,25-vitD at subsequent days from day 0 (monocyte) through day 5 and harvested 24 hours later. The day of harvest is indicated on the graphs. 5-day IDCs were treated with cocktail of proinflammatory cytokines (TNFα, IL-1β, IL-6 and PGE_2) and 1,25-vitD.*

We therefore investigated the expression pattern of CYP27B1 by qPCR and found that it closely matched that of the VDR (Figure 13A). We also investigated the expression of CYP27B1 at the protein level by Western blotting and found that the protein accumulated during the monocyte to IDC differentiation process (Figure 13B). Maturation of DCs is induced by many different stimuli (13, 15), including proinflammatory cytokines and Toll-like receptor (TLR) ligands. We were interested to see how these various stimuli influence the expression of the receptor and the ligand-producing enzyme. Consistent with previous reports (37, 38), we found that proinflammatory cytokines and TLR-ligands proved to be potent activators of

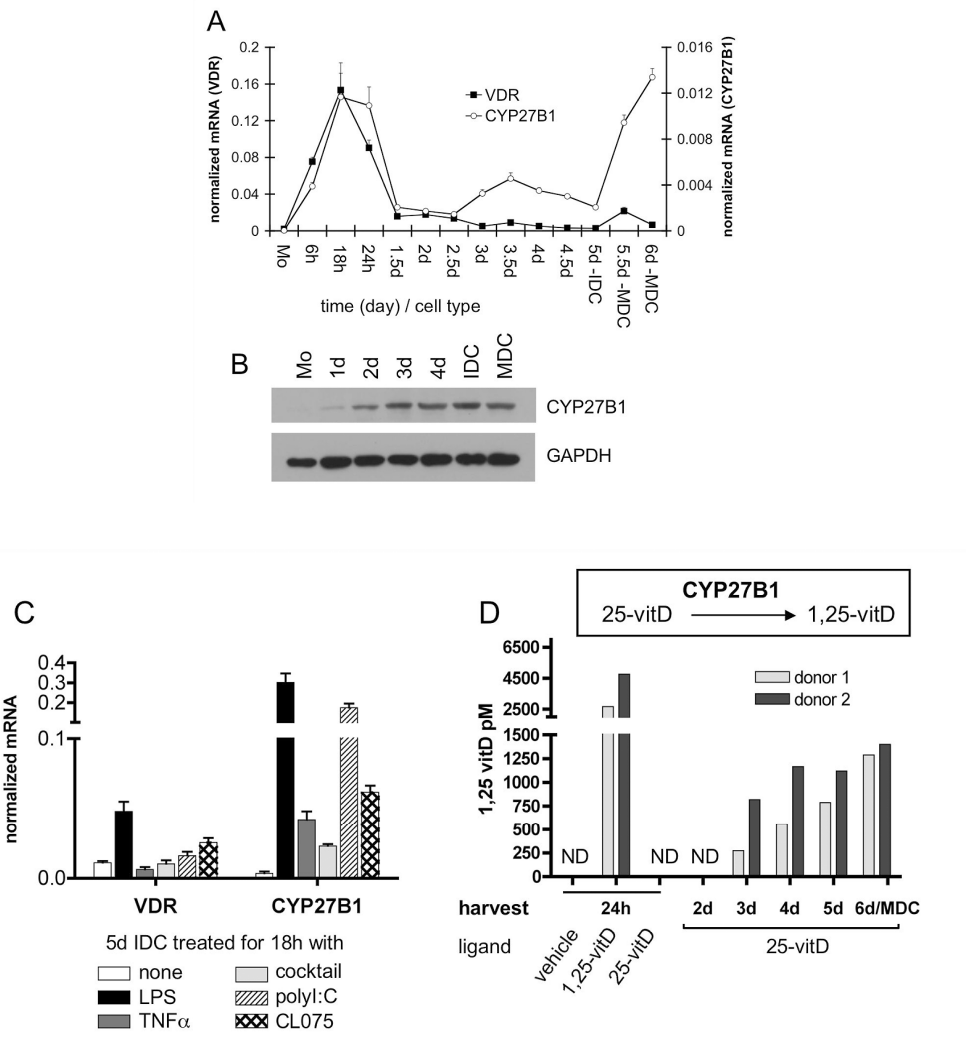

Figure 13. *The inactive 25-vitD is converted to 1,25-vitD by CYP27B1 in DCs. (A) Transcription of VDR and CYP27B1 shows similar kinetics in differentiating human monocyte-derived DCs as determined by qPCR (IDC - immature DC, MDC - mature DC). (B) CYP27B1 protein is accumulated in an early phase during differentiation as shown by Western blot analysis. (C) VDR and CYP27B1 gene expression is induced by a partially overlapping set of stimuli in human monocyte-derived DCs. (Stimuli: LPS; TNFα; cocktail of proinflammatory cytokines: TNFα IL-1β, IL-6 and PGE_2; polyI:C; CL075 – TLR8/7 agonist.) (D) DCs were incubated with 100nM 25-vitD and the produced 1,25-vitD content of the cells was measured by ELISA. For positive and negative controls cells were incubated for 24 hours with 10 nM 1,25-vitD and vehicle, respectively. ND (not detectable) indicates that 1,25-vitD concentration was under the detection limit.*

