Hormones & Vitamins

Orchestrators of Dendritic Cells and Cancer
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Orchestrators of Dendritic Cells and Cancer

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann, volgens besluit van het college van decanen in het openbaar te verdedigen op woensdag 8 januari 2014 om 14.30 uur precies

doors

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Part 1

Introduction
Chapter 1

General Introduction

Partially adapted from:
Ansems, M., Hontelez, S., Karthaus, N., Span, P.N. & Adema, G.J.
Crosstalk and DC-SCRIPT: expanding nuclear receptor modulation
Biochimica et biophysica acta 1806, 193-199 (2010)

Karthaus N., Torensma R., Tel J.
Deciphering the message broadcast by tumor-infiltrating dendritic cells
The American journal of pathology 181, 733-742 (2012)
Hormones and vitamins – orchestrators of dendritic cells and cancer

Hormones and vitamins
Hormones and vitamins are micro-environmental factors that orchestrate the function of cells through downstream signaling via their respective receptors. Hormones are small molecule messengers produced by specialized organs called endocrine glands. The major glands include the sex organs, pancreas, adrenal glands, pituitary glands and thyroid glands each of which secretes a specific repertoire of hormones. In addition to these dedicated organs, hormones are secreted by virtually every cell of the body. Hormones comprise an important class of signaling molecules and play key roles in a variety of physiological processes ranging from growth and reproduction over metabolism to immune responses. Consequently, hormonal malfunctions can lead to a disturbed homeostasis and development of pathologic conditions such as osteoporosis, infertility, diabetes and immune disorders.\textsuperscript{1-4}

Eat your vegetables! A sentence that sets you back to your childhood and that is associated with the question: Why? Vitamins are vital nutrients by definition, essential organic compounds that cannot be synthesized in adequate amounts by an organism and thus must be acquired externally from the diet.\textsuperscript{5} All 13 human vitamins can be obtained from vegetable sources with the exception of vitamin B\textsubscript{12}, which is only present in animal products. As vitamins are essential nutrients, lack thereof is associated with a variety of pathologic conditions affecting organ functions, the nervous and immune systems. Education on natural food sources and chemical supplementation with vitamins has diminished the prevalence of associated diseases. However, even nowadays common deficiencies include Vitamin A and Vitamin D, accounting for mortality, blindness and demineralization of bones, respectively.\textsuperscript{6-11}

Figure 1: Involvement of NRs in physiological functions. Nuclear Receptors (NR) and their ligands (hormones and vitamins) are implicated in a variety of physiological processes and related diseases; i.e. metabolism, nervous system, reproductive system, endocrine system, cell division, immune system and cardiovascular system. Three to four prominent examples of involved NRs are shown per process. Abbreviations see table 1.
The Nuclear Receptor Superfamily

Most hormones and vitamins are ligands for the Nuclear Receptor (NR) superfamily of transcriptional regulators. NRs encompass a unique class of ligand activated transcriptional regulators which is phylogenetically conserved among the metazoan kingdom. Individual gene counts, however, differ a lot between species. The human genome contains 48 genes, similar to the murine genome with 50 genes (Table 1); whereas the genome of Caenorhabditis elegans comprises approximately 270 NR genes. In line with their hormone and vitamin ligands, NRs are key-regulators in a diversity of physiological functions, including reproduction, development, homeostasis, metabolism, cell differentiation and immune responses (Figure 1). Malfunction of receptor mediated signaling therefore is associated with diseases like infertility, diabetes, chronic inflammatory diseases and cancer.

The cloning of the first NR in 1958, the glucocorticoid receptor (GR), was the key to the identification of the NR superfamily. All family members share a common modular organization which allowed for the prediction of new receptors based on their homology. NRs are typically composed of 3 main functional domains, the amino-terminal activation function (AF1) domain, the DNA binding domain (DBD) and the carboxy-terminal ligand binding domain (LBD) (Figure 2A). They are generally divided into three main subclasses. Type I NRs comprise the steroid receptors, including the Estrogen Receptor (ER), Progesterone Receptor (PR), Androgen Receptor (AR) and Glucocorticoid Receptor (GR) (Table 1). Steroid receptors are classically sequestered in the cytoplasm of cells by binding to heat shock proteins. Ligand binding induces dissociation of the chaperone complexes and translocation to the nucleus where the receptors can bind to specific DNA sequences. Binding to these DNA response elements will lead to recruitment of coactivator complexes ultimately resulting in transcriptional activation of the target gene. Type II NRs encompass the Retinoid X Receptor (RXR) heterodimers. Well-known members of this class are the Retinoic Acid Receptor (RAR), peroxisome proliferator-activated receptors (PPAR) and the Vitamin D Receptor (VDR) (Table 1). In contrast to type I NRs, type II receptors generally reside in the nucleus in the absence of ligand. They bind to their response elements and are often in complex with corepressor proteins. Upon ligand binding, conformational changes occur, corepressor proteins are released, coactivators are recruited and transcription is initiated. An additional subfamily are the so called orphan receptors, including the RAR related orphan receptors (ROAR) and the NR4A family members Nur77 (growth factor-inducible immediate early gene nur77 like receptor), NURR1 (NR-related protein 1) and NOR1 (Neuron-derived orphan receptor 1) (Table 1). These receptors have been identified according to sequence homologies and could not be linked to any natural ligand, yet. Orphan receptors often bind DNA as monomers, but are known to dimerize as well. Some of these receptors are known to be constitutively active, whereas induced transcriptional activation may be mediated by yet unidentified natural ligands, changes in expression patterns or alternatively by posttranslational modifications. Phosphorylation is known to target all NRs and alter their transcriptional activity subsequent to ligand stimulation. Such modifications might directly regulate transcriptional activity of orphan receptors, as they potentially miss natural ligands.

The regulation of cellular functions by hormones, vitamins and NRs is not restricted to the described classical mechanisms of transcription repression and activation (Figure 2B). Many NR ligands have been shown to exert non-genomic functions, i.e. rapid activation of plasma membrane ion channels, adenylate cyclase (AC), protein kinases A and C (PKA, PKC), Src tyrosine kinase, phosphatidyl-inositol-3-kinase (PI3K), extracellular-regulated kinase (ERK), p38 mitogen-activated kinase (p38MAPK), c-Jun N-terminal kinase (JNK), phospholipases C and D (PLC, PLD) and several phosphatases (PP1,
The molecular mechanisms underlying these non-genomic functions of NR ligands are still controversial and might be explained by two different theories. On the one hand, studies have shown the existence of membrane bound steroid receptors, like ER and PR. Other steroid hormone receptors such as GR and AR are likely to associate with the membrane too, as ER and PR membrane bound forms most probably depend on palmitoylation, which also occurs in GR and AR. On the other hand, rapid non-genomic actions of NR ligands also occur in NR deficient cells suggesting the existence of non-classical, alternative receptors. In line with this theory, thyroid hormones have been shown to signal through integrins and estrogens are thought to activate G protein coupled oestrogen receptor 1 (GPER1/GPR30). Current literature on both theories, however, provides conflicting data; especially regarding the membrane bound PRs and GPER1/GPR30. Dedicated studies to elucidate the molecular mechanisms of the non-genomic actions of NRs are thus needed and will probably uncover tissue and cell specific receptor pathways.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Family member</th>
<th>Receptor name</th>
<th>NR subclass</th>
<th>Natural Ligand (synthetic agonist)</th>
<th>Human (48)</th>
<th>Mouse (50)</th>
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<td>DAX1</td>
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<td>NR1A1</td>
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<td>x</td>
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<td>x</td>
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<td>x</td>
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<td>x</td>
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<td>x</td>
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<td>Vitamin A (LGD 100268, GW0791)</td>
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<td>NR6A1</td>
<td>Germ cell nuclear factor 1</td>
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X, expressed; NA, not available
**Nuclear Receptor coregulators**

NR activity is critically dependent on the presence of coregulatory proteins that mediate their transcriptional function. More than 300 of these molecules have been described until now, displaying an enormous diversity and establishing a complex regulatory network.\(^\text{39}\) NR coregulators are classically divided into coactivators and corepressors.\(^\text{40}\) Coactivators bind to NRs in a ligand dependent manner through an α-helical motif with a LXXLL consensus sequence.\(^\text{41}\) Transcription initiation by coactivator complexes is generally mediated by the recruitment of histone acetyltransferases (HAT), which cause local acetylation, followed by chromatin decondensation and enhanced promoter accessibility. Corepressors on the other hand do generally interact with NRs in a ligand independent manner and they comprise the conserved negative motif LXXI/HIXXXI/L. Inhibition of transcription by corepressors is mainly mediated by histone deacetylases (HDAC) through local acetylation, condensation of chromatin and hindered entry of e.g. polymerases. In addition to the characteristic coactivators and corepressors, a few versatile coregulators have been identified that were shown to both enhance and dampen NR mediated transcription; these include RIP140, PELP1 and DC-SCRIPT.\(^\text{42-45}\)

DC-SCRIPT (dendritic cell-specific transcript), also known as ZNF366, was first described in 2006 and recently shown to act as a coregulator of multiple NRs.\(^\text{44-48}\) DC-SCRIPT is a highly conserved protein and originally identified in a unique immune cell subset, the antigen presenting dendritic cell (DC). The DC-SCRIPT protein consists of an N-terminal proline-rich region, 11 Cys2His2-type zinc fingers and a C-terminal acidic region. Beyond its zinc fingers, it shares no homology with proteins alike and has a restricted expression pattern, including epithelial cells.\(^\text{45}\) Remarkably, it was shown that DC-SCRIPT could regulate the activity of several subclasses of NRs. DC-SCRIPT overexpression inhibited the activity of the type I NRs ER and PR, whereas, it enhanced the transcriptional activities of the type II NRs RAR/RXR and PPAR/RXR.\(^\text{45}\)

**Figure 2: Nuclear receptor structure and transcriptional regulation.** (A) Domain organization of NRs. The activation function 1 (AF1) domain is involved in constitutive transcriptional activation of NRs. The DNA binding domain (DBD) mediates binding to NR specific genomic response elements. The ligand binding domain (LBD) binds ligand, is involved in dimerization and contains activation function 2, which mediates ligand dependent transcriptional activation and repression. (B) NRs classically regulate gene expression via DNA binding and recruitment of coactivator or corepressor complexes. However, NRs and their ligands also exert non-genomic functions through activation of protein kinases A and C (PKA, PKC), phosphatidyl-inositol-3-kinase (PI3K), Src tyrosine kinase, extracellular-regulated kinase (ERK), mitogen-activated kinases (MAPK), c-Jun N-terminal kinase (JNK), phospholipases C and D (PLC, PLD) and phosphatases (PP1, PP2A). It is still elusive whether these functions are mediated by membrane bound NRs or alternative receptors binding NR ligands.
Chapter 1 - General Introduction

In summary, NRs comprise a large superfamily of ligand activated transcription factors involved in diverse cellular processes. Therefore, NRs and their coregulators play pivotal roles within physiological and pathological functions including cancer- and immunobiology, which will be highlighted in the following paragraphs.

Cancer
Cancer comprises a heterogeneous collection of pathogenic conditions marked by the unregulated proliferation of cells. Its development follows six distinct hallmarks: the self-production of growth hormones, imperviousness to growth inhibitory signals, avoidance of apoptosis, unlimited proliferation, sustained angiogenesis and metastatic capacity. Recently, two additional emerging hallmarks have been added to the list: the reprogramming of energy metabolism and immunosurveillance evasion. Generally, cancer growth is either benign (non-aggressive) or malignant and thus invasive. Moreover, five major cancer categories are recognized based on the histological origin of the malignant cells: sarcoma, myeloma, leukemia, lymphoma and carcinoma. The latter arises from epithelial cells and constitutes 80-90% of all cancer cases. A substantial part of carcinomas is associated with the endocrine system, as hormones and vitamins are important factors in the homeostasis of cell proliferation.

Hormones and vitamins in cancer biology
Malignant cells maintain, transform and utilize the characteristics of their progenitor cells. This concept is well established for carcinomas of glandular organs like liver, pancreatic, ovarian, testicular, prostate and breast cancer, all of which are highly dependent on the presence of steroid hormones, an essential feature of their ancestor cells. Two well studied hormone dependent malignancies are breast and prostate cancer.

Early mammary gland development and cyclic proliferation of mammary duct cells are driven by the steroid hormones estrogen and progesterone. Both hormones are also well established key factors in the development of breast cancer. 70% of breast tumors express stable or elevated levels of the ERα and PR. Estrogen and progesterone augment proliferation of breast tumor cells by inducing the transition from G1- to S-phase. This process is dependent on the hyperactivation of the cycle-related genes c-myc and cyclin D1 (CCND1). Consequently, current therapy regimens include the ER antagonist tamoxifen and aromatase inhibitors, which block the production of estrogens. In line with the importance of the ERα and PR in breast cancer, the expression of transcriptional coregulators of both receptors is also of prognostic significance in breast cancer. In contrast to the progressive role of ERα and PR in breast cancer, RAR and PPARγ are typically referred to as tumor suppressive. Stimulation of RAR with retinoic acid (vitamin A) in breast cancer cells triggers cell cycle arrest in the G1 phase through induction of the cell cycle regulator Btg-2 and reduced expression of cyclins D1 and D3. Activation of PPARγ inhibits cell proliferation of breast cancer cells and promotes apoptosis. Recently, the versatile NR coregulator DC-SCRIPT was shown to dampen the activity of the type I NRs ERα and PR, positive regulators of breast carcinogenesis, whereas it enhanced the activity of the type II NRs RAR and PPARγ, negative regulators of breast tumor development. Moreover, DC-SCRIPT expression positively correlated with disease free survival and was shown to be an independent prognostic factor in breast cancer.

Similar to mammary glands which are dependent on the steroids estrogen and progesterone, the development of the prostate and its proper function are critically dependent on androgens. Moreover, this hormone is an important factor in the development and sustained growth of prostate
cancer and the vast majority of tumors express the AR. Activation of AR in prostate cancer cells inhibits the expression of the cell cycle inhibitor p16 and induces the expression of the cyclin dependent kinases 2 and 4 (CDK2 and CDK4). Therefore, hormonal treatment modalities for prostate carcinoma comprise AR blocking agents and androgen deprivation. Although almost all prostate cancer patients initially respond to anti androgen therapy, most patients will transit into the hormone-refractory stage characterized by androgen independent tumor growth. Besides the involvement of the receptor itself, coregulatory proteins of the AR have also been implicated in prostate cancer. While AR is important in the initiation and progression of prostate cancer another NR, the VDR, has recently been shown to hamper prostate cancer growth. Stimulation of prostate cancer cells with Vitamin D inhibits the transition from $G_1$ into S phase by increased expression of the cell cycle repressor p27Kip1, reduced expression of c-myc and impaired activity of CDK2.

Better understanding of the roles of hormones, vitamins, NRs and their coregulators in hormone dependent cancers, as breast and prostate carcinomas, might stimulate the development of novel treatment modalities. Given the multifunctional regulatory effect of DC-SCRIPT on type I and type II NRs, it is interesting to speculate about the prognostic significance of DC-SCRIPT in prostate carcinoma and other hormone dependent cancers.

**Immune System**

The equilibrium of tolerance and immunity is constantly perturbed by influences from outside and inside the human body like viruses, bacteria and malignant cell formation. Our immune system comprises various regulatory mechanisms on a cellular and molecular level that buffer these perturbations to warrant a proper physiological balance. Generally, the immune system can be divided into the innate and adaptive arm. The innate immune arm constitutes the primary response system which works in a non-specific manner without the need of prior exposure to a certain pathogen.

Innate immune cells comprise granulocytes (eosinophil, basophil and neutrophil), mast cells, macrophages, natural killer (NK) cells, macrophages and dendritic cells (DC). These cells represent the first line of defense; they exert two main functions: 1) direct killing of pathogens and tumor cells 2) phagocytosis of pathogenic material and intracellular break down thereof into peptides. Immune activation of innate cells is mediated by the recognition of pathogen associated molecular patterns (PAMP) through pattern recognition receptors (PRR). Four different classes of PRRs are known based on protein homologies, signaling motifs and localization. The toll like receptor (TLR) family comprises transmembrane receptors located at the plasma membrane or in endosomal compartments. TLRs recognize a variety of ligands like RNA, DNA, bacterial carbohydrates and bacterial peptides. The second class of PRRs are the nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). NLRs are located in the cytoplasm and detect PAMPs of intracellular pathogens. C-type lectin receptors (CLR) form the third family of PRRs; they cover recognition of most human pathogens. Most CLRs are endocytic receptors which bind conserved carbohydrate residues. The last class of PRRs constitute the retinoic acid-inducible gene (RIG)-I-like receptors (RLR). These cytosolic receptors sense intracellular viral replication through binding of viral nucleic acids.

