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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The Examination takes place at
The Department of Medical Chemistry
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INTRODUCTION

Dendritic cells (DCs)

DCs are professional antigen presenting cells, which have the superior ability to stimulate naive T-cells and regulate their functions. DCs are heterogeneous and subtypes differ in location, migratory pathways and immunological function. 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. The migratory DCs in their immature state can be found at body surfaces like in the skin, pharynx, upper esophagus, vagina and anus, and at mucosal surfaces, such as the respiratory and gastrointestinal system. They sense and translate environmental cues by sampling and processing antigens of dying cells and various pathogens. DCs are able to take up antigens via various mechanisms, such as phagocytosis, macropinocytosis and endocytosis. Antigen uptake and maturation signals (e.g. pro-inflammatory cytokines and exogenous microbial products) trigger maturation of DCs. Maturation is a complex process, which includes phenotypic and functional changes. 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. T-cell stimulation and Th1/Th2 polarization are thought to be dependent mainly on three types of DC-derived signals. “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 that promote the development of

Th1 and Th2 cells, respectively. The antigen-presentation of DCs 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. Besides inducing immune responses, DCs could also provoke immunological tolerance. 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.

Nuclear receptors

Nuclear receptors, such as estrogen receptor (ER), peroxisome proliferators-activated receptor (PPAR), glucocorticoid receptor (GR), vitamin D receptor (VDR) and retinoic acid receptor (RAR) are ligand-activated transcription factors that have diverse roles in regulating developmental, homeostatic, metabolic, inflammatory and immune processes. The human genome contains 48 members of this family, and many of them are indispensable for life. Ligands for these receptors are steroids, cholesterol, fatty acids, and fat-soluble vitamins. 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. 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 and thyroid hormone 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, liver X receptor (LXR), PPAR and RXR) in DCs.

The role of nuclear receptors in DC biology

A growing body of evidence suggests that nuclear receptors play an important role in regulation of DC development and functions. GR, VDR and RAR were among the first nuclear receptors for which effects on DCs were documented. We and others also investigated and identified important aspects of other receptors, PPARs, LXRs and RXR, in DC biology. 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. 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. These active metabolites may be involved in autocrine regulation of DCs under certain circumstances. Remarkably, all-trans retinoic acid and 1,25-dihydroxyvitamin D_3 released by DCs contribute to T-cell tropism by regulating integrins and chemokine receptors expressed by T-cells. Interestingly, several functions and pathways are regulated similarly by various nuclear receptors: e.g. differentiation markers (down-modulation of CD1A and up-regulation of CD14 by agonists of GR,

PPAR, VDR and RAR), antigen uptake (enhanced by agonists of PPAR, VDR, RAR and GR), and capacity of T-cell activation (down-modulated by agonists of VDR, GR and LXR). Moreover, our microarray studies also indicated that the transcriptional targets of these nuclear receptors (PPAR vs. RAR and VDR vs. RAR) significantly overlap. Our research group elucidated some aspects of PPAR and RAR crosstalk in DCs, and now we are investigating the molecular mechanisms, which may be behind the overlap between target genes of VDR and RAR.

The role of 1,25-dihydroxyvitamin D_3 (1,25-vitD) in the immune system and DC biology

The influence of 1,25-vitD in the immune system has been known for 20 years. 1,25-vitD inhibits T-cell proliferation, down-modulates the expression of IL-2, IFNγ, and CD8+ T-cell mediated cytotoxicity, while IL-4 production is enhanced. The net result of 1,25-vitD actions on T-cells is to block the induction of Th1 cell and Th17 responses, while promoting Th2 cell and Treg responses. In addition to its inhibitory effects on T-cells, 1,25-vitD decreases B-cell proliferation, plasma cell differentiation and IgG secretion. Interestingly, some of 1,25-vitD effects on monocytes/macrophages are stimulatory: 1,25-vitD can induce the proliferation of human monocytes in vitro, and increase the production of IL-1. TLR activation of human macrophages up-regulates the expression of VDR and the vitamin D-1α hydroxylase genes, leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular Mycobacterium tuberculosis.

A number of studies 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. 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. The effect of 1,25-vitD on inhibiting the maturation of DCs was dependent on VDR. 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. In addition animal studies demonstrated that treatment with 1,25-vitD arrests the development of autoimmune diabetes and mediates tolerance to transplants, supporting a potential pharmacological application for this hormone or its analogs.

We aimed at investigating the role of 1,25-vitD on the DCs in a developmental context. The ways how immunogenic and tolerogenic DCs develop and the mechanisms immunsuppressive drugs can modify the function, differentiation and maturation of DCs are central issues 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 events. Determining whether 1,25-vitD acts via inhibition of immunogenic mechanisms or acts autonomously is an important issue. In particular the interrelationship between the complex processes of differentiation and development of tolerance at the transcriptional level is not clear. This prompted us to investigate the impact of 1,25-vitD treatment on the transcriptome of differentiating DCs. Our studies suggested that ligand-bound VDR acts to a very large degree autonomously, independent of the transcriptional changes dictated by the differentiation and maturation program, leading to a distinct tolerogenic phenotype.