expression. Interestingly, LPS and TLR8/7 ligand CL075 also induced VDR expression, suggesting that upon specific maturation stimuli VDR and CYP27B1 are likely to be co-regulated (Figure 13C). To test if 25-vitD may indeed be actively converted by differentiating DCs to the active form, we cultured differentiating DCs in

the presence of 100 nM 25-vitD and measured the concentration of the produced 1,25-vitD by ELISA (37). We could detect increasing amounts of 1,25-vitD in differentiating DCs (Figure 13D). Consistent with a previous report (38) we found that endogenously produced 1,25-vitD was effective in regulating key markers such as CD14, CD1A and HLA-DR in IDCs, and CD83 and HLA-DR in MDCs (Figure 14).

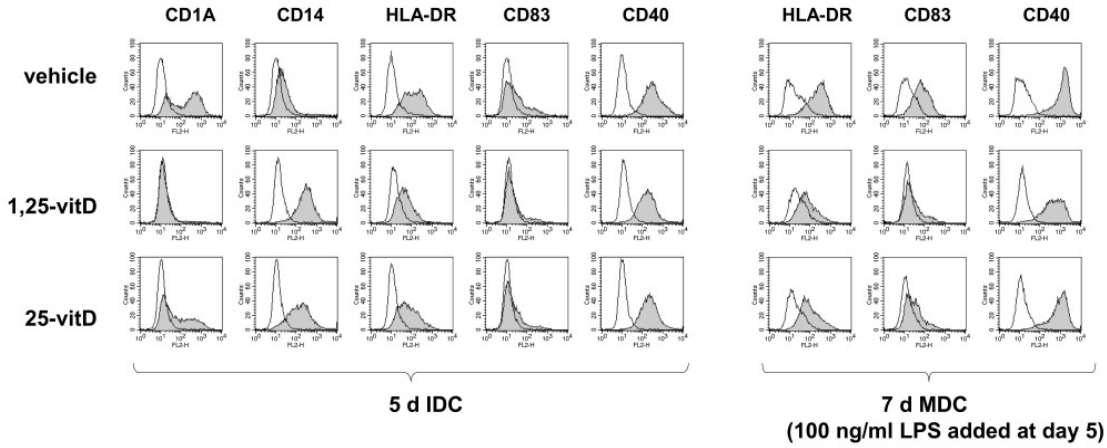

Figure 14. *Effects of 1,25-vitD and 25-vitD on differentiation and maturation markers. Monocytes were cultured with IL-4 and GM-CSF for 5 days in the presence of vehicle, 10 nM 1,25-vitD or 100 nM 25-vitD and cell surface expression of CD1A, CD14, HLA-DR, CD83 and CD40 was measured by flow cytometry. Expression of HLA-DR, CD83 and CD40 was also measured after 7 days in MDC, upon addition of 100 ng/ml LPS at day 5.*

We then investigated if CYP24A1, CCL22 and CD300LF are induced by not only 1,25-vitD, but also by 25-vitD treatment. As expected 25-vitD-treatment resulted in increased transcription of all three genes, showing that the cells converted 25-vitD in sufficient amount to induce these genes (Figure 15A). We also demonstrated by ELISA that transcriptional up-regulation of CCL22 in both 1,25-vitD and 25-vitD-treated cells results in a higher concentration of secreted CCL22 by day 5 (Figure 15B). Similarly, increased CD300LF transcription was manifested as increased cell surface expression of CD300LF on 1,25-vitD and 25-vitD-treated cells as determined by flow cytometry (Figure 15C). Collectively, these data showed that the endogenously produced 1,25-vitD appeared to be sufficient to regulate the identified program.

4.8. Identification of common targets of VDR and RARα

Previously we demonstrated that monocyte-derived DCs treated with PPARγ specific rosiglitazone and RARα specific synthetic agonist, AM580, exhibited several common