The adaptive immune system is characterized by pathogen specific responses mediated by B and T lymphocytes. The development of adaptive immune reactions takes significantly longer (4 to 7 days) than the initiation of the rapid innate reactions (minutes to hours). However, the adaptive immune system is able to build memory responses after the initial encounter with a specific pathogen, providing a more rapid reaction to subsequent infections. B cells are activated through recognition of
pathogen specific antigens via the B cell receptor (BCR) and subsequently differentiate into plasma cells, characterized by the secretion of pathogen specific antibodies (AB). The binding of pathogens by ABs triggers the activation of the complement system, an innate protein cascade facilitating the uptake of pathogens by immune cells and mediating the direct killing of pathogens. Moreover, binding of AB to pathogens activates mast cells, NK cells and neutrophils. The second effector cells of the adaptive immune system, T cells, are stimulated through their specific T cell receptor (TCR). Antigen presenting cells (APC), i.e. B cells, macrophages and DCs, display the TCR matching antigen in major histocompatibility complex (MHC) molecules on their surface, provide appropriate co-stimulation and secrete pro-inflammatory cytokines to activate T cells. Three different subsets of T cells are generally distinguished: 1) CD4$^+$ T helper (Th) cells 2) CD8$^+$ cytotoxic T cells (CTL) and 3) CD4$^+$CD25$^+$ regulatory T cells (T reg). Naïve Th cells are activated through presentation of antigen in the context of MHC class II molecules on APCs and subsequently differentiate into different subsets, i.e. Th1, Th2, and Th17. Th1 cells mainly produce the cytokine interferon γ (IFNγ) thereby assisting CTL responses and macrophage activation. Th2 cells support the AB production by B cells through secretion of interleukins (IL) 4 and 5. Th17 cells are characterized by their secretion of IL17 and thought to be involved in protection from viral, bacterial and fungal infections. Naïve CTLs, the second T cell subset, are activated through antigen bound MHC class I molecules on the surface of APCs. Activated CTLs recognize and kill virally infected cells through secretion of cytotoxic substances like granzyme B and perforin. T regs constitute a special T cell subset as they can inhibit the functions of Th cells and CTLs through cell-cell contact dependent mechanisms. This suppression is further assisted by the secretion of the anti-inflammatory cytokines IL-10 and TGF-β. The inhibitory T regs are pivotal for the regulation of excessive immune responses and therewith for the prevention of auto-immunity.

**Dendritic cells**

DCs are the most potent professional APCs of the immune system which instruct innate immune cells and antigen-specific B and T cells to initiate adaptive immunity. DCs sample and process material from their microenvironment. They ingest proteins, break them down into peptides and present them in MHC molecules. Upon infection or inflammation, immature DCs get activated by danger signals (e.g., bacteria, viruses, apoptotic cells and cancer peptides) through PRR signaling and differentiate into mature DCs. After maturation, they migrate to the lymph nodes, where they present the peptides to T cells. Endogenous antigens (self or viral) are processed by the proteasome and loaded onto MHC class I molecules, whereas exogenous antigens are broken down in endosomes and loaded onto MHC class II molecules. Moreover, DCs have the unique ability to present exogenous antigen in the context of MHC class I molecules, a process referred to as cross-presentation. The efficient cross-priming of CTLs with MHC class I bound exogenous peptides is crucial for the induction of immune responses against viruses which do not infect DCs and tumor cells. The immunological response mediated by DCs strongly depends on the integration of three distinct signals: the recognition of the peptide-MHC complex (signal 1), stimulation via co-stimulatory molecules (signal 2) and cytokines (signal 3). Immature DCs provide only signal 1, leading to immune tolerance via T cell anergy or the induction of T reg cells, whereas, fully matured DCs provide all three signals and induce the full-scale activation of immune-competent effector T cells. The nature of cytokines secreted by DCs thereby greatly determines the polarization of Th cells (Th1, Th2, Th17 or T reg). Recent studies have demonstrated that innate immune signals are important for the skewing of DCs themselves and therewith subsequently determine the polarization of Th cells. INFγ, e.g.
secreted by NK cells, was shown to enhance IL-12 production by DCs, the key cytokine for Th1 polarization. Skewing towards Th2 differentiation is driven by the nature of the pathogen, i.e. helminthes, and cytokines like IL-4 and the epithelial cell secreted thymic stromal lymphopoietin (TSLP). Fungal PAMPs recognized by the c-type lectin receptor dectin-1 have been shown to induce a Th17 polarizing phenotype in DCs, i.e. the secretion of IL-6 and IL-23. Lastly, binding of bacterial products to CD11b/CD18 on DCs induces the secretion of IL-10 and subsequent induction of T reg differentiation. Besides these innate signals, recent data suggest that the nature of the DC subset is an additional important factor in the polarization of T cells.

**DC subsets**

Despite their indispensable role in raising an immune response, DCs are a rather rare and heterogeneous type of immune cell. DC subtypes differ in phenotype and function, which may also depend on their localization. Langerhans cells (LCs) are DCs that are predominantly found in epidermal layers of the skin where they are mainly involved in the uptake and presentation of microbial antigens. This specific tissue subset is characterized by the expression of langerin. Human peripheral blood comprises two more main subtypes of DCs. CD11c expressing conventional DCs (cDCs, also referred to as myeloid DCs) and CD11c negative (human) or low (mouse) plasmacytoid DCs (pDCs). CD11c+ cDCs are recognized as the classical DC subset; they rapidly and very efficiently take up and process antigen to subsequently prime B- and T-lymphocytes. In man, cDCs can further be characterized by the expression of general myeloid markers, such as CD13 and CD33. They lack lineage specific markers (CD3, CD14, CD19 and CD56), but express high levels of MHC class II. Blood residing cDCs can further be subdivided based on differential surface expression of CD1c (BDCA-1) and BDCA-3. Both of these cDC subsets have the capacity to produce interleukin-12 (IL-12) in response to microbial stimuli through TLRs. However, they clearly differ in their expression of surface molecules, PRRs and their potency to stimulate T cells. BDCA-1+ cDCs play a central role in the recruitment of other immune cells via production of IL-8, whereas BDCA-3+ cDCs were shown to efficiently cross-present exogenous antigen to CTLs, a process that is essential for the induction of anti-tumor immune responses. In mice, at least three cDC subsets are distinguished in the spleen: CD8α+/CD4+, CD8α–/CD4+ and double negative CD8α–/CD4– cDCs. These three murine cDC subsets express distinct PRR profiles and respond differently to pathogenic stimuli. CD8α+/CD4+ are generally regarded as the most efficient cross-presenting subset, similar to the BDCA-3+ cDCs in human. However, recent data indicate that all DC subsets are commonly able to cross-present antigen, whereas the efficiency critically depends on the nature of antigens and activation signals. pDCs are clearly different from cDCs in shape, physiological presence, activation pattern, migration and function. They play a distinctive role at the interface of innate and adaptive immunity. Human pDCs are distinctively identified by their expression of BDCA-4, BDCA-2 and CD123. In the mouse, pDCs express the cell specific surface molecules Siglec-H and PDCA-1 (also known as BST-2). pDCs comprise a highly specialized DC subset which is characterized by the rapid secretion of large amounts of type I IFNs in response to unmethylated CpG oligonucleotide motifs derived from bacterial and viral DNA/RNA. In addition, upon activation they are able to acquire DC morphology, present antigen and efficiently stimulate T cells.

The heterogeneous phenotypes of the different DC subsets endows them to distinctly respond to the danger signals that they encounter in their microenvironment (Figure 3). Besides the pathogen derived triggers, chemokines and cytokines are known to modulate DC function and thereby the outcome of immune responses (see also above “Dendritic cells”). In addition, recent data
demonstrate that the unique composition of a given tissue microenvironment can influence the function of local DCs.

**Hormones and vitamins in dendritic cell biology**

The important role of the tissue microenvironment on the phenotype and activation state of DCs is well established for mucosal organs like the gut and skin. Optimal cDC function in the microenvironment of the gut and in the local mesenteric lymph nodes is dependent on the VitA derivative retinoic acid (RA). The resident, tolerogenic CD103+ intestinal DCs are imprinted by RA, secreted by epithelial cells of the gut and stromal cells of the local MLNs. RA conditioned CD103+ DCs express the RA metabolizing enzyme retinal aldehyde dehydrogenase (RALDH) which enables them to produce RA themselves, a pivotal factor for the attraction of gut-homing T regs. Similar to the gut, optimal function of DCs in the skin is directed by sunlight induced VitD. DCs in the skin express the VitD metabolizing enzymes Cyp27A1 and Cyp27B1 and convert VitD precursors to the active ligand calcitriol. The secretion of calcitriol subsequently enables the infiltration of skin homing T cells. In addition to the important role of VitA and VitD in the imprinting of DCs to attract T cells, different hormones, vitamins and their NRs have been shown to direct immune responses of DCs, both towards tolerance and immunity (Figure 3). DC mediated immune responses can be enhanced by the stimulation of RAR, e.g. langerhans cells that were treated with RA have augmented T cell activating potential. Moreover, RA stimulated moDCs secrete increased levels of the pro-inflammatory cytokines IL-6 and TGF-β. The suppressive effects of NR stimulation (i.e. GR, VDR, LXR (oxysterol receptor) and PPARγ) are particularly well studied in DCs. GR inhibits DC maturation by repression of co-stimulatory molecules and pro-inflammatory cytokines on the one hand, and strong induction of the anti-inflammatory cytokine IL-10 on the other hand. GR agonists (i.e. corticosteroids) are widely used immunosuppressive drugs for the treatment of autoimmune diseases, like rheumatoid arthritis systemic lupus erythematosus and transplant graft rejection. Stimulation of VDR with VitD is well established to impair the T cell activating capacity of DCs, particularly moDCs. Moreover, both LXR and PPARγ have initially been shown to inhibit DC maturation, however, recent studies provide conflicting data showing enhanced DC activation and skewed DC polarization. The effect of LXR and PPARγ on DC function is thought to be dependent on the given tissue context and thus additional signals like PAMPs and cytokines, though the exact interplay of these factors remains elusive. 

Hormones and vitamins alter DC mediated immunity and play a critical role in tissue specific immune responses. Still, little is known regarding the effect on DC subsets, enhancement of DC activation, DC polarization and the integration of NR signaling with additional micro-environmental factors.
Figure 3: Dendritic cells are shaped by their microenvironment. Dendritic cells integrate pathogenic signals recognized by pattern recognition receptors (PRR) and microenvironmental cues recognized by nuclear receptors (NR). The combination of received signals will lead to the differentiation of tolerogenic or immunogenic DCs. The glucocorticoid receptor (GR) and vitamin D receptor (VDR) and induce tolerogenic responses marked by increased IL-10 and decreased IL-12, TNFα, CD86 and CD80. The retinoid acid receptor (RAR) enhances the immunogenic phenotype of DCs with increased IL-6, TGFβ and CD1d (important for NK cell activation). The peroxisome proliferator activated receptor (PPARγ) and liver x receptor (LXR) have been implicated in both tolerance and immunity.
Chapter 1 - General Introduction

Scope of this thesis

Hormones, vitamins and their nuclear receptors control various physiological processes like metabolism, neurological responses, endocrine signals, cell growth and immune responses. Consequently, aberrant signaling via NRs is associated with corresponding diseases including cancer and autoimmunity. NRs are activated by hormones and vitamins, small compounds which are easy to synthesize and NRs therefore represent ideal drug targets. Profound knowledge of the effects of NR signaling on distinct cell types is a pre-requisite for the rationale design and application of NR based treatment modalities. The scope of this thesis was to further elucidate the role of hormones and vitamins in dendritic cell and cancer biology.

Part II Despite the importance of both NRs and DCs within the immune system, still little is known regarding the NR expression profile in DC subsets in general and in pDCs in particular. In chapter 2 we assessed and compared the NR expression profile of murine cDCs and pDCs. We show that both subsets express the same repertoire of NRs in vitro and ex vivo from different lymphoid organs. mRNA expression levels of distinct receptors however, differed between pDCs and cDCs especially upon maturation. Chapter 3 investigates the effect of VitD treatment on murine and human pDCs. VitD is known to have immunosuppressive functions and applied in the clinics for the treatment of autoimmune diseases. We show that in addition to cDCs, VitD also impairs pDCs in their ability to activate T cells in a VDR dependent manner.

Part III The transcriptional activity of NRs is critically dependent on the presence of coregulatory proteins. Most coregulators either enhance (coactivators) or inhibit (corepressors) NR mediated transcription. DC-SCRIPT is a versatile NR coregulator which exerts both of these functions. In chapter 4 we studied the role of DC-SCRIPT in DC biology. We show that DC-SCRIPT is expressed early upon differentiation of monocytes to DCs and also in different circulating DC subsets. Functionally, knockdown of DC-SCRIPT in moDCs results in increased production of IL-10 and decreased levels of IL-12. Chapter 5 deals with the interplay of DC-SCRIPT with the glucocorticoid receptor in DCs. DC-SCRIPT interacts with GR and inhibits GR mediated transcription. Furthermore, knockdown of DC-SCRIPT leads to the enhanced expression of the GR target gene GILZ in DCs, a known hallmark of tolerogenic DCs.

DC-SCRIPT has been shown to be an independent prognostic marker in breast cancer. In chapter 6 we further extend the role of DC-SCRIPT in cancer biology and address its function in prostate cancer. Early malignant transformation of normal prostate epithelial cells is characterized by the loss of DC-SCRIPT expression. Moreover, we show that DC-SCRIPT represses the function of AR and enhances transcription mediated by VDR, two important NRs in prostate carcinoma.

Part IV Finally, in chapter 7, I summarize the results of this thesis and further discuss the implications and future perspectives of hormones and vitamins as orchestrators of dendritic cells and cancer.
Part 2

Nuclear Receptors
Chapter 2

Nuclear Receptor expression patterns in murine plasmacytoid and conventional dendritic cells

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Molecular Immunology (2013) 55 (3-4), 409-417
Nuclear Receptor expression patterns in murine plasmacytoid and conventional dendritic cells

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Abstract
Dendritic cells (DC) play a central role in the immune system. They can either induce immunity or promote tolerance. The DC family is generally comprised of two functionally distinct DC subsets. Conventional dendritic cells (cDC) are the classical antigen presenting cells; plasmacytoid dendritic cells (pDC) are the main producers of type I interferons thereby serving innate immunity. Upon activation DCs are able to present antigen and stimulate T cells. The immune modulatory functions of DCs largely depend on the recognition of soluble cues. Besides pathogen derived cues, recent data indicate that the tissue micro-environment i.e. of the gut and skin affects cDC function. Many of these micro-environmental factors are ligands for the nuclear receptor (NR) family of transcription regulators known to affect immunity and tolerance. Whether pDC function is also influenced by tissue derived cues, like hormones, vitamins and metabolic products, is largely unknown. Here, we investigated the NR expression profile of murine pDCs and cDCs. We assessed the mRNA levels of 19 NRs of in vitro derived as well as ex vivo isolated DCs from four different lymphoid tissues. We observed that cDCs and pDCs expressed the same repertoire of NRs. Expression levels, however, differed between the two subsets, especially upon maturation of DCs. These data imply that NR ligands do impact pDC function and that their activity might be regulated in a DC-specific manner.

Keywords: Nuclear Receptor, Dendritic cell, Dendritic cell subset, plasmacytoid dendritic cell

Introduction
Dendritic cells (DCs) are the most powerful professional antigen presenting cells of the immune system. DCs are generally divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs form the classical DC subset, they are key regulators in the balance of immunity and tolerance due to their ability to prime B- and T-lymphocytes, the effector cells of an immune response (1-3). pDCs are clearly different from cDCs in shape, physiological presence, activation pattern, migration and function. They play a distinctive role at the interface of innate and adaptive immunity (4). pDCs are the main producers of type I interferons thereby inducing efficient anti-viral responses (5, 6). In addition, upon activation they are able to present antigen and stimulate T cells thereby inducing specific immune responses (4, 5).