MATERIALS AND METHODS

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 3.5×10^5 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.

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. For activation of RARα receptor, synthetic agonist,

AM580 was used at 100 nM.

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.

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).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qPCR) was conducted using TaqMan probes (Applied Biosystems). 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.

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.

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.

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).

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).

Immunohistochemistry

Cells were collected by centrifugation, fixed in buffered formalin, and embedded into paraffin. Sections (5 µm) were immunostained with anti-human 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.

RESULTS

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 in which we and others have previously mapped nuclear hormone receptor mediated transcriptional events. 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. 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. The VDR protein was located in the nuclei of IDCs as determined by immunohistochemistry. We thus demonstrated that VDR is expressed rapidly and at high levels in differentiating DCs.

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 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 that the differentiation of myeloid cells from precursors will lead to differential expression of several thousand genes. 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. 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. 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. These results thus strongly suggest that 1,25-vitD should not be simply viewed as a general and global inhibitor of differentiation.

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. 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. Importantly, this category was significantly overrepresented among the functional classes at both time points. We chose two approaches to

validate these findings. First, we selected a set of genes previously reported 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 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 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. 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. 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.

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. This comparison proved that 1,25-vitD and differentiation regulated "immunity

and defense” gene sets only partially overlap, similarly to the entire gene sets. 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 time points. The majority of genes 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. In addition, many 1,25-vitD-regulated genes were not affected by differentiation at all, providing evidence to suggest that the differentiation program and the 1,25-vitD-induced program are indeed, non-overlaping.

Many early “immunity and defense” genes are autonomously regulated in differentiating DCs and blood myeloid DCs

Interestingly, the expression profile of ALOX5 suggested that genes showing opposite regulation during differentiation and upon 1,25-vitD-treatment may also be regulated independent of differentiation. The fact that ALOX5 (arachidonate 5-lipoxygenase) is a direct VDR target 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 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, 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 dependent of differentiation 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, providing further evidence that many genes are regulated by 1,25-vitD-treatment independent of differentiation signals.

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 of VDR), chemokine ligand 22 (CCL22, a chemokine attracting regulatory T-cells) and CD300LF (an inhibitory receptor, 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. 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. 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, and could show that treatment with ZK159222 has significantly repressed the 1,25-vitD-elicited transcriptional response of all three genes. 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. 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. However, CCL22 was not induced significantly in MDCs probably due to its already high expression level.

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

The physiological serum levels of 1,25-vitD (~ 40-130 pM) are unlikely to be sufficient to turn on 1,25-vitD signaling in DCs. However, previous studies revealed that 1,25-vitD can be generated endogenously. We therefore sought to determine whether and when the >1,000 times more abundant 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. We therefore investigated the expression pattern of CYP27B1 by qPCR and found that it closely matched that of the VDR. 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. Maturation of DCs is induced by many different stimuli, 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, we found that proinflammatory cytokines and TLR-ligands proved to be potent activators of CYP27B1 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. 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. We could detect increasing amounts of 1,25-vitD in differentiating DCs. Consistent with a previous report 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. 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. 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. 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. Collectively, these data showed that the endogenously produced 1,25-vitD appeared to be sufficient to regulate the identified program.

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 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. 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 (e.g. TGM2; fructose-1,6-bisphosphatase 1, FBP1; vascular endothelial growth factor, VEGF; and nuclear receptor interacting protein 1, NRIP1). This raised the question whether and how the programs initiated by RARα and VDR overlap.

In our microarray analysis, differentiating DCs were treated, in addition to vehicle and 1,25-vitD, with AM580. 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. When we tested the overlap of genes sensitive to the two ligands at day 5, we found that ~50% of 1,25-vitD regulated genes were also regulated by AM580 and vice versa. 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. 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. 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).

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 direct monocyte-derived macrophage differentiation towards an anti-inflammatory type macrophage. Likewise the primary role of PPARγ in regulating lipid metabolism was established in monocyte-derived DCs. 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.

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. 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* 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, 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 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.

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. 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. 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.

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 it was previously thought. These changes include induction of inhibitory receptors and secreted cytokines and chemokines. This observation is consistent with a recent report 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 as 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). 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.

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. 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.

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 supports 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. If the effect of 1,25-vitD

were mediated through the suppression of the differentiation program, we would expect that 1,25-vitD mostly regulates 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.

Second, the set of genes regulated both by the differentiation and by 1,25-vitD are not necessarily regulated in 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 did not comply with this rule.

Third, the set of genes regulated in opposite direction by the differentiation and by 1,25-vitD 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. 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. 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. 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 NFAT/AP-1 and NF-κB, which results in inhibited expression of IL-2 and IL-12, respectively. Yet, in our system 1,25-vitD could regulate almost

200 “immunity and defense” genes, including many previously identified targets 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. 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. 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.

Potential mechanisms behind the common regulation by VDR and RARα

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 RARα and VDR via hormone response elements. Unfortunately, very few direct target genes have been identified for both receptors. 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 characterized only for one receptor. 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.

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 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.

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.
IF: 6.068

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.
IF: 3.539

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.
IF: 5.808

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.

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