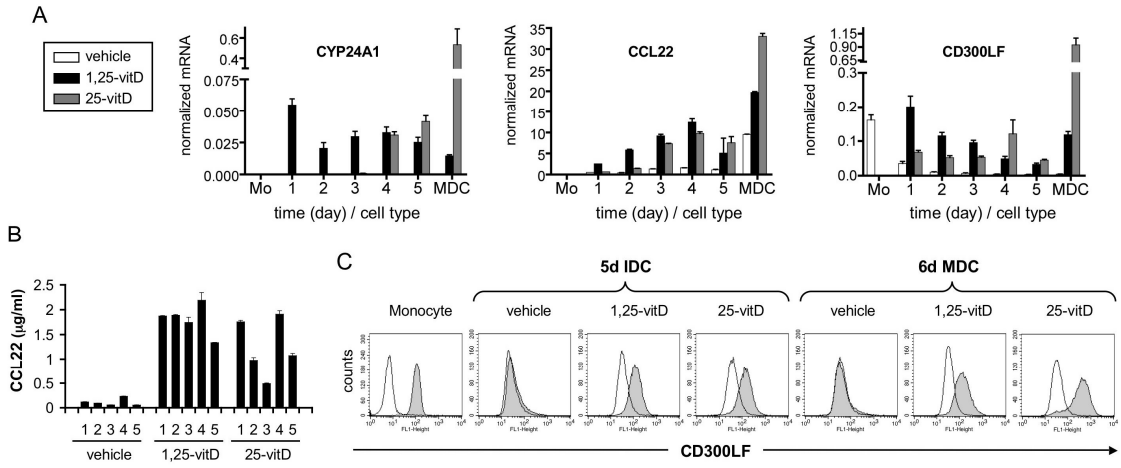

Figure 15. *Endogenously produced 1,25-vitD regulates the expression of CYP24A1, CCL22 and CD300LF. (A) Cells were cultured with 10nM 1,25-vitD or 100nM 25-vitD for various times. Mature DCs (MDC) were generated with a cocktail of proinflammatory cytokines. The expression of CYP24A1, CCL22 and CD300LF in differentiating, immature and mature DCs was determined by qPCR. (B) Elevated level of secreted CCL22 was detected by ELISA in supernatants of IDCs differentiated from monocytes obtained from 5 different donors after a 5-day 10 nM 1,25-vitD or 100 nM 25-vitD treatment. (C) Up-regulation of CD300LF (gray histograms) in the presence of 1,25-vitD and 25-vitD was confirmed by flow cytometry. An irrelevant isotype-matched immunoglobulin was used as a negative control (shown by white histograms). One representative experiment of four performed is shown.*

characteristics, e.g. similar regulation of the expression of transglutaminase 2 (TGM2), CD1A and CD1D and promotion of the expansion and activation of iNKT-cells (41). As we discussed in the Introduction, this phenomenon was partly due to the fact that PPARγ could instruct DCs to produce ATRA from its precursors by induction of the expression of genes involved in retinoic acid biosynthesis.

We also measured the expression of several genes regulated by 1,25-vitD to test whether these genes are also regulated by other nuclear receptor ligands. Interestingly, we found that several genes regulated by 1,25-vitD were also affected by AM580, a synthetic RARα agonist (e.g. transglutaminase 2, TGM2; fructose-1,6-bisphosphatase 1, FBP1; vascular endothelial growth factor, VEGF; and nuclear receptor interacting protein 1, NRIP1) (Figure 16.) This raised the question whether and how the programs initiated by RARα and VDR overlap.

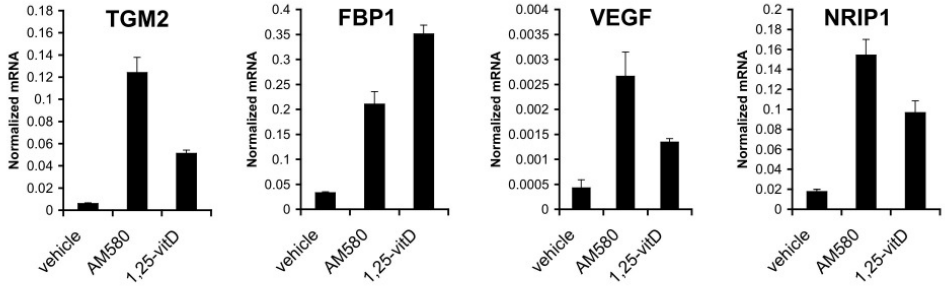

Figure 16. *1,25-vitD and RARα specific agonist, AM580 regulate the transcription of common targets. Monocytes were cultured in the presence of IL-4 and GM-CSF. Fourteen hours after setting up the culture the differentiating cells were treated with 10 nM 1,25-vitD, 100 nM AM580 or vehicle. The expression of transglutaminase 2 (TGM2), fructose-1,6-bisphosphatase 1 (FBP1), vascular endothelial growth factor (VEGF) and nuclear receptor interacting protein 1 (NRIP1) was determined by qPCR.*

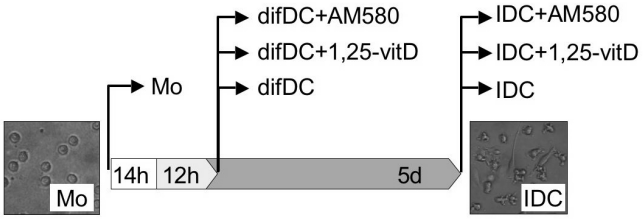

Figure 17. *Experimental setup of microarray analysis for identification of 1,25-vitD and AM580 common targets. Monocytes were cultured in the presence of IL-4 and GM-CSF. Fourteen hours after setting up the culture the differentiating cells were treated with 10 nM 1,25-vitD, 100 nM AM580 or vehicle for twelve hours or 5 days. The transcriptomes of monocytes, differentiating DCs (difDC) or 5-day-immature DCs (IDC) treated with 10 nM 1,25-vitD, 100 nM AM580 or vehicle were analyzed by Affymetrix microarrays.*

In fact, the experimental setup of our microarray analysis was more complex than it is illustrated in Figure 7. Differentiating DCs were treated, in addition to vehicle and 1,25-vitD, with AM580 (Figure 17). In this way we could identify the transcriptional program regulated by RARα parallel with the 1,25-vitD-regulated one.

For analysis of significantly regulated genes we filtered probe sets as described in materials and methods. For statistical analysis we used ANOVA t test. Regulated genes were identified in the samples treated with RARα and VDR specific ligands for 12 hours and 5 days.