DCs constantly scan their environment for possible threads, like bacteria or viruses. Upon encounter of pathogens they recognize specific molecular patterns via binding to their pattern recognition receptors (PRRs), like toll like receptors (TLRs). The cells then become activated and initiate an
immune response. DCs are present in essentially every tissue. Besides the pathogen derived triggers, chemokines and cytokines are known to modulate DC function (7). In addition, recent data demonstrate that the unique composition of a given tissue microenvironment can influence the function of tissue-resident cDCs. Various studies indicate that hormones, vitamins and metabolic products are part of the tissue-derived signals that affect DC function (8-11). These factors are all ligands for the nuclear receptor (NR) family of transcription regulators (12, 13). Nuclear receptors are key-regulators in a diversity of physiological functions including reproduction, development, homeostasis, metabolism, cell differentiation and immune responses (14). Several NR agonists are known to modulate the immune system in general and to alter cDC phenotype and function in particular (8, 11, 15). The retinoid all-trans-retinol i.e. was shown to be necessary for optimal cDC function in mesenteric lymph nodes in the microenvironment of the gut (16-19). Despite the importance of both NRs and DCs within the immune system, still little is known regarding the NR expression profile in DC subsets including pDCs. Only recently, the hormone progesterone was shown to regulate IFNα production in pDCs (20). Furthermore, Abe et al. showed that the GR ligand dexamethasone inhibited differentiation of murine pDC and increased their apoptotic death (21). These data imply that NR ligands do impact pDC function and that their activity is regulated in a DC specific manner. Therefore, we compared the NR expression profiles of murine plasmacytoid and conventional DCs. *In vitro* cultured pDCs and cDCs as well as freshly isolated cells from four different lymphoid tissues were analyzed for mRNA levels of 28 NRs. Moreover, we assessed changes in NR expression upon maturation of pDCs and cDCs with TLR ligands.

**Materials & Methods**

*In vitro and ex vivo purification of pDCs & cDCs*

Dendritic cells were generated from murine bone marrow isolated from femur/tibia of female C57BL/6N mice (Charles River, Germany). Mice were used at 6-12 weeks of age. Animal studies were approved by the Animal Ethics Committee of the Nijmegen Animal Experiments Committee. Cells were cultured at a density of 1.5-2*10^6/ml for 8-10 days (37°C, 10% CO\textsubscript{2}) in RPMI 1640 supplemented with 10% FCS (Gibco-BRL Life Technologies), 0.5% antibiotic-antimycotic (Gibco/Invitrogen), 1% Ultra-glutamine (Lonza), 50mM β-ME (Sigma-Aldrich) and 200ng/ml human rFlt3L (Preprotech).

To increase pDC and cDC cell counts in murine lymphoid organs, C57BL/6N mice were injected subcutaneously on their left flank (height of spleen) with 5-7.5*10^6 B16Flt3L tumor cells (22). Tumors were grown for 10-14 days and lymphoid organs (bone marrow, spleen, mesenteric and skin draining lymph nodes) were harvested subsequently.

Single cell suspensions were labeled with anti-SiglecH-FITC (eBiosciences) and anti-CD11c-APC (Biolegend) antibodies for pDCs and cDCs, respectively. pDCs were positively sorted with anti-FITC microbeads. The negative fraction was subjected to positive selection with anti-APC microbeads (both Miltenyi Biotec, Germany) to obtain cDCs.

**Flow cytometry**

The purity of isolated DC subsets was determined by flow cytometry. The following monoclonal anti-mouse antibodies and appropriate isotype controls were used: anti-CD11b-PE, anti-B220-PerCP, anti-CD11c-APC, ratIgG2a-PerCp (all from Biologend), anti-PDCA1-PE, hamster IgG1-APC (all BD Pharmingen), rat IgG2b-PE, rat IgG2b-FITC, anti-siglecH-FITC (all from eBioscience). Samples were
**Part 2 - Nuclear Receptors**

acquired on a CyAn™ ADP Analyzer (Beckman Coulter) and data were analyzed with FlowJo software (TreeStar).

**Stimulation experiments**

Purified DC subsets were cultured at a concentration of 1*10^6 cells/ml in 6 well cell culture cluster plates (Corning Incorporated). Cells were stimulated with 1µg/ml CpGB oligonucleotide (1668, Sigma Aldrich), and 4µg/ml Resiquimod (R848, Axxora) for 16 hours. After O/N incubation cells were harvested and subjected to density gradient centrifugation (PAA) to remove dead cells.

**RNA isolation, RT-PCR & Quantitative PCR**

Total RNA was isolated with RNeasy™ MiniPrep Kit (Qiagen Benelux) according to manufacturer’s instructions. The optional on column DNase treatment was applied. RNA was transcribed into cDNA with the RT^2 First Strand cDNA Kit (SABiosciences, Qiagen Benelux) according to manufacturer’s instructions. The optional on column DNase treatment was applied. RNA was transcribed into cDNA with the RT^2 First Strand cDNA Kit (SABiosciences, Qiagen Benelux) according to manufacturer’s instructions. cDNA was subjected to Custom Mouse RT^2 profiler™ PCR Arrays (CAPM09438D-24, SABiosciences, Qiagen Benelux). Samples were read on a CFX96 Real Time System (BioRad). mRNA levels were normalized to the house keeping gene PBGD (detected by in house qPCR) and are expressed as 2^{(Ct gene of interest minus Ct PBGD)}. mRNA levels of PBGD, TLRs (Table 1) and selected NRs (primer sequences from www.nursa.org) were detected by in house qPCR with FastStart SYBR Green Master (Roche Applied Science). Samples were read on a CFX96 Real Time System (BioRad). mRNA levels were normalized to the house keeping gene PBGD and are expressed as 2^{(Ct gene of interest minus Ct PBGD)}. Statistical significance was tested with two tailed, paired students T-test. *= P<0.05; **= P<0.01; ***= P<0.001.

**Table 1: Primer sequences (in house qPCR)**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequences (fw/rev)</th>
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<tbody>
<tr>
<td>mPBGD</td>
<td>5’CCTACCATACCTCTCCTGCGTTTAC 5’TTTGGTGGAAGACACAGCAT</td>
</tr>
<tr>
<td>mTLR3</td>
<td>5’GGGGTCCACTGGAGAACTCTTAT 5’CCGGGGAAGACCTCTTTAAGTGGG</td>
</tr>
<tr>
<td>mTLR7</td>
<td>5’TCTTACCTTCATGACATCAACACA 5’CCCCAGTAGAACAGGTACACA</td>
</tr>
<tr>
<td>mTLR9</td>
<td>5’ACTCCGACTTCGTCCTCACCT 5’GGCTCAATGTCATGCTGCA</td>
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**Results**

In vitro generated pDCs and cDCs express the same repertoire of NR

The in vitro differentiation of DC subsets from mouse BM is a widely used approach for the simple generation of large amounts of cells. To assess the expression of NRs in pDCs and cDCs, cells were differentiated in vitro from murine whole bone marrow (BM) (Experimental design, Figure 1A and suppl. Figure 1). Both subsets were sorted from the same culture with purities exceeding 95%. pDCs were detected as B220^+\text{SiglecH}^+ (CD11c^+, CD11b^+\text{B220}^+) and cDCs were defined as CD11c^+, CD11b^+ (B220^+ , SiglecH^+) (Figure 2A) (5). To further confirm purity of the sorted subsets, TLR3, 7 and -9 mRNA expression levels were assessed by qPCR. In line with published data, we observed that pDCs did not express TLR3 but high levels of TLR7 and 9, whereas cDCs did highly express TLR3 but lower levels of
TLR7 and 9 (Figure 2B) (23).

Total mRNA of the purified subsets was subjected to qPCR-array based expression analysis of 28 NRs. We tested the expression of selected receptors based on the availability of ligands and potential influence in immunobiology. Overall, we observed that in vitro cultured, bone marrow derived pDCs and cDCs expressed the same repertoire of 15 NRs out of 28 tested (Figure 2C). The expression profile of both subsets included at least one member of each NR subfamily analyzed. Strikingly, each subfamily was represented with the same member in pDCs as in cDCs. Both DC subsets expressed the type II NR’s RARα, RARγ, PPARβ/δ, PPARγ, Rev-Erbβ, RORα, VDR and CAR. mRNA levels of their hetero-dimeric partner RXRα could not be detected on the array. Independent qPCR analysis of all three RXR isotypes (NURSA consortium curated primers) did show expression of RXRα and RXRβ, but not RXRγ in both pDCs and cDCs (Figure 2D). The steroid type I NRs were less prominently represented; DCs showed expression of ERRα and GR, only two of eight analyzed receptors. Interestingly, mRNA of the sex hormone responsive NRs (ER, PR and AR) was not detected in 3-4 independent samples. Individual qPCR analysis (NURSA consortium curated primers) of these receptors in the same samples confirmed the lack of expression (data not shown). The recently identified nerve growth factor IB-like receptors Nur77, NURR1 and NOR1 were all expressed by pDCs and cDCs. Interestingly, while the repertoire of NRs was identical in pDCs and cDCs, expression levels of individual receptors were significantly different. pDCs expressed significantly higher levels of the RXR heterodimer VDR and the orphan receptor TR4 compared to cDCs. Furthermore, pDCs had significantly lower mRNA levels of Nur77, a constitutively active orphan receptor. In conclusion, in vitro derived murine pDCs and cDCs expressed the same repertoire of NRs. However, the expression levels of specific NRs (TR4, VDR, Nur77) differed significantly between the two subsets.

Figure 1. Experimental design
(A) DCs were generated in vitro from murine BM. Subsequent to culture, cells were sorted into pDC and cDC fractions by MACS. mRNA expression levels of 28 NRs were analyzed in fresh cells or TLR matured cells. (B) Primary pDCs and cDCs were isolated from four different lymphoid tissues. The mRNA levels of 28 NRs were analyzed in these cells.
DC Maturation affects NR expression in a subset specific manner (in vitro)

The maturation of DCs via the TLR pathways is known to be affected by several NR ligands. Therefore, the effect of DC maturation on the expression of NRs was investigated.

In vitro cultured pDCs and cDCs were sorted and matured for 16 hr with a combination of the TLR7/8 ligand R848 and the TLR9 ligand CpG. The maturation status of both cell types was confirmed by analysis of co-stimulatory molecule expression (CD86) and cytokine secretion (type I IFN, IL-6) (data not shown).

Maturation of pDCs and cDCs augmented the mRNA levels of some NRs while expression levels of others remained unchanged (Figure 3 and suppl. Figure 2). The retinoic acid responsive NRs RARα and RARγ both were significantly downregulated in mature pDCs. In contrast, mature cDCs only downregulated the expression of RARα. Immature pDCs had higher expression levels of VDR compared to cDCs. Interestingly, maturation induced the downregulation of VDR in pDCs, whereas it led to increased VDR mRNA levels in cDCs. Expression of Nur77 was significantly decreased in both subsets upon maturation. On the contrary, NURR1 and both NURR1 and NOR1 were upregulated in mature pDCs and cDCs, respectively. In conclusion, the TLR induced maturation of pDCs and cDCs altered the expression levels of certain NRs in a subset specific manner.
NR mRNA levels in pDCs and cDCs vary between lymphoid organs

To confirm that the expression profiles of the in vitro derived DCs match their in vivo counterparts, we isolated pDCs and cDCs from four different lymphoid organs – spleen, bone marrow (BM), skin draining lymph nodes (SLN) and mesenteric lymph nodes (MLN) (Experimental design: Figure 1B). The purity of cells isolated from spleen and BM was higher than 95% as analyzed by surface marker expression (Figure 4, A). DC subsets isolated from SLN and MLN were 80-90% pure. qPCR based expression analysis of TLRs further confirmed the purities on mRNA level (Figure 4B). pDC populations did not express TLR3 and TLR 13, which are expressed by CD8 positive cDCs and both CD8 negative and positive cDCs, respectively (23, 24).

The NR expression profiles of pDCs and cDCs ex vivo in the BM, spleen, SLN and MLN were consistent with those of the in vitro, BM derived cell subsets (Figure 5 and Figure 6). DCs from both sources, the lymphoid tissues and in vitro BM cultures, expressed the same repertoire of NRs. Except for the orphan receptor RORγ, that was detected in freshly isolated cells from all four tissues, but not in the in vitro generated DC. Additionally, the expression levels of all NRs were highly comparable between in vitro and ex vivo DCs. The freshly isolated DCs from the lymphoid tissues showed the same trend as the in vitro differentiated pDCs and cDCs with respect to differential expression levels of TR4, VDR and Nur77 mRNA. Moreover, the differences in mRNA levels of all NRs between pDCs and cDCs presented the same pattern in all four tissues. However, significant changes varied between BM, spleen and LNs. pDCs from the BM expressed significantly lower levels of RARγ and RORγ than cDCs (Figure 5, A). Splenic pDCs had higher mRNA levels of RORα than cDCs (Figure 5B). In SLNs, cDCs showed higher expression levels of RARγ, Nur77 and NURR1 than pDCs (Figure 5C). MLN pDCs had higher mRNA levels of PPARβ/δ, while they did not express RORα compared to cDCs (Figure 5D).

To evaluate the effect of the microenvironment on mRNA levels of NRs we compared the expression profiles of DCs freshly isolated from spleen, BM, SLN and MLN. pDCs and cDCs from all four analyzed

| Figure 3. Maturation of pDCs and cDCs augments NR expression levels |
|---|---|---|
| RARα | RARγ | VDR |
| pDC immature | pDC mature | cDC immature | cDC mature | pDC immature | pDC mature | cDC immature | cDC mature |
| relative expression | relative expression | relative expression |

pDC and cDC were sorted from Flt3L BM cultures. Purified pDCs and cDCs were stimulated for 16hrs with a combination of 4µg/ml R848 and 1µg/ml CpG. Expression levels of RARα, RARγ, VDR, Nur77, NURR1 and NOR1 were readily detected by qPCR analysis (RT² profiler PCR array; SA Biosciences). mRNA levels are shown relative to expression of mouse PBGD. Data shown are mean values of at least three independent experiments. * = P<0.05; ** = P<0.01; *** = P<0.001. Mean +/- SEM.
lymphoid organs expressed the same repertoire of NRs (Figure 6 and Figure 5). SLN resident pDCs had slightly higher mRNA levels of the retinoic acid responsive receptor RARα compared to their splenic and BM counterparts (Figure 6A). Splenic pDCs on the other hand had a trend towards higher expression levels of both PPARγ and Nur77. Yet, none of these differences in pDCs from different organs reached statistical significance. Expression levels in cDCs were also largely similar between organs (Figure 6B). However, the mRNA level of RORalpha was significantly higher in MLN cDCs compared to cells from BM, spleen and SLN. RXRα and β were expressed in comparable levels in both, pDCs and cDCs (Figure 6C). In conclusion, pDCs and cDCs in the BM, spleen, SLNs and MLNs expressed the same repertoire of NRs. The differences in the NR mRNA expression levels of pDCs compared to cDCs on the other hand varied between organs. The mRNA levels of most NRs within one subset were relatively stable in cells from different lymphoid tissues in steady state conditions.