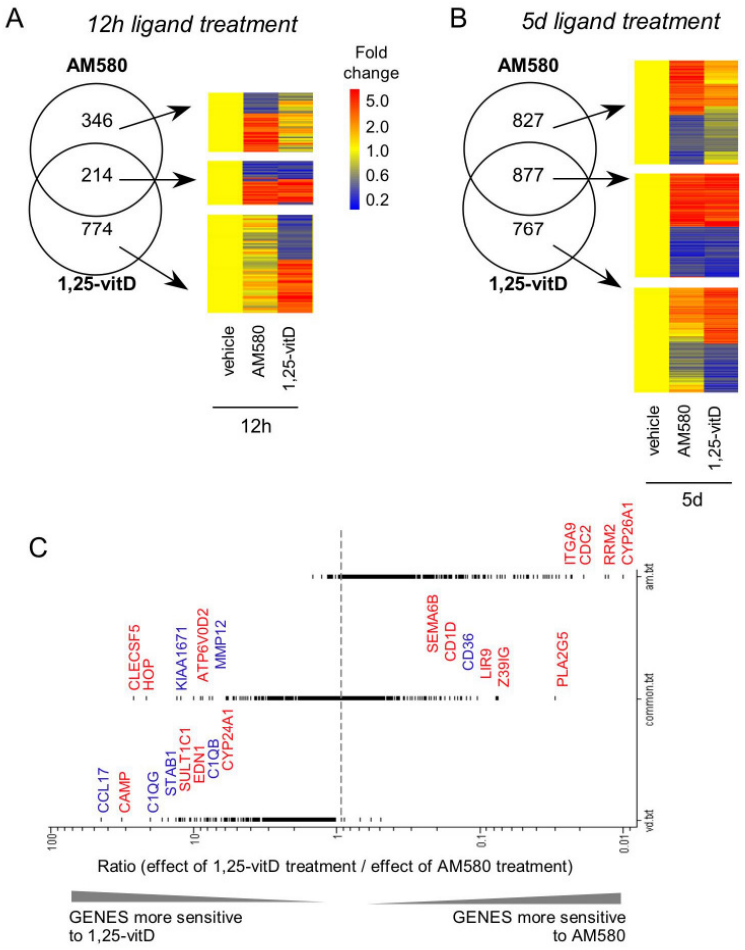

Figure 18. *The probe sets affected by 1,25-vitD and AM580 significantly overlap at both time points. (A, B) Probe sets regulated by 1,25-vitD-treatment and/or AM580-treatment in differentiating DCs (12h) and IDCs (5d) were identified and compared. The results are visualized as a Venn-diagram and heat maps. In heat maps columns represent expression profiles of mean of three independent donors for the two studied time points. Color intensities reflect the ratios of signal intensities as shown. (C) Genes regulated by 1,25-vitD and/or AM580 are indicated. The fold changes upon 1,25-vitD-treatment and AM50-teatment were divided and the ratios were plotted. Genes with high ratio values (bottom, on the left) are very sensitive to 1,25-vitD but not sensitive to AM580. Similarly, genes with low ratio values (top, on the right) are very sensitive to AM580 but not sensitive to 1,25-vitD. There are genes regulated by both agonists but they were much more sensitive to AM580 or 1,25-vitD (in the middle, on the right and on the left, respectively).*

When we tested the overlap of genes sensitive to the two ligands, we found that at day five ~50% of 1,25-vitD regulated genes were also regulated by AM580 and vice versa at day 5. At 12h the number of genes regulated by the two ligands was less (214), representing ~20% and ~38% of genes regulated by 1,25-vitD and AM580, respectively (Figure 18). We performed the *in silico* functional analysis of regulated

genes using Panther classification system. We found that, similarly to 1,25-vitD regulated genes, the immune function related genes were also overrepresented among AM580-sensitive genes (data not shown). When we tested the regulation of genes important for various DC functions we found that there are examples of common regulation from all tested categories (e.g. antigen uptake, chemokines, cytokines and their receptors as well as inhibitory receptors) (Table 3).

Function, Gene clusters	AM580 specific genes	Regulated by both ligands	1,25-vitD specific genes
Antigen uptake	CD209 (DC-SIGN) FCGR2A (CD32)	CD36 FCGR1A (CD64) FCGR2B (CD32) FCGR3A (CD16A)	ITGA5 ASGR2
HLA	-	HLA-DPA1 HLA-DRB3 HLA-DPB1	HLA-DMB HLA-DOA HLA-DPA1 HLA-DPB1 HLA-DQA1 HLA-DQB1
CD1	-	CD1A CD1D	CD1C CD1E
Costimulatory molecules	-	CD80	CD86
Chemokines/ chemokines receptors	CCL24 CXCL16	CCL18 CCL2 CCL23 CCL5 CCL7 IL8	CCR7 CCL13 CCL17 CCL22
Other cytokines/ cytokine receptor	IL27RA GDF15	CSF1 CSF2RB CSF3R IL6 IL18 IL6R TNFRSF21	CSF1 CSF2RA IL13RA1 IL6R IL7R INHBA SPP1
Inhibitory receptors	LAIR1 LILRB3 SIGLEC7	ACVR1B ILT8 LILRB1 LILRB2 LILRB3 LILRB4 NKIR	SIGLEC5 TLR8
MMP	MMP14	MMP12 MMP19	-
Other enzymes	DHRS3 CYP26A1 CYP27A1	TGM2 FBP1 ALOX5	CYP24A1

***Table 3**. Functional clusters of genes regulated significantly by 1,25-vitD and/or AM580 after 5-day-ligand treatment.*

5. DISCUSSION

Complex patterns of gene expression as determined by microarray analysis have been previously used to map interactions between biological processes. These investigations revealed new aspects in the regulation of immune functions by nuclear hormone receptors. Glucocorticoids acting through GR (glucocorticoid receptor) direct monocyte-derived macrophage differentiation towards an anti-inflammatory type macrophage (45). Likewise the primary role of PPARγ (peroxisome proliferator-activated receptor gamma) in regulating lipid metabolism was established in monocyte-derived DCs (42). Here we provide an analysis of the 1,25-vitD-induced changes in differentiating DCs and establish that this receptor regulates the tolerogenic program largely autonomously, e.g. independent of the differentiation and maturation.

5.1. An *ex vivo* model of *in vivo* DC development

The *in vivo* relevance 1,25-vitD signaling is clearly demonstrated by studies on $VDR^{-/-}$ mice (94). In this study we determined the transcriptional targets of 1,25-vitD in *ex vivo* differentiating primary human DCs. The combined treatment of $CD14^{+}$ monocytes with GM-CSF and IL-4 *in vitro* (30, 42, 43, 55, 58, 96) results in a non-proliferating and very homogenous population of cells, an ideal subject of transcriptome analysis. These cells have DC morphology and share functional characteristics for IDCs. Although a recent study documented that Langerhans cells arise from monocyte *in vivo* during inflammation (119), we need to acknowledge that it is not known to what extent *ex vivo* differentiation of monocyte-derived DCs recapitulates the *in vivo* differentiation of DCs. Nonetheless monocyte-derived DCs are successfully introduced in clinical studies (120, 121) underscoring the *in vivo* relevance of the cell type of our choice. Our model and experimental approach was further validated by the concordance of data from monocyte-derived DCs and blood myeloid DCs (Figure 11B).

A key issue in analyzing the activity of a nuclear hormone receptor is the source of the endogenous ligand. Previous studies documented that 1,25-vitD can be generated in DCs, particularly after maturation induced by LPS and other maturation stimuli (37, 38). We demonstrated here that 25-vitD is converted to 1,25-vitD even in

differentiating DCs and the produced ligand appeared to be sufficient to regulate the identified program (Figure 13, 14 and 15). Further studies are needed to clarify whether the polarization of DCs to a more tolerogenic direction by 1,25-vitD or its precursor(s) may occur during differentiation or it is restricted to the maturation phase *in vivo*.

5.2. 1,25-vitD treatment leads to the transcriptional regulation of many genes implicated in the tolerogenic phenotype of DCs

By using monocyte-derived DCs we determined the transcriptional targets of 1,25-vitD-treatment by microarrays. Our aim was not to characterize the potential targets in detail; this will be the subject of future studies. However, studying the 1,25-vitD-regulated gene set, we made two noteworthy observations. First, our microarray data suggest that up-regulation of target genes appears to be more prevalent for tolerogenic phenotype than was previously thought (Figure 19A). These changes include induction of inhibitory receptors and secreted cytokines and chemokines. This observation is consistent with a recent report (45) on the role of GR in macrophage differentiation, where the authors provided evidence to question the long-held theory that the immunosuppressive glucocorticoid action is primarily mediated by transrepression of inflammatory genes. Second, many 1,25-vitD regulated genes with similar functions appear to be coordinately controlled or co-regulated (e.g. antigen presentation, co-stimulation, cytokines and chemokines contributing to enhancement of regulatory T-cells, inhibitory receptors). Interestingly, some of these genes form clusters or are located on the same chromosomal region in the genome (e.g. MHC class II, CD1 and LILRB clusters) (Figure 19A). These data suggest that entire gene clusters or even large genomic regions may be co-regulated by 1,25-vitD-bound VDR. Deciphering the molecular background of this phenomenon requires further work.

5.3. 1,25-vitD initiates an autonomous transcriptional program in DCs

Previous studies documented that 1,25-vitD-treatment suppressed the induction of DC differentiation and maturation markers (CD1A, MHC class II molecules, CD83, co-stimulatory molecules, etc.) and suppressed the down-regulation of monocyte marker CD14 (58, 95-97). If 1,25-vitD mainly acted through the inhibition of the differentiation and the maturation program, it would most likely act through

suppressing/antagonizing the effect of transcription factors driving DC differentiation and maturation. In this way, the sets of genes regulated by differentiation and 1,25-vitD would overlap to a very large degree. Our data, however, do not support the scenario that the effect of 1,25-vitD is mostly restricted to the transcriptional regulation of "master transcription factors" or antagonism of transcription factors activated during maturation.