**Discussion**

Increasing evidence shows that ligands for the NR superfamily shape the phenotype of DCs. Particularly activation of RAR, VDR, PPARγ, GR and more recently LXR has been shown to influence the immune response elicited by cDCs (8, 11). While cDCs are appreciated to be shaped by NR ligands, little is known about the role of NRs in pDCs. Interestingly, pDCs express the novel transcriptional regulator DC-SCRIPT, which modulates the activity of multiple members of the NR family of proteins (25-28). In the present study, we evaluated the expression of 28 NRs in murine pDCs and cDCs. Both cell types expressed the same repertoire of 15 receptors *in vitro*. TLR mediated maturation of DCs significantly augmented the expression levels of the retinoid acid receptors in immature pDCs and cDCs. In the gut, retinoid acid was shown to be important in the imprinting of resident CD103+ DCs. Stimulation of these cells in the lamina propria by dietary retinoid acids is pivotal for their tolerogenic properties (18, 19). Populations of pDCs are found in the lamina propria and the local mesenteric lymph nodes. Their role within the tolerogenic environment in the
small intestine, however, has not yet been investigated. The expression of RARα and RARβ in immature and mature pDCs suggests a role for retinoid acid stimulation also for this DC subset. Expression levels of the universal heterodimeric receptor RXRα were rather low in both pDCs and cDCs. Despite these low mRNA levels the receptor is likely to be active as both cells types responded to stimulation with 1,25 dihydroxyvitamin D3, the ligand for the RXR-heterodimer VDR (data not shown). We found that the RXR heterodimer VDR is expressed in immature and mature pDCs and cDCs. VDR is well known to induce tolerogenic properties in murine and human cDCs (29, 30). In contrast, stimulation of human pDCs with VDR ligands did not induce a tolerogenic phenotype (31).
However, pDC specific functional consequences, like enhanced activation or altered cytokine production, of VDR stimulation have not yet been thoroughly investigated. All three members of the NR4A family of NRs were expressed in immature and mature cDCs and in pDCs. Recently, the NR4A family of orphan receptors is proposed to be involved in immune regulation and inflammation, including immune disorders like rheumatoid arthritis, psoriasis and artherosclerosis (32). Moreover, NR4A receptor genes have been shown to be rapidly induced in activated macrophages (33). Overexpression of Nur77 in murine RAW264.7 macrophages led to increased transcription of inflammatory, apoptosis and cell cycle control genes. In accordance with our data, Ng et al. found NOR1 upregulated and Nur77 downregulated in virally infected murine DCs (34). Additionally, human DCs have increased NOR1 mRNA levels upon maturation. This high expression was correlated to an increase in apoptosis (35). Yet, the functional role of all three NR4A members in DC biology, and specifically in different DC subsets, remains to be elucidated.

pDCs and cDCs were matured with the TLR7/8 ligand R848 and TLR9 ligand CpGB as both subsets express significant amounts of TLR7 and TLR9. It would be interesting to assess the effect of other TLR ligands, i.e. for TLR2 or TLR4 which are more specific for cDCs, and their effects on NR expression. In addition to in vitro derived pDCs and cDCs, we investigated the NR expression pattern in primary DCs isolated from four different lymphoid tissues. Given the low abundance of pDCs in these tissues, we injected mice with Flt3I expressing B16 tumors to increase DC numbers. The B16 tumor model is poorly immunogenic and DCs isolated from these mice had an immature phenotype. We therefore consider these conditions to be steady state. pDCs and cDCs in the BM, spleen, SLNs and MLNs expressed the same repertoire of NRs. These results show that in vitro, Flt3L – differentiated, murine DCs resemble in vivo pDCs and cDCs in the lymphoid tissues with respect to their NR expression pattern. Therefore, they represent a very suitable system for isolated analysis of individual NR ligand

Figure 6. NR expression profiles of pDCs and cDCs from different microenvironments
pDC and cDC were isolated from bone marrow (BM), spleen, skin draining lymph nodes (SLN) and mesenteric lymph nodes (MLN). Expression levels of 28 selected NRs in (A) pDC and (B) cDC were readily detected by qPCR analysis (RT² profiler PCR array; SA Biosciences). (C) Expression of RXRα, β and γ in pDC and cDC from lymphoid organs as detected with NURSA curated primers. mRNA levels are shown relative to expression of mouse PBGD. Data shown are mean values of three independent experiments. * = P<0.05; ** = P<0.01; *** = P<0.001. Mean +/- SEM.
effects. Although the NR repertoire of pDCs and cDCs was similar between organs, the mRNA expression levels differed. The distinct DC subsets might therefore specifically react to NR ligands present in different microenvironments. Interestingly, pDCs in the gut draining LNs expressed significantly higher levels of PPARβ/δ than cDCs whereas cDCs expressed more PPARγ. Hously et al. showed that PPARγ is essential for the imprinting of tolerogenic cDCs and consequently regulatory T cell (Treg) induction (36). PPARβ/δ might be functionally important for pDCs in the mesenteric LNs.

Conclusion
Different DC subsets can exert specified functions due to cell-intrinsic properties, like differential expression of PRRs and downstream signaling proteins (37). Moreover, each DC subset can specifically react to microenvironmental factors, including NR ligands. Our data show that NR expression is regulated in a cell specific manner in pDCs and cDCs, which indicates that hormones and vitamins differentially affect pDC and cDC function.

Acknowledgements
This work was supported by a PhD grant from the Radboud University Nijmegen Medical Centre (to N.K.), the Kidney Foundation (IP11.31 to A.B.vS.), the Netherlands Organization for Scientific Research (NWO ZonMW; Vici Grant 918.66.615 to G.J.A.; Vidi Grant 864.11.006 to A.B.vS.) and the Dutch Cancer Society (KWF2011-5229 to M.A. and G.J.A.).

References
Part 2 - Nuclear Receptors


Suppl. Figure 1: DC subset purification procedure

Cells were labeled with anti-SiglecH-FITC and anti-CD11c-APC antibodies for pDCs and cDCs, respectively. pDCs were positively sorted with anti-FITC microbeads. The negative fraction was subsequently subjected to positive selection with anti-APC microbeads to obtain cDCs.
Suppl. Figure 2: NR expression levels in mature pDCs and cDCs

pDCs and cDCs were sorted from Flt3L BM cultures. Purified pDCs and cDCs were stimulated for 16hrs with a combination of 4µg/ml R848 and 1µg/ml CpGB. Expression levels of 28 selected NRs in (A) pDCs and (B) cDCs were readily detected by qPCR analysis (RT² profiler PCR array; SA Biosciences). (C) Expression of RXRα, β and γ in pDCs and cDCs from lymphoid organs as detected with NURSA curated primers. mRNA levels are shown relative to expression of mouse PBGD. Data shown are mean values of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.
Chapter 3

Vitamin D controls murine and human plasmacytoid dendritic cell function

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Journal of Investigative Dermatology, In press
Vitamin D controls murine and human plasmacytoid dendritic cell function

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Abstract
Topical application of the Vitamin D (VitD) analog calcipotriol is a highly effective standard treatment modality of psoriatic skin lesions. However, the immune modulatory effects of the treatment are incompletely understood. VitD is well known to induce tolerogenic responses in conventional DCs (cDC). Plasmacytoid DCs (pDCs) comprise a specialized, naturally occurring DC subset known to be important in autoimmune diseases including psoriasis. pDCs from the blood rapidly infiltrate psoriatic skin and are key to the initiation of the immune mediated pathogenesis of the disease. We now demonstrate that pDCs express various proteins of the VitD receptor (VDR) pathway, including the VitD metabolizing enzymes Cyp27B1 and Cyp24A1, and that VDR is transcriptionally active in pDCs. Moreover, VitD impairs the capacity of murine and human pDCs to induce T cell proliferation and secretion of the Th1 cytokine, IFN\(\gamma\). The inhibitory effect of VitD is dependent on the expression of the VitD receptor in the DCs. This study demonstrates that VitD signaling can act as a natural inhibitory mechanism on both cDCs and pDCs which may instigate the development of VitD based therapeutic applications for psoriasis and other inflammatory skin diseases.

Introduction
Psoriasis is a common autoimmune disease of the human skin, affecting 2% of the population worldwide (1). Disease onset and chronic states are mediated by autoreactive T cells and secretion of Th1 cytokines which induce the hyperproliferation of keratinocytes (1, 2). Recent studies have demonstrated that the early pathogenesis of psoriasis is marked by the infiltration of plasmacytoid dendritic cells (pDCs), a specialized subset of dendritic cells (DCs). pDCs are attracted to chemerin chemokines and subsequently exacerbate T cell activation, thereby driving disease development(3-5).

Current standard treatment modalities of psoriatic skin plaques include the topical use of the vitamin D (VitD) analog calcipotriol (5-7). VitD refers to a group of metabolites which exert their function through the VitD receptor (VDR). The active metabolite calcitriol (1,25-dihydroxyvitamin D3) is metabolized from precursors during a multistep process starting in the skin (8). Here, UVB induces the conversion of 7-dehydrocholesterol into vitamin D3 (cholecalciferol). Subsequently,
cholecalciferol is hydroxylated in the liver by the enzymes Cyp2R1 or Cyp27A1 to calcifediol (25-hydroxyvitamin D), the main circulating derivative of VitD. Finally, calcifediol is hydroxylated by the enzyme Cyp27B1 towards the active ligand calcitriol, in the kidneys. Importantly, the metabolism of calcitriol is not restricted to these specific tissues and can also take place in different cell types expressing the enzymes, including conventional dendritic cells (cDC) (9). Although VitD therapy is very effective in resolving skin plaques, the exact underlying mechanisms of action are not yet completely understood.

In psoriatic lesions, VitD is known to inhibit the epidermic hyperproliferation and promote differentiation of keratinocytes. Moreover, VitD has been shown to be a potent regulator of immune responses in general and also in psoriatic skin (10, 11). The two main target cells of VitD in the immune system are T cells and DCs. Stimulation of T cells with VitD impairs their proliferation, pro-inflammatory cytokine secretion (IFNγ, IL-17) and promotes development of Th2 and regulatory T cells (Treg) (12, 13). VitD treated in vitro differentiated DCs express decreased levels of co-stimulatory molecules and increased levels of inhibitory receptors. Moreover, they secrete lower amounts of pro-inflammatory and higher amounts of anti-inflammatory cytokines (14-16). Consequently, VitD treated DCs are less potent in their induction of T cell responses and induced the generation of Tregs. Few studies described a tolerogenic response of VitD treated naturally occurring blood DCs (17-19). The data from Penna and colleagues pointed towards a marked inhibitory effect of VitD on cDCs, exclusively. They showed that production of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules by pDCs was unaffected after treatment with VitD (17).

pDCs are the main producers of type I interferon; additionally, when activated, they too are potent antigen presenting cells and inducers of T cell responses (20, 21). The precise effect of VitD on the antigen presenting capacities of pDCs has not been elucidated to date. Here, we show that stimulation of murine and human pDCs with VitD prior to and during maturation significantly impairs their T cell stimulatory capacities. The inhibitory effect of VitD was dependent on the expression of the vitamin D receptor (VDR). We conclude that VitD signaling acts as a natural inhibitory mechanism on pDCs and cDCs.

Materials and Methods

Mice

C57BL/6n and Balb/C mice (6–12 weeks old, Charles River Wiga (Sulzfeld, Germany) were maintained in individually ventilated cage (IVC) units at the Central Animal Laboratory (Nijmegen, The Netherlands). Drinking water and standard laboratory food pellets were provided ad libitum. Experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee.

Leuven Vdr KO mice were described elsewhere (43, 44). The animals were bred in the animal housing facilities of the Proefdierencentrum Katholieke Universiteit Leuven, received a normal diet (1.1% calcium, 0.8% phosphorus, 0% lactose (Standard; Carfil, Oud-Turnhout, Belgium), and had free access to tap water. The Animal Ethics Board of the KUL approved all experimental procedures.

Murine pDCs and cDCs

DCs were generated from murine BM isolated from femur/tibia. Cells were cultured at a density of 1.5-2 * 10^6/ml for 8-10 days (37°C, 10% CO_2) in RPMI 1640 + 10% FCS (Gibco-BRL Life Technologies), 0.5% antibiotic-antimycotic (Gibco/Invitrogen), 1% Ultra-glutamine (Lonza), 50mM β-ME (Sigma-
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Aldrich) and 200ng/ml human rFlt3L (Preprotech). Before MACS sorting cells were harvested and subjected to density gradient centrifugation (PAA, Germany) to remove dead cells.

To increase pDC and cDC counts in murine lymphoid organs, mice were injected subcutaneously on their left flank (height of spleen) with 5-7.5*10^6 B16Flt3L tumor cells (45). Tumors were grown for 10-14 days and lymphoid organs were harvested subsequently.

Single cell suspensions were labeled with anti-SiglecH-FITC (eBiosciences) and anti-CD11c-APC (Biolegend) antibodies for pDCs and cDCs, respectively. pDCs were positively sorted with anti-FITC microbeads. The negative fraction was subjected to positive selection with anti-APC microbeads (both Miltenyi Biotec, Germany) to obtain cDCs.

Human moDCs and pDCs

DCs were isolated from buffy coats obtained from healthy volunteers after written informed consent per the Declaration of Helsinki and according to institutional guidelines. Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats via ficoll density gradient centrifugation (Lucron Bioproducts). moDCs were cultured as described previously (46). Plastic–adherent monocytes were cultured for 6 d in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) with 1% ultra-glutamine (PAA Germany), 0.5% antibiotic–antimycotic (PAA, Germany), 10% (v/v) FCS (Greiner Bio-one), IL-4 (300 U/ml), and GM-CSF (450 U/ml) (both Cellgenix). IL4 and GM-CSF were added again at day3.

pDCs were purified from PBLs (non-adherent fraction) by positive selection using anti–BDCA-4–conjugated magnetic microbeads (Miltenyi Biotec). As previously described, pDC purity was assessed by double staining BDCA2/CD123 for pDCs (> 95%; all Miltenyi Biotec) (47). pDCs were cultured in X-VIVO-15 medium (Lonza) with 2% human serum. 10ng/ml human rIL3 (Cellgenix) was added if indicated.

Flow cytometry

VDR protein was detected by intracellular antibody staining. Briefly, cells were stained with fixable viability dye efluor780 (eBioscience). Subsequently, murine organ suspensions and human pDCs were stained for appropriate membrane markers (see below). Cells were fixed/permeabilized with Cytofix/Cytoperm Kit (BD Bioscience), and blocked with 1% normal goat serum for mouse and 1% human serum for human cells. VDR was stained and samples were acquired on a CyAn™ ADP Analyzer (Beckman Coulter). Data were analyzed with FlowJo software (TreeStar).

The following antibodies and isotype controls were used 1) Murine: rat-anti-CD11b-PE, rat-anti-B220-PerCP, hamster-anti-CD11c-APC, ratIgG2a-PerCp (all from Biologend), rat-anti-PDCA1-PE, hamster IgG1-APC (all BD Biosciences), rat IgG2b-PE, rat IgG2b-FITC, rat-anti-SiglecH-FITC (all from eBioscience); 2) human: mouse-anti-BDCA2-FITC, mouse-anti-CD123-APC (both Miltenyi Biotec), mlgG1-FITC and mlgG1-APC (eBioscience).

For murine and human samples VDR was detected with either m-anti-VDR or m-anti-VDR-biotin at 4µg/ml (both clone D6, Santa Cruz Biotechnology) and secondary detection antibodies goat-anti-mlg-PE or streptavidin-PE (both BD Biosciences), respectively.

RNA isolation, RT-PCR & Quantitative PCR

Total RNA was isolated with RNeasy™ MiniPrep Kit (Qiagen Benelux) or Quick-RNA MiniPrep (ZymoResearch) according to manufacturer’s instructions. RNA was transcribed into cDNA with the RT^2 First Strand cDNA Kit (SABiosciences, Qiagen Benelux). Alternatively, RNA was treated with
DNase I (amplification grade; Invitrogen) and reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Invitrogen). mRNA levels of genes of interest (see suppl. table 1) were detected by qPCR with FastStart SYBR Green Master (Roche Applied Science). Samples were read on a CFX96 Real Time System (BioRad). mRNA levels were normalized to the housekeeping gene PBGD and are expressed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference in Ct values.

Western blotting

$5\times10^6$ murine in vitro derived cDCs and pDCs were lysed in 1% SDS and 62.5 mM Tris pH 6.8. Cell lysates were mixed with sample buffer (5% glycerol, 6% SDS, 125mM Tris-HCL pH 6.8, 0.1 mg/ml bromophenol blue and 10% β-ME), heated to 95°C for 5 min, resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (4°C, 30mA) (Schleicher and Schuell). Membranes were blocked with 3% BSA and 1% milk powder in PBST (1 hr RT) and subsequently stained with 1 μg/ml m-anti-VDR (clone D6, Santa Cruz Biotechnology) and rb-anti-actin (Sigma Aldrich) (both in ¼ PBST, 1 hr RT). After 3 x wash with PBST secondary Abs goat-anti-mouse IRDye 800CW and donkey-anti-rabbit IRDye 680RD (Li-cor Biosciences) were added (¼ PBST, 1hr RT). Membranes were washed 3x in PBST and scanned with an Odyssey Infrared Imaging System (Li-cor Biosciences) to visualize proteins.