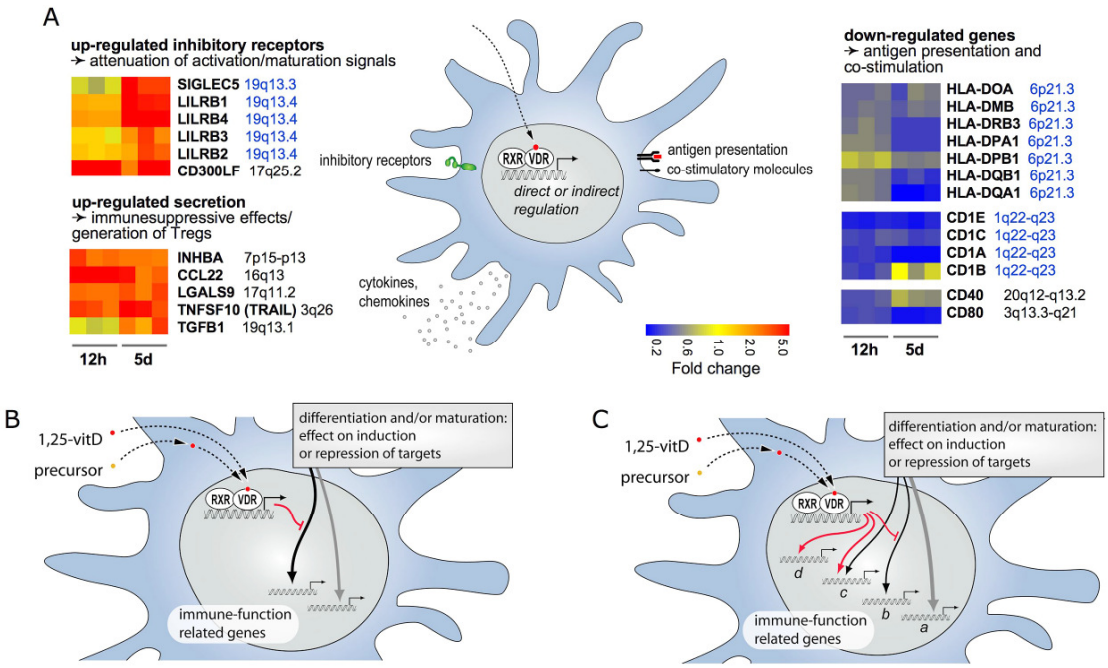

Figure 19. *The proposed model how exogenous or endogenously produced 1,25-vitD regulates gene expression in developing DCs. (A) 1,25-vitD regulated clusters of genes implicated in the tolerogenic phenotype (LILRB inhibitory receptors, HLA, CD1 molecules) identified by microarray analysis. The heatmaps show the expression levels of the indicated genes from three donors at 12 hours and 5 days. The chromosomal location of the genes are also indicated. The effect of 1,25-vitD is only partially mediated by suppression of target genes (e.g. HLA, CD1 and costimulatory molecules), and the induction of target genes (inhibitory receptors, chemokines and cytokines) is likely more important than previously thought to achieve the tolerogenic phenotype. (B) Previous studies investigated the 1,25-vitD-effect in developmental context, documented that 1,25-vitD leads to transcriptional changes that are opposite to those brought upon by differentiation and maturation. (C) Our data suggests a complex role for 1,25-vitD in inducing the tolerogenic phenotype. The majority of genes changing during differentiation are not a target of 1,25-vitD regulation (a). A fraction of genes is likely to be regulated, especially in maturing DCs, as suggested earlier via inhibition of other signaling pathways by controlling "master regulators", antagonizing the action of other transcription factors (b). A significant fraction of 1,25-vitD regulated genes appears to be regulated independently by 1,25-vitD and the differentiation program, even if the effect of 1,25-vitD and the differentiation programs are opposite (c). There are several 1,25-vitD regulated genes not affected during differentiation (d).*

Our comparative analysis of the transcriptomes of monocytes, differentiating DCs and IDCs differentiated in the presence or absence of 1,25-vitD suggest that the 1,25-vitD elicited transcriptional program is an autonomous one that runs parallel or as a module with the differentiation and/or maturation transcriptional program, as soon as cells become 1,25-vitD responsive and are exposed to the ligand. Several lines of evidence presented in this study support this claim. First, the set of genes regulated by differentiation, and by 1,25-vitD overlap only partially, e.g. there are many genes that are regulated by 1,25-vitD, but not the differentiation and vice versa (Figures 7E). If the effect of 1,25-vitD were mediated through the suppression of the differentiation program, we would expect that 1,25-vitD mostly regulate a subset of the genes regulated by the differentiation process. Our data did not support this hypothesis. Furthermore, this is not only true for the whole regulated gene set, but also for the "immunity and defense" subset (Figures 9A). Second, the set of genes regulated both by the differentiation and by 1,25-vitD are not necessarily regulated in an opposite manner. If 1,25-vitD acted through suppression of the differentiation program, it would regulate gene expression into the opposite direction than differentiation. A large fraction of the genes are indeed regulated in opposing directions by the two programs (e.g. CD1A, CD14) as predicted by the earlier results, but another significant fraction (Clusters 3 and 4, Figure 10B) did not comply with this rule. Third, the set of genes regulated in opposite directions by the differentiation and by 1,25-vitD (Clusters 1 and 6, Figure 10B) contains genes that can be autonomously regulated by 1,25-vitD. We showed that many genes that are up-regulated early by 1,25-vitD, are also up-regulated by late application of the hormone (Figure 11A). The developmental context therefore did not prove to be essential for most investigated genes. Very importantly, the differentiation independent regulation was also demonstrated in blood myeloid DCs for several genes (Figure 11B). A more detailed characterization of CD300LF, a gene oppositely regulated by differentiation and 1,25-vitD, showed that this gene is regulated similarly to CYP24A1 and CCL22 (Figure 12). Finally, 1,25-vitD is capable of initiating the 1,25-vitD dependent transcription program in the absence of maturation signals. There are several inflammatory stimuli, including LPS and many proinflammatory cytokines, which trigger DC maturation. 1,25-vitD has been reported to inhibit this maturation. 1,25-vitD is indeed documented to antagonize the