Stimulation experiments DCs

Human and murine DC subsets were cultured at a concentration of $1\times10^6$ cells/ml. For target gene detection cells were stimulated with 100nM calcitriol (1,25-(OH)2D3), 100nM calcifediol (25(OH)D3) (both Sigma Aldrich), a combination of both or vehicle (EtOH). For the FACS staining of co-stimulatory molecules, human pDCs were pre-stimulated with 100nM 1,25(OH)2D3, 100nM 25(OH)D3 for two hours and subsequently VitD derivatives and 0.5 μg/ml ODN-CpG C (M362; Axxora) were added for additional incubation overnight.
Mixed Leukocyte reaction murine DCs

Isolated pDCs and cDCs were pre-stimulated with vehicle or 100nM Calcifediol and 100nM Calcitrol for 2 h. Subsequently, additional VitD derivatives and 0.1 µg/ml CpGB (1668, Sigma Aldrich) or 0.4 µg/ml Resiquimod (R848, Axxora) were added for additional incubation O/N. Supernatant was collected, DCs were washed with PBS and 50^4 CFSE-labeled allogeneic splenocytes (depleted for CD11c and B220) were added in a ratio of 1:2 and 1:6. Cells were incubated round bottom 96 well cluster plates (Corning).

ELISA

Human IFNγ levels were measured with the ELISA Ready-SET-Go!*RSG kit according to manufacturer’s instructions (eBioscience, Vienna, Austria). Murine IFNγ levels were measured by sandwich Elisa with rat-anti-mIFNgamma (Clone R4-6A2, BD), anti-mIFNgamma-biotin (Clone XMG 1.2, Biolegend), streptavidin-HRP (Invitrogen) and TMB (Sigma-Aldrich) substrate.

Statistics

Data were analyzed with one or two tailed, students T-test using GraphPad Prism 5.0. *= P<0.05; **= P<0.01; ***= P<0.001.

Results

Murine pDCs and cDCs respond to stimulation with calcitriol and its precursor calcifediol

In a qPCR based screen for NR expression in DC subsets, we found expression of VDR and its heterodimeric partner RXR in murine pDCs (22). To corroborate these findings, we assessed the mRNA levels of VDR in independent pDC and cDC samples freshly isolated from bone marrow (BM) and spleen or derived from in vitro progenitor cell cultures with Flt3L. These data confirmed that in vitro derived and lymphoid tissue resident pDCs expressed VDR mRNA levels (Figure 1A). In accordance with the mRNA levels, also VDR protein was expressed in in vitro cultured pDCs (Figure 1B and C) and in pDCs in the BM and spleen (Figure 1C).

Figure 2. Murine pDCs and cDCs react to stimulation with calcitriol and its precursor calcifediol

pDCs and cDCs were sorted from Flt3L bone marrow cultures. Expression of (A) the co-regulators Drp205, Hairless, (B) the vitamin D metabolizing enzymes Cyp27A1, Cyp2R1, Cyp27B1, and the VDR target genes VDR (C) and Cyp24A1 (D) in pDCs and cDCs were detected with qPCR. mRNA levels are shown relative to expression of mouse PBGD. Data shown are mean values of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.
The transcriptional activity of VDR is affected by the presence of the active ligand calcitriol, Vitamin D (VitD) metabolizing enzymes and transcriptional co-regulators. We assessed the mRNA levels of the VDR co-activator, DRIP205, and the co-inhibitor, Hairless (23, 24). Both co-regulators were expressed at comparable levels in Flt3L derived pDCs and cDCs (Figure 2A). cDCs are known to be able to actively synthesize the active VDR ligand calcitriol from its precursors cholecalciferol and calcifediol, mediated by the enzymes Cyp27A1 or Cyp2R1 and Cyp27B1, respectively (9, 25). Our data show that both murine pDCs and cDCs express similar levels of Cyp27A1 and Cyp2R1 as well as Cyp27B1 (Figure 2B). As pDCs and cDCs expressed co-regulators and enzymes of the VitD pathway, we investigated whether VDR was transcriptionally active and whether both subsets could respond to the VitD precursor calcifediol. To this extent, pDCs and cDCs were stimulated with calcifediol or calcitriol and mRNA levels of the known VDR target genes VDR itself and the enzyme Cyp24A1 were measured. pDCs and cDCs both rapidly up-regulated the expression of VDR two hours after stimulation with calcitriol (Figure 2C). VDR mRNA was back to baseline levels 16 hours after stimulation. Moreover, both subsets also strongly up-regulated VDR in response to calcifediol stimulation, indicating that calcifediol is processed into active calcitriol and thus that the enzyme Cyp27B1 is active in DCs. Expression of the target gene Cyp24A1 was induced 16 hours after stimulation with calcitriol, albeit...
at low levels (Figure 2D). Calcifediol alone induced very low expression of Cyp24A1 in pDCs only. Moreover, both pDCs and cDCs responded to stimulation with the clinical VitD analog calcipotriol (suppl. Figure 1). Taken together, these data show that both pDCs and cDCs express transcriptionally active VDR protein and respond to stimulation with the VitD precursor calcifediol and the active VDR ligand calcitriol.

**Vitamin D impairs the T cell stimulatory capacities of murine pDCs**

VitD is part of the standard treatment of psoriatic skin lesions and different studies suggest that VitD therapy modulates the immune response in skin plaques. Since pDCs are implicated in the initiation of psoriatic pathogenesis we investigated the T cell stimulatory capacities of VitD stimulated pDCs. DCs were pre-treated with calcitriol and calcifediol and subsequently stimulated with CpG. After stimulation, DCs were added to an allogeneic mixed leukocyte reaction (MLR). cDCs are known to be impaired by VitD and as expected, VitD stimulated cDCs were much less potent in the induction of T cell proliferation compared to vehicle treated cells (Figure 3A). Intriguingly, also pDCs showed a remarkably decreased T cell activating capacity when treated with VDR ligands (Figure 3B). The inhibitory effect of VDR ligand stimulation was most pronounced in unstimulated DCs compared to CpG stimulated cells in both pDCs and cDCs and essentially absent when DCs were matured with the potent TLR ligand R848 (suppl. Figure 2A). VDR deficient (VDR-/-) pDCs and cDCs were generally slightly less effective T cell stimulators compared to wildtype (WT) DCs. However, upon stimulation with the VDR ligands VDR-/- DCs were

![Figure 4](image)

**Figure 4. Vitamin D impairs the capacity of murine pDCs and cDCs to activate T cells**

(A) pDC and (B) cDC were sorted from Flt3L BM cultures of either WT or VDR-/− mice. Purified pDCs and cDCs were pre-stimulated for 2 hrs with 100nM calcitriol and 100nM calcifediol. Subsequently, cells were stimulated again with 100nM calcitriol, 100nM calcifediol and vehicle or 100ng/ml CpG for additional 16hrs. Cells were washed and co-incubated with splenocytes (CD11c, B220 depleted) for 5 and 6 days. IFNγ levels in the supernatant were measured with Elisa. Data shown are mean values of at least three independent experiments. ns = non significant; *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.
not impaired in their T cell activating potential, indeed demonstrating that the inhibitory effect of VitD was dependent on the presence of VDR in DCs (Figure 3).

Psoriasis is a Th1 driven disease; therefore we next assessed the secretion of cytokines in the allogeneic MLR. In line with the impaired proliferation, also levels of the Th1 cytokine IFNγ were significantly decreased when pDCs and cDCs were pretreated with VDR ligands (Figure 4). Moreover, inhibition of IFNγ production was more prominent in unstimulated pDCs compared to the CpG stimulated cells. Robust stimulation of pDCs and cDCs with R848 lead to small but significant impairment of IFNγ secretion (suppl. Figure 2B). Importantly, VDR deficient DCs were not hampered in their capacity to induce IFNγ in T cells when stimulated with VD ligands confirming that the inhibitory effect of VitD is dependent on VDR expression (Figure 4). Furthermore, the secretion of IL5, IL10 and IL17 was not changed significantly between vehicle and calcitriol/calcifediol treatment (data not shown) indicating that VDR stimulation did not alter T cell skewing in this setting.

In conclusion, stimulation of the VDR inhibits the T cell stimulatory capacity of both conventional and plasmacytoid murine dendritic cells.

**Human pDCs respond to stimulation with calcitriol**

Our data show that murine pDCs exhibit impaired T cell stimulatory capacities upon pretreatment with VDR ligands. To extrapolate these findings to men, we assessed the activity of VDR in human pDCs (hPDC). Freshly isolated pDCs from human buffy coats expressed high levels of VDR protein (Figure 5A). Moreover, in line with the murine data both human moDCs and hPDCs expressed considerable mRNA levels of the VitD metabolizing enzymes Cyp27A1, Cyp2R1 and Cyp27B1 (Figure 5B). To address the functionality of VDR, moDCs and hPDCs were stimulated with either calcifediol alone or the combination of calcitriol and calcifediol. Subsequently, mRNA levels of the VDR target gene Cyp24A1 were measured. mRNA levels of Cyp24A1 were significantly induced in moDCs and hPDCs upon stimulation with calcitriol/calcifediol (Figure 5C). Treatment with calcifediol alone led to significant expression of Cyp24A1 in moDCs after 16hrs, indicating that the cells metabolize the precursor. hPDCs did not express Cyp24A1 when stimulated with calcifediol alone (data not shown).

These data show that human moDCs and pDCs express a functionally active VDR.

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**Figure 5. Human PDCs and moDCs react to stimulation with calcitriol**

Human PDCs were sorted from PBLs. moDCs were cultured from monocytes for 6 days in the presence of IL-4 (300 U/ml) and GM-CSF (450 U/ml). Expression of (A) VDR protein was detected by intracellular antibody staining. (B) The VitD metabolizing enzymes Cyp27A1, Cyp2R1 and Cyp27B1 and (C) the VDR target gene Cyp24A1 in hPDCs and moDCs were detected with qPCR. (C) moDCs and hPDCs were stimulated with 100nM calcitriol and 100nM calcifediol for indicated timepoints. mRNA levels are shown relative to expression of human PBGD. Data shown are (A) representative or (B,C) mean values of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.
Vitamin D stimulated human pDCs have impaired T cell stimulatory capacities

As VDR was transcriptionally active in hPDCs we next looked at the effect of VDR ligand stimulation on their T cell activating capacity. hPDCs were pre-stimulated with calcifediol and calcitriol, subsequently, cells were stimulated again with 100nM calcitriol, 100nM calcifediol and vehicle or 100ng/ml CpG for additional 5hrs. Cells were washed and co-incubated with allogeneic PBLs for 7 days. IFNγ production by PBLs was measured by Elisa. Data shown are mean values of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.

Discussion

Topical application of the VitD analog calcipotriol is a highly effective standard treatment of psoriatic skin lesions (26). However, the underlying mechanisms of action are still incompletely understood. Psoriasis is a immune driven disease and VitD is thought to modulate inflammation in diseased skin (Reichrath et al., 1997). Within the immune system, VitD has pronounced tolerogenic effects on T cells and cDCs (27-29). pDCs rapidly infiltrate psoriatic skin and are at the initiation of disease pathogenesis (3). Here, we show that the VDR is active in and potently inhibits murine and human pDCs in their ability to induce T cell proliferation and activation.

pDCs expressed various proteins of the VDR pathway, including the coactivator Drip205, the coinhibitor Hairless and the VitD metabolizing enzymes Cyp27A1, Cyp27B1 and Cyp24A1. VDR was transcriptionally active in pDCs as shown by induced target gene expression after stimulation with VDR ligands. Moreover, murine pDCs that were treated with calcitriol/calcifediol prior to and during activation with the TLR9 ligand CpG were clearly reduced in their capacity to stimulate T cell proliferation. Production of the Th1 cytokine IFNγ in the allogeneic MLR was also significantly decreased when hPDCs were stimulated with VDR ligands (Figure 6B). These data indicate that VitD is able to modulate the function of hPDCs and acts to decrease their capacity to induce T cell activation.

Figure 6. Vitamin D stimulation of human PDCs reduces their ability to activate T cells

Human PDCs were sorted from PBLs and pre-stimulated for 2 hrs with 100nM calcitriol and 100nM calcifediol. Subsequently, cells were stimulated again with 100nM calcitriol, 100nM calcifediol and vehicle or 100ng/ml CpG for additional 5hrs. Cells were washed and co-incubated with allogeneic PBLs for 7 days. IFNγ production by PBLs was measured by Elisa. Data shown are mean values of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001. Mean +/- SEM.
reduced by VitD pre-treatment of pDCs and cDCs, whereas, secretion of the T cell cytokines IL5, IL17 and IL10 was unaffected (data not shown). VitD stimulated DCs were previously shown to induce the generation of tolerogenic T cells (14, 29, 30). However, most of these studies have been performed with VitD treatment during the differentiation of monocytes towards DCs. These experiments also showed marked reduction in the expression of CD80, CD86 and diminished secretion of pro-inflammatory cytokines IL6 and IL12. We could not detect a significant inhibition of these co-stimulatory molecules and cytokines in pDCs and cDCs stimulated with VitD during maturation. The inhibitory mechanisms of VitD during DC differentiation therefore are likely to follow different kinetics compared to the inhibitory mechanisms during DC maturation.

The impairment of allogeneic T cell activation was most pronounced in unstimulated pDCs and less in CpG treated cells. In addition, strong activation of murine pDCs and cDCs with the TLR7/8 ligand R848 was not affected by stimulation with VitD. Consequently, VitD mediated suppression of T cell priming seems to be an immune regulatory mechanism that is most important during mild infections and that can be overcome by strong immune stimuli. VitD metabolism is a multistep process initiated in the skin with the photoinduced conversion of 7-dehydrocholesterol and resulting in the production of the active ligand calcitriol. Recent studies report an alternative VitD activation pathway that is dependent on Cyp11A1. This pathway comprises a range of bioactive VitD derivatives with potential therapeutic interest due to their low calcemic activity (31-33). The skin is a part of the first line of defense that harbors various dendritic cells (34, 35). In this context, VDR ligands can act as a steady state regulatory system that may prevent autoimmune responses while allowing immune activation towards severe pathogenic manifestations. This hypothesis is supported by the evident epidemiologic correlation of VitD deficiency with psoriasis (36) and other autoimmune disorders like systemic lupus erythematosus (SLE) (37, 38). Moreover, rapid skin infiltrations of pDCs are causally linked to the early pathogenesis of psoriasis (39) and SLE (40).

In man, VitD is known to inhibit the differentiation and maturation of moDCs (15). Moreover, Penna et al. reported that calcitriol inhibits the maturation of human circulating cDCs, whereas hPDCs were not affected (17). In line, we also observed that stimulation of hPDCs with calcitriol did not impair the production of IFNα (data not shown) or the expression of CD80, CD86, MHC-II and ILT3 (suppl. Figure 3). However, stimulation with VitD clearly impaired the ability of hPDCs to induce T cell proliferation and secretion of the Th1 cytokine IFNγ.

The tolerogenic agent VitD has been studied and exploited for the treatment of psoriasis and other cutaneous diseases (39) but the underlying molecular mechanisms are only partly understood. Recent data demonstrate that VitD analogs alter the expression of antimicrobial alarmins, i.e. decreased HBD2, HBD3 and increased cathelicidin, in psoriatic skin (41). Interestingly, the cathelicidin peptide LL-37 has been shown to induce pDC activation when complexed with self DNA, suggesting increased inflammatory responses upon VitD treatment (42). However, VitD analogs did not result in increased IFNα levels in psoriatic skin. The diverse functions of LL-37 in psoriasis are still incompletely understood and future studies will further clarify its seemingly paradoxical effects.

pDCs are important players within the immune system; while they are absent in the skin during steady state, they rapidly infiltrate upon skin injury and during pathologic skin conditions (39). We now demonstrate that VitD potently inhibits the ability of pDCs to induce T cell activation in mouse and man. Exploitation of the tolerogenic properties of pDCs in therapeutic applications may therefore benefit from the clinical applicability of VitD for psoriasis and other skin pathologies.

Conflict of interest: The authors state no conflict of interest.
**Part 2 - Nuclear Receptors**

**Acknowledgements**

This work was supported by a PhD grant from the Radboud University Nijmegen Medical Centre (to N.K.), the Netherlands Organization for Scientific Research (NWO ZonMW; Vici Grant 918.66.615 to G.J.A.; Vidi Grant 864.11.006 to A.B.v.S.), the fund for Scientific Research-Flanders (G.0573.13 to G.C.) and the Dutch Cancer Society (KWF2011-5229 to M.A. and G.J.A.).

**References**


Chapter 3 - Vitamin D controls pDC function


Supplementary Table 1: Primer sequences

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Supplementary Figure 1. pDCs and cDCs were sorted from Flt3L bone marrow cultures. Cells were stimulated with 100nM calcipotriol for 4hrs. Expression of the VDR target genes mVDR itself was detected with qPCR. mRNA levels are shown relative to expression of mouse PBGD. Data shown are mean values of at least three independent experiments. *= P<0.05; Mean ±/− SEM.
**Supplementary Figure 2.** pDC and cDC were sorted from Flt3L BM cultures of either WT or VDR-/− mice. Purified pDCs and cDCs were pre-stimulated for 2 hrs with 100nM calcitriol and 100nM calcifediol. Subsequently, cells were stimulated again with 100nM calcitriol, 100nM calcifediol and vehicle or 400ng/ml R848 for additional 16hrs. Cells were washed and co-incubated with CFSE labeled splenocytes (CD11c, B220 depleted) for 5 and 6 days. (A) T cell proliferation was measured as indicated by CFSE dilution. Numbers indicate the percentage of proliferation. (B) IFNγ levels in the supernatant were measured by Elisa. Data shown are (A) representative or (B) mean values +/- SEM of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001.

**Supplementary Figure 3.** Human pDCs were sorted from PBLs. Expression of (A) CD80, CD86 and HLA-DR was detected by antibody staining. (B) Expression of hILT3 was detected with qPCR. mRNA levels are shown relative to expression of human PBGD. Data shown are (A) representative or (B) mean values +/- SEM of at least three independent experiments. *= P<0.05; **= P<0.01; ***= P<0.001.
Part 3

Nuclear Receptor Coregulators
Chapter 4

Dendritic cell-specific transcript: dendritic cell marker and regulator of TLR-induced cytokine production

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Journal of Immunology (2012) 189 (1), 138-145
DC-SCRIPT: DC marker and regulator of TLR induced cytokine production

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Abstract
Dendritic cells (DCs) are the professional antigen presenting cells of the immune system that dictate the type and course of an immune response. Molecular understanding of DC biology is important for the design of DC based immunotherapies and optimal clinical applications in vaccination settings. Previously, we isolated and characterized the cDNA encoding DC-SCRIPT (dendritic cell–specific transcript, also known as ZNF366). DC-SCRIPT mRNA expression in the immune system was confined to DCs and was reported to be an early hallmark of DC differentiation. Here, we demonstrate IL-4 to be the dominant factor for DC-SCRIPT expression in human monocyte derived DCs (moDCs). In addition we show, for the first time, endogenous DC-SCRIPT protein expression in human DCs both in vitro and in situ. DC-SCRIPT protein is detected early upon differentiation of monocytes into DCs and is also present in multiple freshly isolated DC subsets. Maturation of DCs with TLR ligands further increased DC-SCRIPT mRNA expression, suggesting a role in DC maturation. Indeed, siRNA mediated knock-down of DC-SCRIPT affected the cytokine response upon TLR stimulation. These DCs displayed enhanced IL-10 and decreased IL-12 production, compared to wild-type DCs. Silencing of IL-10 in DC-SCRIPT knock-down DCs rescued IL-12 expression, suggesting a primary role for DC-SCRIPT in the regulation of IL-10 production.

Introduction
Dendritic cells (DCs) are the professional antigen presenting cells of the immune system and play an essential role in the initiation and modulation of immune responses. DCs reside in the tissue in an immature state, and are capable of recognizing and capturing microbial antigens through specific receptors. Upon infection or inflammation, they undergo a complex process of maturation, where they change from antigen-capturing cells into antigen-presenting cells(1). With the expression of co-stimulatory or co-inhibitory molecules and the secretion of pro- or anti-inflammatory cytokines, DCs generate either immunity or tolerance through T-lymphocyte stimulation(2). The type of molecules that are expressed greatly depends on the activation status of the DC, and is affected by environmental stimuli(3-5).

A broad range of DC subsets have been described, including the in vitro monocyte derived DCs (moDCs) and the in vivo blood derived myeloid and plasmacytoid DCs (mDCs and pDCs, respectively). Myeloid DCs act as sentinels in the periphery and have a specialized function depending on their location and Pattern Recognition Receptors (PRRs) expression profile. The cellular cues present at
different locations, e.g. gut, skin or other organs, inflicted by local invading pathogens direct the mDCs towards a specific response. Plasmacytoid DCs are considered the front line of defense in antiviral immunity as they rapidly produce massive amounts of type I interferon in response to viral infection and prime T cells against viral antigens(6-8). In vitro, DCs can be generated from monocytes through stimulation with IL-4 and GM-CSF(9). These cytokines trigger DC differentiation while inhibiting macrophage and osteoclast differentiation(10-12).

The differentiation of the DC subsets from their precursors is a highly complex process. Genetic analyses have identified different transcription factors, including IRF4, RelB and PU.1, to be crucial in the development of specific DC subsets in lymphoid organs(13-17). DC differentiation and maturation requires a complete change in the DC gene expression profile, mediated by the combinatorial effect of a few key transcription factors and chromatin re-organization(18).

In 2006 we identified and characterized a new DC expressed transcription factor, termed dendritic cell–specific transcript (DC-SCRIPT; also known as ZNF366). DC-SCRIPT mRNA is present in all DC subsets tested so far, including moDCs, mDCs, pDCs and LCs (Langerhans cells). Interestingly, expression was not detected among other leukocyte populations(19), suggesting an essential role of DC-SCRIPT in DC biology. Outside the immune system, DC-SCRIPT has also been detected in epithelial cells in the breast and in tumors derived thereof(20, 21). DC-SCRIPT is located on human chromosomes 5q13.2(22) and is encoded by an 8 kb messenger RNA. It is well conserved in evolution, with the human and mouse genes both located in syntenic chromosomal regions, sharing 80% amino acid sequence homology(23). The protein consists of a proline rich region, 11 C2H2-type zinc fingers and an acidic region. In addition, it bears a functional CtBP1 motif and an LxxLL Nuclear Receptor (NR) interaction motif(19, 24). NRs are ligand-inducible transcription factors that bind specific DNA-regulatory response elements. NRs and their co-regulators have been described to play an important role in a wide variety of biological processes including immunobiology and cancer biology(25-29). Moreover, we demonstrated that DC-SCRIPT is a unique modulator of NR function and a strong and independent prognostic marker in breast carcinoma(20).

Thus far, the expression and function of DC-SCRIPT in DCs remains largely unknown. Here, we characterized the endogenous DC-SCRIPT protein expression dynamics in the in vitro monocyte derived DCs as well as in primary blood derived DCs and studied its functional role in DC maturation.

**Material & Methods**

**Generation of human DCs**

Human monocyte derived Dendritic Cells (moDCs) were generated from PBMCs as described previously(30). Monocytes were derived from buffy coats. Plastic-adherent monocytes were cultured for 6 days in Phenol red free RPMI-1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultra-glutamine (Cambrex, Wiesbaden, Germany), 0,5% antibiotic-antimycotic (Invitrogen, Breda, The Netherlands), 10% (v/v) FCS (Greiner, Kremsmuenster, Austria), IL-4 (300 U/ml), and GM-CSF (450 U/ml) both from cellgenix. During day 3 moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 48 hour stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Human Myeloid Dendritic Cells (mDCs) were isolated from PBMCs using the CD1c (BDCA-1) Dendritic Cell Isolation Kit (Miltenyi Biotec, Leiden, The Netherlands). Human Plasmacytoid Dendritic Cells (pDCs) were isolated from PBMCs using the CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotec, Leiden, The Netherlands). Purity (>90%) of the freshly isolated mDCs and pDCs were ensured by FACS staining.
RNA isolation and quantitative PCR

Total RNA was isolated from cells using an RNA isolation kit (Zymo research). RNA quantity and purity were determined on a NanoDrop spectrophotometer. RNA was treated with DNase I (amplification grade; Invitrogen) and reverse-transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad, Veenendaal, The Netherlands) with SYBR Green (Roche, Woerden, The Netherlands) as the fluorophore and gene specific oligonucleotide primers. The primers for DC-SCRIPT and PBGD were described previously (20) Other used primers (forward, reverse): IL-12 (5'-ATGGCCCTGTGCCCTAGTAGT-3', 5'-CGGTTCTTCAAGGGAGGATTTT-3'), IL-6 (5'-GCTATGAACTCTTCTCCACAAGCG-3', 5'-ATCCATCTTTTTCAGCCATCTTTTG-3'), TNF (5'-ATGAGCAGCTGAAGACATGATCC-3', 5'-GAGGGCTGATTAGAGAGGTC-3'), IL10 (5'-TCAAGGGCGCATGTAACCTCC-3', 5'-GATGTCAAACTCACTCATGGCT-3'). Reaction mixtures and program conditions were used that were recommended by the manufacturer (Bio-Rad). Quantitative PCR data were analyzed with the CFX Manager V1.6.541.1028 software (Bio-Rad) and checked for correct amplification and dissociation of the products. mRNA levels of the genes of interest were normalized to mRNA levels of the housekeeping gene porphobilinogen deaminase (PBGD) and were calculated according to the cycle threshold method (31).

Immunohistochemistry

Snap-frozen tonsil specimens were obtained from the Department of Pathology, RUNMC St, Radboud and approved by the institutional ethics committee of the RUNMC. The specimens were embedded in OCT embedding matrix (CellPath, Newtown, UK) and sectioned in 5 μM thick tissue sections. The sections were placed on Superfrost slides (Thermo Scientific, Etten-Leur, the Netherlands), fixed with acetone, and incubated with 4 μg/mL goat anti-human DC-SCRIPT antibody (R&D Systems, Abingdon, UK), 4 μg/mL mouse anti-human DC-SIGN (AZN-D1), followed by incubation with a biotinylated horse anti-goat IgG or horse anti-mouse (Vector Laboratories), and signal development was performed using a Vectastain ABC-HRP Kit (Vector Laboratories, Burlingame, CA) and DAB (Sigma Aldrich, Zwijndrecht, the Netherlands). Isotype-matched goat IgG (R&D Systems) and mouse IgG1 (BD Bioscience) were used as controls. Sections were counterstained with hematoxylin to visualize the cell nuclei and analyzed by using a Leica DM LB microscope (Leica Microsystems B.V., Rijswijk, the Netherlands).

Western blotting

Cells were lysed in 1% SDS and 62,5mM Tris pH 6.8 and the protease inhibitors 2 μg/mL leupeptin (Sigma Aldrich), 2 μg/mL apro tinin (Roche), and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cell lysates were mixed with sample buffer containing 5% glycerol, 6% sodium dodecyl sulfate, 125 mM Tris–HCl (pH 6.8), 0.1 mg/mL bromophenol blue (Gebr. Schmid GmbH + Co, Freud enstadt, Germany), and 10%-mercaptoethanol (Sigma Aldrich); heated at 95°C for 5 minutes; and then cooled on ice. The proteins were resolved by electrophoresis on an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) and transferred overnight to Protran nitrocellulose transfer membranes (Schleicher and Schuell, ‘s-Hertogenbosch, the Netherlands) at 30 mA and 4°C. To block nonspecific protein binding, the membranes were incubated in 1% skimmed milk powder and 3% bovine serum albumin in PBST. The membranes were then incubated for 1 hr with 2,5 μg/mL goat anti-human DC-SCRIPT antibody (R&D Systems, Abingdon, UK), washed three times in PBST, and subsequently incubated for 1 hour with the secondary antibody IRDye 800CW donkey anti goat IgG.
(1:5000 dilution; Li-cor Biosciences, Bad Homburg, Germany) to detect DC-SCRIPT. To detect actin, the membranes were incubated with a mouse anti-actin (1:20,000 dilution, Sigma clone AC-40), washed three times in PBST, and incubated for 1 hour with the secondary antibody Alexa Fluor 680–conjugated Donkey-anti-mouse IgG (1:5000 dilution; Invitrogen). All membranes were then washed three times in PBST. After staining, the membranes were scanned by using an Odyssey Infrared Imaging System (Li-cor Biosciences) to visualize the proteins.

**Confocal Laser Scanning Microscopy**

Round ø 12 mm cover slides (Thermo Scientific, Braunschweig, Germany) were coated with Poly-L-Lysine (Sigma Aldrich). Immature and mature moDCs (day 6), mDCs and pDCs were seeded on cover slides (50,000 cell/slide) and adhered for 2 hours in serum free, phenol red free RPMI-1640 supplemented with 1% ultra-glutamine, 0.5% antibiotic-antimycotic, IL-4 (300 U/ml) and GM-CSF (450 U/ml). DCs were fixed using 1% paraformaldehyde extra pure DAC 1 (MERCK, Haarlem, The Netherlands) in PBS for 15 min at RT. DCs were permeabilized with 100% ice cold Methanol (Boom, Meppel, The Netherlands) for 5 minutes at 4°C, washed with PBS, blocked for 1 hour with 3% BSA (Roche) and 1% Normal Donkey Serum (Sigma Aldrich) in PBS, stained 1 hour with 2.5 μg/ml Goat-anti-human DC-SCRIPT (R&D Systems, Abingdon, UK) and 1 hour with 1/400 Alexa Fluor 488 Donkey anti-Goat IgG (Invitrogen). The nucleus was stained for 5 minutes with 0.3 μg/ml DAPI (Sigma Aldrich) or 1 μg/μl Propidium Iodide (ITK, Uithoorn, The Netherlands), washed with PBS and mounted on 76 x 26 mm microscope slide (Thermo Scientific) with mowiol + 2.5% azide (Calbiochem, San Diego, US). Confocal laser scanning microscopy (CLSM) was carried out with an Olympus FV1000 Confocal Laser Scanning Microscope with an Argon (457, 488, 515nm), and 405, 559 and 635 diode lasers at the Microscopic Imaging Facility of the Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. The N/C ratio (mean Nuclear values/mean Cytoplasmic values) of DC-SCRIPT expression was calculated using a custom written quantitative image analysis algorithm in Fiji/ImageJ software (http://fiji.sc/).

**Goat-anti-DC-SCRIPT validation**

Immature moDCs were stained with 2.5 μg/ml goat-anti-human DC-SCRIPT or mouse-anti-human GR (Abcam, Cambridge, UK) for 40 minutes and 1 hour with 1/400 Alexa Fluor 488 Donkey-anti-goat or Goat-anti-mouse. Prior to staining the primary antibodies were supplemented with vehicle (2.9 μl 10 mM HCl + 2.9 μl 10mM NaOH) or 9.96 μg/ml recombinant human DC-SCRIPT (R&D Systems, Abingdon, UK).

**siRNA mediated knock-down**

For DC-SCRIPT silencing a 23 nucleotide Custom ZNF366 siRNA termed SC38 targeting the DC-SCRIPT gene at position 2349-2369 was used (Dharmacon, Lafayette, Colorado, US). For IL-10 silencing the ON-TARGETplus SMARTpool IL10 (Dharmacon) containing 4 different IL-10 targeting siRNA oligos each 21 nucleotides long was used. The irrelevant siRNA ON-TARGETplus Non-Targeting siRNA1 (Dharmacon) was used as control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 μg of siRNA was transferred to a 4-mm cuvette (Bio-Rad), and 10 × 10⁶ DCs were added in 200 μL of OptiMEM and incubated for 3 minutes before being pulsed with an exponential decay pulse at 300 V, 150 μF in a GenePulser Xcell (Bio-Rad) as described previously (32). Immediately after electroporation, the cells were transferred to warm (37°C) DC culture medium without AA and supplemented with 1% ultra-glutamine, 0.5% antibiotic-antimycotic,
10% (v/v) FCS, IL-4 (300 U/ml), and GM-CSF (450 U/ml). Day 6 (72 hours after transfection) DCs were stimulated with vehicle, 200 ng/ml LPS, 4 μg/ml R848 (Axxora, Raamsdonkveer, The Netherlands) or 20 μg/ml PolyI:C (Sigma Aldrich) for 24 hours. RNA was isolated with the Quick-RNA MiniPrep kit (Zymoresearch). Total lysates were prepared 72 hours after transfection, lysing 50,000 cells in 50 μl 1% SDS lysis buffer containing 1% SDS and 62.5 mM TRIS pH 6.8 plus the protease inhibitors 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride.