"inflammatory" transcription factors like NFATp/AP-1 and NF-κB, which results in inhibited expression of IL-2 (122) and IL-12 (123), respectively. Yet, in our system 1,25-vitD could regulate almost 200 "immunity and defense" genes (Figure 9A), including many previously identified targets (Figure 9B-C) in IDC, demonstrating that the 1,25-vitD induced transcriptional program can be initiated in the absence of (inflammatory) maturation. These arguments collectively imply a more complex role for 1,25-vitD in the regulation of transcriptional targets in DCs than previously thought (Figure 19B-C). According to our data, approximately 80% of probe sets representing genes playing a role in IDC differentiation is not a target of 1,25-vitD regulation and 40% of probe sets regulated by 1,25-vitD is not affected by differentiation (Figure 7E). A fraction of genes is likely regulated, especially in maturing DCs, as suggested earlier via inhibition of other signaling pathways. A significant fraction of genes is likely to be regulated independently by 1,25-vitD and the differentiation, even if the effect of 1,25-vitD and the differentiation program are opposite.

Several lines of evidence suggest that IDCs can give rise to distinct types of MDCs depending on stimuli from the environment and/or other cell types to become tolerogenic or immunogenic. Our interpretation of the presented data is that 1,25-vitD initiates an autonomous transcriptional program that is to a large part independent of differentiation and maturation. These findings also let us propose that the tolerogenic phenotype is the result of an active process and unlikely to be the consequence of the inhibition of differentiation and maturation. Thus an independent DC differentiation/maturation program could be complemented by either of two competing transcriptional programs, an immunogenic one initiated by TLR-receptors, proinflammatory cytokines or other immunogenic signals and a tolerogenic one initiated by tolerogenic signals, including 1,25-vitD or other immunosuppressive agents. This finding is providing further support to the recent shift in paradigm concerning tolerogenic and immunogenic DCs.

5.4. Potential mechanisms behind the common regulation by VDR and RARα

In our second study we aimed at investigating the overlap of regulated genes by VDR and RARα. We found that AM580 can regulate a large set of genes, which was regulated by 1,25-vitD. There are several mechanisms that may contribute to the

significant overlap of the genes sensitive to both ligands, e.g. the two receptors may regulate the same transcription factors, enzymes, etc. involved in the control of the differentiation program. Subsets of genes can also be regulated directly by RAR and VDR via hormone response elements. Not surprisingly, when we searched for genes meeting two criteria: (1) regulated in developing DC by both ligands and (2) having response elements for both receptors, we were not able to identify many genes. In fact, we found only one gene that fulfilled these two criteria, fructose-1,6-bisphosphatase 1 (FBP1). Interestingly, this gene is regulated by the same response element, meaning that VDRE=RARE for this gene. However, we could identify a series of genes, which were regulated by both ligands, but the response element was also characterized only for one receptor (Figure 20). Using the list of these genes we aimed at identifying whether these genes are regulated by both receptors using the same response element, similarly to FBP1. In the future we will perform transient transfection assays as well as band shift assays using the identified response elements.

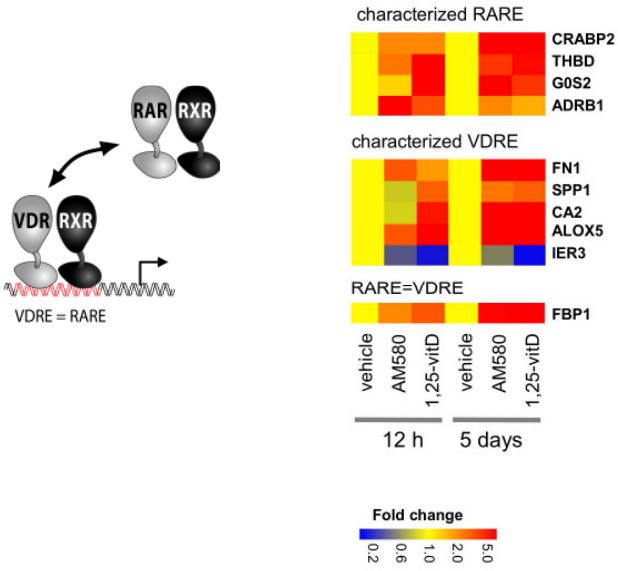

Figure 20. *Heat maps of genes with identified response elements and a proposed model how RARα and VDR regulate common targets are shown. In heat maps columns represent expression profiles of mean of three independent donors for the two studied time points. Color intensities reflect the ratios of signal intensities as shown. Response element of RAR (RARE) has been identified in the promoters of CRABP2, THBD, G0S2 and ADRB1. Similarly, response element of VDR (VDRE) has been characterized in the promoter of FN1, SPP1, CA2, ALOX5 and IER3. Both receptors can bind the response element of FBP1*