**ELISA**

Cytokines were measured in the supernatants 24 hours after induction of maturation. IL-12p70 production was measured using a standard sandwich enzyme-linked immunosorbent assay (ELISA; Pierce Biotechnology, Etten-Leur, The Netherlands). IL-6 was measured using PeliPair human IL-6 ELISA kit (Sanquin, Amsterdam, The Netherlands), TNF was measured using Human TNF ELISA Set (BD Biosciences, Breda, The Netherlands), IL-10 was measured using Human IL-10 Module Set (Bender MedSystems, Vienna, Austria). Differences in cytokine production were assessed using t tests. Two-sided P values less than .05 were a priori considered to be statistically significant.

**Mixed Leukocyte Reaction**

DCs were electroporated at day 4 of differentiation with siSC or control siRNA and seeded in a 96-well plate (50000 cells/well). At day 7 DCs were stimulated with vehicle or 4 μg/ml R848 for 8 hours, after which the medium was replaced with fresh DC-medium. At day 8, 24 hours after R848 stimulation, PBLs were added to the DCs, in a ratio of 1:1, and co-cultured for 120 hours. After 4 days of co-culture cells were pulsed with [3H]-thymidine for 15 hours, harvested, and [3H]-thymidine incorporation was determined as a measure for T-cell proliferation. To analyze the T helper cell profile, supernatants were collected after 2 days of DC-PBLs coculture. Cytokine production in the supernatant were analyzed with a human Th1/Th2 Multiplex kit (eBioscience, Vienna, Austria) according to manufacturer’s instructions.

**Figure 1. DC-SCRIPT mRNA expression in moDCs**

(A and B) DC-SCRIPT mRNA expression relative to PBGD mRNA in monocytes as determined by quantitative polymerase chain reaction. Monocytes were cultured in the presence of vehicle (white bars), IL-4 (light grey bars), GM-CSF (dark grey bars) or IL-4 and GM-CSF (black bars) and harvested at the indicated time points. Day 6 DCs were stimulated with vehicle or LPS for 48 hours to obtain immature and mature day 8 DCs, respectively. Representative data from 1 out of 3 donors.
Results

IL-4 induces DC-SCRIPT mRNA expression in monocytes
Within the immune system, human DC-SCRIPT mRNA has been found to be preferentially expressed by DCs(19) To obtain more insight into the expression characteristics of DC-SCRIPT, we investigated DC-SCRIPT mRNA expression during differentiation of monocytes into DCs. Hereto, adherent monocytes were cultured in the presence of IL-4(33, 34) GM-CSF or the combination of both cytokines. Cells were analyzed at different time points after start of differentiation. In the absence of cytokines (vehicle) essentially no DC-SCRIPT mRNA expression could be detected. In the presence of GM-CSF alone, only small amounts of DC-SCRIPT mRNA were discerned. Incubation with IL-4 and GM-CSF or IL-4 alone resulted in DC-SCRIPT mRNA expression within 2 hours after the start of stimulation, indicating IL-4 as the dominant factor for DC-SCRIPT induction. An increase in DC-SCRIPT mRNA levels was observed up to 8 hours after stimulation (figure 1A). At later time points, DC-SCRIPT mRNA levels decreased somewhat but remained stable from day 3 to day 8. DCs matured with LPS demonstrated an increase in mRNA expression levels (figure 1B), suggesting a role for DC-SCRIPT in DC maturation.

DC-SCRIPT protein is expressed in different DC subsets
To confirm endogenous DC-SCRIPT protein expression, cell lysates were prepared from monocytes at different time points after the onset of differentiation towards DCs. Within 4 hours, DC-SCRIPT protein expression could be observed. Protein levels steadily increased during differentiation to DCs and remained constant from day 6 onwards (Figure 2A). In line with its mRNA expression, DC-SCRIPT protein expression is also dependent on IL-4 (data not shown).
To prove that DC-SCRIPT protein is also present in freshly isolated blood mDCs and pDCs, cell lysates were prepared from purified mDCs and compared to total PBMC (peripheral blood mononuclear cells). DC-SCRIPT protein could not be detected in total PBMCs (figure 2B, lane 1), nor could it be detected in the mDC negative fraction (figure 2B, lane 2). However, in the mDC fraction DC-SCRIPT protein expression was readily observed (figure 2B, lane 3). Similarly, DC-SCRIPT protein was present in pDCs, albeit at lower levels compared to moDCs and mDCs (figure 2C, lane 3). No DC-SCRIPT expression could be detected in the total PBL (peripheral blood leukocyte) fraction (figure 2C, lane 1) and the pDC negative fraction (figure 2C, lane 2). These data show for the first time that DC-SCRIPT is endogenously expressed at protein level in freshly isolated mDCs and pDCs.

DC-SCRIPT sub-cellular distribution varies among DC subsets
The localization of endogenous DC-SCRIPT protein in the different subsets of DCs was investigated with confocal laser scanning microscopy (CLSM). Hereto, DCs were stained with anti-DC-SCRIPT antibodies recognizing the C-terminal part of DC-SCRIPT. The specificity of the antibody was validated by DC-SCRIPT peptide blocking experiments (supplemental figure S1). Our data show that DC-SCRIPT is predominantly localized in the nucleus of moDCs, and reveal that localization does not change upon maturation of the cells with LPS (figure 3A). Some DC-SCRIPT expression could be discerned in the cytoplasm. In fresh mDCs, DC-SCRIPT localization is also most pronounced in the nucleus of the cells. In pDCs, expression levels of DC-SCRIPT were apparently lower compared to mDC and moDC subsets. DC-SCRIPT staining could be found in both the cytoplasm and nucleus of pDCs, depending on the donor. Quantification of DC-SCRIPT expression in the nucleus and the cytoplasm was used to confirm localization differences between pDC donors, and between mDCs and pDCs. Between pDC donors the nucleus to cytoplasm (N/C) ratio varied between 1,1 and 2,4 (mean: 1,5 +/- 0,59) In
contrast, mDCs displayed a N/C ratio of 2.5, whereas moDCs showed an average ratio of 3. The variation in N/C ratio within pDCs and mDCs from a single donor was minimal. These data show that the DC-SCRIPT localization is predominantly nuclear in moDCs and mDCs, while in pDCs a more pronounced cytoplasmic DC-SCRIPT staining is observed that varies between different pDC donors.

To further confirm DC-SCRIPT protein expression in DCs in immunological tissue, frozen tonsil sections were analyzed for DC-SCRIPT expression. The presence of DCs was confirmed by staining the consecutive section with the DC marker DC-SIGN (figure 3B). As expected, DCs with myeloid appearance were mainly present in the T-cell area in between the germinal centers, as shown by the DC-SIGN staining. The area in which DC-SCRIPT positive cells were found overlapped with the area containing DC-SIGN, a previously defined marker for myeloid DCs (35). Furthermore, also DC-SCRIPT expression was observed in cells with myeloid DC morphology located in the T-cell area, further substantiating its protein expression in mDCs. In situ, DC-SCRIPT expression in the observed myeloid DCs appeared to be mostly confined to the nucleus. The low expression levels of DC-SCRIPT in pDCs, and their low abundance in lymph nodes, did not allow proper assessment of pDCs with this approach.

**DC-SCRIPT knock-down affects IL-10 secretion by moDCs**

DC-SCRIPT mRNA and protein is expressed during the complete lifecycle of moDCs, including in mature DCs. To investigate its function in DC maturation, DC-SCRIPT was silenced using a siRNA oligo (siSC) targeting the acidic region of the DC-SCRIPT gene at position 2349-2369. DCs treated with non-targeting siRNA oligos as well as non-treated DCs were used for comparison. Subsequently, immature DCs were stimulated for 24 hours with ligands for TLR4, -7/8 and -3, respectively LPS, R848 and PolyI:C. Our data demonstrate an efficient knock-down of DC-SCRIPT protein expression in siSC treated DCs at day 6 of differentiation prior to stimulation, but not control siRNA-treated and non-treated DCs (figure 4A).

The effect of DC-SCRIPT knock-down on DC maturation was examined by investigating cell surface maturation marker expression and cytokine secretion in the supernatant, at respectively 48 hours and 24 hours after stimulation. As expected, non-electroporated DCs markedly increased expression
Figure 3. DC-SCRIPT localization in different DC subsets
CLSM analysis of DC-SCRIPT expression in different DC subsets. DC-SCRIPT in immature and mature DCs (A) or pDCs was stained with a goat-anti-DC-SCRIPT (green), the nucleus was visualized by staining with DAPI (blue). DC-SCRIPT in myeloid DCs was stained with a goat-anti-DC-SCRIPT (green), the nucleus was visualized by staining with PI (red). The scale bar represents 10 µm. (B) Immunohistochemistry staining of DC-SIGN and DC-SCRIPT in frozen tonsil sections. Cells were counterstained with hematoxiline to visualize the nuclei.
of both maturation markers CD80 and CD83 upon TLR stimulation (figure 4B). DCs electroporated with siSC or irrelevant siRNA (control) also enhanced CD80 and CD83 expression upon activation, at equal intensities. Relative to untreated DCs, electroporated DCs showed some disparity in CD83 expression upon LPS stimulation, possibly due to the variable maturation effects by LPS.

DC maturation was also qualified by analyzing cytokine secretion (figure 4C). Secretion of the pro-inflammatory cytokines IL-6 and TNF by non-stimulated DCs electroporated or not, could not be detected, in agreement with their immature status. Stimulation with TLR ligands differentially induced IL-6 and TNF secretion, with highest levels found upon R848 treatment, and lowest levels after PolyI:C stimulation. Both electroporated and not electroporated DCs secreted equal amounts of IL-6 and TNF. No significant effect of DC-SCRIPT silencing was detected relative to control siRNA treated and non-treated DCs. Variation after LPS treatment, was again detected between donors. In line with the absence of pro-inflammatory cytokine expression, also secretion of the anti-inflammatory cytokine IL-10 was minimal in all conditions in immature DCs. As expected, TLR

![Figure 4. Cytokine secretion by DC-SCRIPT knock-down moDCs](image)

Day 4 moDCs were not electroporated (non-treated), or electroporated with control siRNA or siSC oligos. At day 6, vehicle, LPS, R848 or PolyI:C were used to mature the DCs. (A) DC-SCRIPT expression at day 6, prior to stimulation, was analyzed by western blot analysis. DC-SCRIPT was visualized by immunoblotting with anti-DC-SCRIPT and anti-actin as a loading control. Maturation of non-treated DCs (white bars), control siRNA treated DCs (grey bars) and siSC treated DCs (black bars) was analyzed by measuring maturation markers CD80 and CD83 by means of FACS (B) and secretion of IL-6, TNF and IL-10 in the supernatant by means of ELISA (C), respectively 48 hours and 24 hours after stimulation. Data from at least 3 donors. Error bars correspond to +/- SEM.
mediated maturation did induce only a minimal amount of IL-10 expression in both siRNA control DCs and non-treated DCs. Surprisingly, DCs electroporated with siSC displayed a significant increase in IL-10 secretion after treatment with LPS or R848. Little or no effect of DC-SCRIPT silencing could be detected upon Poly(I:C) stimulation. Hence, these data demonstrate that DC-SCRIPT expression in DCs is important for repression of IL-10 secretion during TLR4 and -7/8 induced maturation.

**Increased IL-10 secretion in siSC DCs impairs IL-12 secretion**

The anti-inflammatory cytokine IL-10 is known to impair DC maturation, including IL-12 production (2). To gain more insight into the kinetics of cytokine production in siRNA control and DC-SCRIPT knock-down DCs, cytokine mRNA and protein levels were monitored in time. To this end, IL-6, TNF, IL-12 and IL-10 mRNA and protein expression of control siRNA or siSC electroporated DCs was measured at 0-, 2-, 4-, 8-, 16- and 24 hours after R848 stimulation (figure 5). Both siSC and control siRNA treated cells demonstrated maximum mRNA expression between 2 and 16 hours for all cytokines, which decreased at later time points. Maximum levels of IL-6 and TNF expression were detected 4 hours after stimulation, whereas IL-12p35 and IL-10 mRNA expression peaked at 8 hours. The protein expression of IL-6, TNF and IL-10 followed the mRNA expression kinetics, reaching maximum levels at later time points, after which expression remained relatively stable. When comparing control siRNA and siSC treated DCs, no effect was found for IL-6 or TNF secretion. In contrast, siSC and control siRNA DCs differed greatly in the expression of IL-10 and IL-12. In addition to the increase in IL-10 production, IL-12 production was significantly impaired at both the mRNA and the protein level in siSC DCs. The impaired IL-12 expression observed in siSC DCs was preceded by the increased IL-10 secretion, suggesting a role for IL-10 in reducing IL-12 levels in siSC DCs.

**IL-10 silencing rescues IL-12 secretion in DC-SCRIPT knock-down DCs**

In order to confirm the role of enhanced IL-10 secretion on the expression of IL-12, both DC-SCRIPT and IL-10 expression were silenced. To this end, DCs were electroporated with either control siRNA, siSC with control siRNA or siSC with IL-10 targeting siRNA (siIL-10). Day 6 DCs were stimulated with R848 for 24h after which cytokine production was measured in the supernatant. As an additional

**Figure 5. Kinetics of cytokine expression**

Day 4 moDCs were electroporated with control siRNA (black) or siSC oligos (grey). At day 6 DCs were stimulated with vehicle or R848. Messenger RNA and protein were measured at the indicated time points for IL-10, IL-12, IL-6 and TNF expression by means of Q-PCR and ELISA, respectively. Representative data from 1 out of 3 donors.
control, untreated DCs stimulated with R848 received $10^6$ U/ml IL-10 or vehicle 4 hours after R848 stimulation, in order to mimic the enhanced IL-10 secretion in siSC DCs.

Again, efficient knock-down of DC-SCRIPT protein expression was detected in all conditions electroporated with siSC prior to stimulation (figure 6A). IL-10 protein expression in the supernatant was measured 24 hours after R848 by means of ELISA. Data from 5 donors. Error bars correspond to +/- SEM.

(C) Analysis of IL-10, IL-12, IL-6 and TNF secretion in the supernatant of control siRNA DCs (white bars), siSC + control siRNA DCs (grey bars) and siSC + siIL-10 DCs (black bars), 24 hours after stimulation, by means of ELISA. Data from at least 3 donors. Error bars correspond to +/- SEM.

**DC-SCRIPT knock-down impairs T-cell responses**

Next, we investigated the biological consequences of DC-SCRIPT silencing on DC-mediated T-cell responses in an allogeneic Mixed Leukocyte Reaction (MLR). SiSC or control siRNA electroporated DCs were stimulated at day 7 with vehicle or R848 for 8 hours, after which the medium was replaced with fresh DC-medium. Twenty-four hours after stimulation, PBLs were added and both T-cell proliferation and cytokine secretion were determined as a measure of T-cell activation. As shown (figure 7A), T-cell proliferation was readily detected upon stimulation with control siRNA treated DCs and was significantly impaired after stimulation with siSC treated DCs. The effect of R848 stimulation on T cell proliferation was limited, possibly reflecting the overall immune activation at these allogeneic conditions. In contrast to T cell proliferation, IFNγ secretion by T-cells in these co-cultures was largely dependent on the presence of R848 (figure 7B). Strikingly, DC-SCRIPT silenced DCs showed an impaired capacity to induce IFNγ secretion by T-cells relative to control siRNA silenced DCs. No significant differences were detected for the pro-inflammatory cytokine levels of IL-6 and...
Collectively, these data strengthen the finding that DC-SCRIPT plays an important role during DC maturation and the induction of T cell responses.

**Discussion**

Previously, we have isolated and characterized the cDNA encoding the transcription regulator DC-SCRIPT (19, 23) that is preferentially expressed in DCs within the immune system. In the present study, DC-SCRIPT mRNA and protein expression were found to be induced early in DC differentiation and were dependent on IL-4. Silencing of DC-SCRIPT expression affected DC maturation and induced IL-10 secretion in mature DCs, which consequently impaired IL-12 secretion by these cells. Furthermore, DC-SCRIPT silenced DCs were shown to have a significantly impaired capacity to induce T-cell proliferation and IFNγ responses. Hence, DC-SCRIPT appears to be an important factor in regulating DC maturation.