6. SUMMARY

Dendritic cells (DCs) are conductors of the adaptive immune system with a capacity to activate naive T-cells and regulate their functions. The integration of environmental signals will lead to at least two distinct, immunogenic and tolerogenic, DC immunophenotypes. How these stereotypic immunophenotypes are achieved at the transcriptional level is not well understood. A member of nuclear hormone receptor family, vitamin D receptor (VDR) is implicated in the development of tolerogenic DC phenotype. We have performed microarray studies to identify transcriptional programs regulated by vitamin D receptor (VDR), retinoic acid receptor α (RARα) and the differentiation process in developing DCs. Using these datasets we aimed at clarifying the connection of the VDR-coordinated program to the differentiation process as well as to the RARα-regulated transcriptional program. (1) Previous studies suggested that 1,25-dihydroxyvitamin D_3 (1,25-vitD) could inhibit the changes brought about by differentiation and maturation of DCs. However, it has not been explored how 1,25-vitD-regulated genes, particularly the ones bringing about the tolerogenic phenotype, are connected to differentiation. Using the global gene expression analysis followed by comprehensive quantitative PCR validation we could clarify the interrelationship between 1,25-vitD and differentiation-driven gene expression patterns in developing human monocyte-derived and blood myeloid DCs. We found that 1,25-vitD regulates a large set of genes that are not affected by differentiation. Interestingly, several genes, impacted both by the ligand and by differentiation, appear to be regulated by 1,25-vitD independently of the developmental context. Our data collectively suggest that exogenous or endogenously generated 1,25-vitD regulates a large set of its targets autonomously and not via inhibition of differentiation and maturation, leading to the previously characterized tolerogenic state. (2) In our ongoing study we have shown that RARα and VDR initiated similar phenotypic and functional changes. Using microarray analysis we have found that ~50% of 1,25-vitD regulated genes were also regulated by AM580 (RARα agonist) at the late stage of DC differentiation. We aim at identifying the potential molecular mechanisms that may be responsible for this phenomenon. Most importantly, we would like to identify target genes that are regulated by the same response elements of the two receptors.

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9. PUBLICATIONS AND FIRST AUTHORED POSTERS

This thesis is built on the following publications:

1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype.
Széles L, Keresztes G, Töröcsik D, Balajthy Z, Krenács L, Póliska S, Steinmeyer A, Zuegel U, Pruenster M, Rot A, Nagy L.
J Immunol. 2009 Feb 15;182(4):2074-83.

PPARgamma in immunity and inflammation: cell types and diseases.
Széles L, Töröcsik D, Nagy L.
Biochim Biophys Acta. 2007 Aug;1771(8):1014-30. Review.

VDR and RARα regulate similar subsets of genes in developing dendritic cells.
Széles L, Nagy L (in preparation)

Other publications:

At the crossroads of lipid metabolism and inflammation.
The role of PPARgamma, a lipid-activated transcription factor.
Széles L, Töröcsik D, Nagy L.
B. I. F. Futura (Boehringer Ingelheim Fonds) 2006, 21:79-85. Review.

Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells.
Szatmari I, Vámosi G, Brazda P, Balint BL, Benko S, **Széles L**, Jeney V, Ozvegy-Laczka C, Szántó A, Barta E, Balla J, Sarkadi B, Nagy L.
J Biol Chem. 2006 Aug 18;281(33):23812-23.

First authored posters on the following international meetings:

Széles L, Szatmári I, and Nagy L: Identification of target genes of PPAR nuclear receptor isotypes. Affymetrix User Group Meeting, Geneve, Switzerland, May 24-26, 2004.

Széles L, Szatmári I, and Nagy L: Nuclear receptors and ligand-dependent repression. EMBO-HHMI Joint meeting, Budapest, Hungary, February 7-9, 2005.

Széles L, Szatmári I, and Nagy L: Nuclear Receptors and ligand-dependent repression. 30th FEBS Congress, Budapest, Hungary, July 2-7, 2005.

Széles L and Nagy L: Nuclear Receptors and ligand-dependent repression. EMBO conference, Villa Alba, Gardone Riviera, Lake Garda, Italy, September 29 - October 1, 2005.

Széles L and Nagy L: Nuclear receptors and ligand-dependent repression. 9th International Conference, DENDRITIC CELLS – 2006, Edinburgh, UK, September 16-20, 2006.

Széles L, Szatmári I, and Nagy L: Investigation of the role of vitamin D receptor and retinoic acid receptor in human monocyte-derived dendritic cells. Spetses Summer School on Nuclear Receptor Signalling: From Molecular Mechanisms to Integrative Physiology, Island of Spetses, Greece, August 26 - 31, 2007.