In human moDCs, DC-SCRIPT expression was dependent on the presence of IL-4. Control experiments demonstrated no DC-SCRIPT expression in PBLs upon IL-4 stimulation (data not shown). In addition, although in vivo studies previously demonstrated DC-SCRIPT expression in breast epithelial cells (20), IL-4 stimulation of the DC-SCRIPT negative MCF-7 breast carcinoma cells did not induce DC-SCRIPT expression (data not shown). These data therefore suggest that the IL-4 mediated induction of DC-SCRIPT is related to the differentiation of monocytes to DCs. It is therefore important to further deduce the expression and function of DC-SCRIPT in the DC differentiation process itself.

Next to moDCs, DC-SCRIPT expression was readily detected in mDCs and pDCs. Previous reports demonstrated DC-SCRIPT mRNA expression in all DC subsets tested, including LCs, mDCs and pDCs (19). Here we show, for the first time, endogenous DC-SCRIPT protein expression in both mDCs and pDCs. Moreover, in vivo DC-SCRIPT expression was observed in DCs present in T-cell areas of tonsil tissue. In IHC- and CLSM slides DC-SCRIPT is predominantly localized in the nucleus of moDCs and mDCs, concomitant with the presence of a nuclear localization motif. Interestingly, some expression was also detected in the cytoplasm of moDCs and mDCs. This was even more pronounced in pDCs, however, it must be noted that expression is lower and that variations between donors were
observed for this subset. In addition, our recent work demonstrated a predominant cytoplasmic expression of DC-SCRIPT in breast epithelial cells(20). This suggests that DC-SCRIPT might also have important functions outside the nucleus.

Further insight into the cytoplasmic expression of DC-SCRIPT might be gained from the NR biology. In a previous publication we have characterized DC-SCRIPT as a NR co-regulator(20). NRs and many of their co-regulators are found both in the nucleus as well as in the cytoplasm. The type I NRs are classically sequestered in the cytoplasm, and translocate to the nucleus upon ligand binding, where they bind to specific DNA sequences(26, 36-39). NR function is controlled by NR co-regulators, such as N-CoR and SMRT. Recent evidence suggests shuttling of these co-regulators between the nucleus and cytoplasm, triggered by changes in signaling at the cell surface(40). Further research is necessary to fully elucidate the pattern and dynamics of DC-SCRIPT expression in different DC subsets. Investigating the effects of various extracellular signals, such as NR ligands, will provide more insight into the DC-SCRIPT localization characteristics in DCs.

In addition, our data uncovered an important role for DC-SCRIPT in DC maturation. DCs with silenced DC-SCRIPT expression displayed enhanced IL-10 and decreased IL-12 cytokine production upon maturation. Silencing IL-10 expression rescued the IL-12 secretion in DC-SCRIPT knock-down DCs, suggesting that DC-SCRIPT primarily affects the expression of IL-10. No effect was detected on IL-6 and TNF secretion, or after PolyI:C maturation. Interestingly, TLR4 and -7/8 signal via the adaptor protein MyD88, whereas TLR3 activates TRIF mediated signaling(41), suggesting that DC-SCRIPT primarily functions in the MyD88 pathway. Functionally, DC-SCRIPT silenced DCs are less capable of inducing T-cell proliferation and IFNγ secretion in a co-culture with allogeneic PBLs.

Unraveling the underlying molecular mechanisms by which DC-SCRIPT can regulate IL-10 expression in DCs will be an important next step. In DCs, IL-10 transcription is induced through NF-κB activation(42). One way of enhanced and prolonged IL-10 transcription is NF-κB acetylation(43). It would therefore be of great interest to see whether, and how DC-SCRIPT is involved in NF-κB acetylation. Furthermore, besides immunostimulatory DCs, future studies on DC-SCRIPT expression and function should also include tolerogenic DCs (tolDCs). These immunosuppressive DCs are known to secrete elevated levels of IL-10, while having diminished IL-12 secretion, thereby preventing T-cell proliferation(44). Studying tolDCs is even more interesting as we have recently found that DC-SCRIPT also affects the function of the Glucocorticoid Receptor (GR) (Hontelez et al., submitted). GR is well known for its central role in the generation of tolerogenic DCs, and known to induce IL-10 production. Moreover, the IL-10 promoter contains a Glucocorticoid Responsive Element (GRE), that could serve as a binding site for GR to stimulate IL-10 transcription(45, 46).

DCs are in the centre of the immune system, controlling the type and course of an immune response. They regulate both innate and adaptive immunity and serve as a bridge between both systems. Therefore, DCs are regularly used in immunotherapy. Detailed understanding of DC differentiation and maturation will allow for the generation of the best suitable DC for these therapies. Collectively, our data provide important insight in the DC biology, highlighting DC-SCRIPT as an essential factor in DC maturation.

Acknowledgements
We thank Marjolein Meddens for technical assistance regarding the quantification of DC-SCRIPT expression in the CLSM data.
Chapter 4 - DC-SCRIPT regulates TLR signaling in DCs

References


Figure S2. DC-SCRIPT knock-down does not affect IL-6 and TNF secretion in a MLR
Effect of DC-SCRIPT silencing on IL-6 and TNF secretion in an allogeneic mixed leukocyte reaction (MLR). Day 4 moDCs were electroporated with control siRNA or siSC oligos. At day 7 DCs were stimulated with vehicle (not shown) or R848 for 8 hours. Day 8 DCs were co-cultured with PBLs in a ratio of 1:1. Data from 3 donors. Error bars correspond to +/- SEM. Data indicated with N.S. (not significant) did not meet the criteria in the t test to be considered statistically significant.
Chapter 5

DC-SCRIPT regulates Glucocorticoid Receptor function and expression of its target GILZ in dendritic cells

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Journal of Immunology (2013) 190 (7), 3172-3179
DC-SCRIPT regulates Glucocorticoid Receptor function and expression of its target GILZ in dendritic cells

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Abstract

Dendritic cells (DCs) play a central role in the immune system; they can induce immunity or tolerance depending on diverse factors in the DC environment. Pathogens, but also tissue damage, hormones and vitamins affect DC activation and maturation. In particular glucocorticoids (GCs) are known for their immunosuppressive effect on DCs, creating tolerogenic DCs (tolDCs). GCs activate the Type I Nuclear Receptor (NR) Glucocorticoid Receptor (GR), followed by induced expression of the transcription factor GILZ (Glucocorticoid Inducible Leucine Zipper). GILZ has been shown to be necessary and sufficient for GC induced tolDC generation. Recently, we have identified the dendritic cell specific transcript (DC-SCRIPT) as a NR co-regulator, suppressing type I steroid NRs ER (estrogen receptor) and PR (progesterone receptor). Here, we analyzed the effect of DC-SCRIPT on GR activity. We demonstrate that DC-SCRIPT co-exists with GR in protein complexes, and functions as a co-repressor of GR mediated transcription. Co-expression of DC-SCRIPT and GR is shown in human monocyte derived DCs, and DC-SCRIPT knock-down enhances GR dependent up-regulation of GILZ mRNA expression in DCs. This demonstrates that DC-SCRIPT serves an important role in regulating GR function in DCs, co-repressing GR dependent up-regulation of the tolerance inducing transcription factor GILZ. These data imply that by controlling GR function and GILZ expression, DC-SCRIPT is potentially involved in the balance between tolerance and immunity.

Introduction

Dendritic cell (DC) maturation occurs upon pathogen recognition and inflammation in the peripheral tissue, and involves up-regulation of co-stimulatory molecules and inflammatory cytokine secretion. Fully matured DCs migrate to T-cell areas in the lymph nodes, inducing T-cell activation (1). In addition, DCs can also be activated to become tolerogenic, suppressing inflammation. Both human and murine studies demonstrated that this process involves impaired DC activation, which can be triggered by ligand dependent activation of the Glucocorticoid Receptor (GR) prior to pathogen recognition (2). Within DCs, GR activation impairs STAT, NF-κB, AP-1, 14-3-3, Raf-1 and Ras signalling, thereby preventing up-regulation of co-stimulatory molecules upon DC maturation. Expression of the GR target gene GILZ is induced upon glucocorticoid (GC) stimulation, and serves an important role in mediating the immunosuppressive effects by GR. In fact, expression of GILZ in the absence of GR activation was demonstrated to be sufficient for the generation of tolDCs, whereas silencing GILZ expression prevented tolerance (3). GC treated DCs displayed suppressed MHC-I restricted antigen presentation (4, 5), impaired up-regulation of co-stimulatory molecules (6, 7) and reduced secretion of pro-inflammatory cytokines. Secretion of immunosuppressive cytokines was enhanced in these
Collectively, these effects result in altered DC mediated T-cell activation, with reduced Th1 responses and selective expansion of T regulatory cells (10, 11). This does not only apply to monocyte derived DCs (moDCs), also myeloid DCs (MDCs) (12) and plasmacytoid DCs (PDCs) (13) have been shown to become tolerogenic following GC exposure.

The immunosuppressive effects of GCs are typically mediated by GR, a Type I Nuclear Receptor. The ligand-free form of GR predominantly resides in the cytoplasm, complexed to chaperone proteins. Conformational changes through ligand binding release GR from the chaperone complex, allowing for nuclear translocation and transcription initiation (14).

The human GR is encoded by a single gene, and is expressed in virtually all cell types. Various GR isoforms have been described, and tissue specific effects are currently attributed to variation in GR isoform expression (15-17). GR pre-mRNA can be alternatively spliced generating the transcriptional active GRα and the repressor GRβ, differing in sequence only at the C-termianus ligand binding domain (LBD). In contrast to GRα, GRβ expression is confined to the nucleus, where it antagonizes GRα dependent gene transcription (18). Additionally, translation reinitiation occurs at seven AUG-start sites at the mRNA 5’-end, generating 8 different GR polypeptides. These isoforms, termed GR-A, -B, -C1, -C2, -C3, -D1, -D2 and -D3, differ in length at the N-terminus and in glucocorticoid responsiveness, differentially affecting target gene expression. The GR-A, -B and -C isoforms are localized in the cytoplasm, translocating to the nucleus upon ligand binding where they initiate target gene transcription. In contrast, GR-D isoforms are, independent of ligand binding, confined to the nucleus, and have markedly lower transcriptional activity (16).

Recently, we have identified DC-SCRIPT (DC-Specific transCRIPT) as a transcription factor and a NR co-regulator in human breast and prostate carcinoma tissue. DC-SCRIPT was found to be present in Type I and Type II NR protein complexes, repressing Type I NRs ER, PR or AR (androgen receptor) mediated transcription, while enhancing Type II NRs RARα/RXR, VDR/RXR or PPARγ/RXR function (19-22). In the immune system DC-SCRIPT is specifically expressed in DCs, and in contrast to other DC markers, identifies all DC-subsets tested to date (23, 24). Interestingly, we have demonstrated an important role for DC-SCRIPT in TLR4 and TLR7/8 mediated DC maturation. DC-SCRIPT knock-down in moDCs increased secretion of the anti-inflammatory cytokine IL-10, which subsequently impaired both the production of the pro-inflammatory cytokine IL-12, as well as T-cell proliferation (25). Here, we investigated the putative role of DC-SCRIPT in controlling the anti-inflammatory function of GR in moDCs. Our data show the presence of DC-SCRIPT in GR protein complexes, and demonstrate altered GR mediated transcription in the presence and absence of DC-SCRIPT. Our findings therefore suggest active regulation of GR function by DC-SCRIPT in DCs.

Material and Methods

Cell lines

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing GlutaMAX (Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS (Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotic – antimycotic (Invitrogen). Human hepatocellular carcinoma Hep3B cells were cultured in IMDM (Invitrogen) supplemented with 10% heat-inactivated FCS and 0.5% antibiotic-antimycotic (Invitrogen).

Generation of human Dendritic Cells

Human moDCs were generated from PBMCs as described previously (26). Monocytes were derived
Part 3 - Nuclear Receptor Coregulators

from buffy coats from healthy donors (Sanquin, Nijmegen, The Netherlands). Buffy coats were obtained from healthy volunteers after informed consent and according to institutional guidelines. Plastic-adherent monocytes were cultured for 6 days in DCs culture medium (Phenolred free RPMI-1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 1% ultra-glutamine (Cambrex, Wiesbaden, Germany), 0,5% antibiotic-antimycotic (Invitrogen, Breda, The Netherlands), 10% (v/v) FCS (Greiner Bio-one, Alphen a/d Rijn, the Netherlands), IL-4 (300 U/ml), and GM-CSF (450 U/ml) both from cellgenix). During day 3 moDCs were supplemented with new IL-4 (300 U/ml) and GM-CSF (450 U/ml). Mature moDCs were generated from day 6 immature moDCs through 24 hr stimulation with vehicle (0,1% EtOH) followed by 24 hour stimulation with 200 ng/ml LPS (InvivoGen, Toulouse, France). Tolerogenic moDCs were generated from day 6 immature moDCs through 24 hour stimulation with 100 nM dexamethasone and subsequent 24 hours with 200 ng/ml LPS. DC maturation was ensured by FACS staining.

Confocal Laser Scanning Microscopy

Round ø 12 mm cover slides (ThermoScientific, Braunschweig, Germany) were coated with Poly-L-Lysine (Sigma Aldrich, Zwijndrecht, the Netherlands). Immature, mature and tolerogenic moDCs were seeded on cover slides (50.000 cell/slide) and adhered for 2 hours in serum free, phenolred free RPMI-1640 supplemented with 1% ultra-glutamine, 0,5% antibiotic-antimycotic, IL-4 (300 U/ml) and GM-CSF (450 U/ml). DCs were stimulated for 1 hour with 100 nM dexamethasone or vehicle (0,1% EtOH). DCs were fixed using 1% paraformaldehyde extra pure DAC 1 (Merck, Haarlem, The Netherlands) in phosphate-buffered saline (PBS) for 15 min at RT. DCs were permeabiliized with 100% ice cold Methanol (Boom, Meppel, The Netherlands) for 5 minutes at 4°C, washed with PBS, blocked for 1 hour with 3% BSA (Roche, Woerden, The Netherlands) and 1% Normal Donkey Serum (Sigma Aldrich) in PBS, stained 1 hour with 2,5 μg/ml (moDCs) Goat-anti-human DC-SCRIPT (R&D Systems, Abingdon, UK) and 2,5 μg/ml (moDCs) Mouse-anti-human GR (Abcam, Cambridge, UK) and 1 hour with 1/400 Alexa Fluor 488 Donkey-anti-Goat IgG and 1/400 Alexa Fluor 647 Rabbit-anti-Mouse IgG (Invitrogen). The nucleus was stained 5 minutes with 0,3 μg/ml DAPI (Sigma Aldrich), washed with PBS and mounted on 76 x 26 mm microscope slide (Thermo Scientific) with mowiol + 2,5% azide (Calbiochem, San Diego, US). Confocal laser scanning microscopy (CLSM) was carried out with an Olympus FV1000 Confocal Laser Scanning Microscope with an Argon (457, 488, 515nm), and 405, 559 and 635 diode lasers at the Microscopic Imaging Facility of the Department of Cell Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

Plasmids

The nuclear receptor isoforms GR, GR-C3 and GR-D3 (National Center for Biotechnology Information accession number) (NM_001018077) were isolated and cloned into pHA-n3 (described previously in (20) generating pHA-n3/GR, pHA-n3/GR-C3, pHA-n3/GR-D3). The vectors pCATCH and pCATCH-DCSCRIPT were described previously (24). pCATCH-DCSCRIPT was used to generate pCATCH-DCSCRIPTΔCtBPdm and pCATCH-DCSCRIPTΔLxxLL. The transcription reporter plasmid pMMTV-luc containing the mouse mammary tumor virus (MMTV) promoter was kindly provided by Prof. Dr. H. Stunnenberg (Department of Molecular Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands). pMMTV-luc was used to generate pMMTVΔ-luc and pMMTVΔNF-1/Oct1-luc. pGRE-luc (Stratagene, La Jolla, United States) was used to generate pHRE1-luc, pHRE2-luc, pHRE3-luc and pHRE4-luc.
Co-Immunoprecipitation assay
HEK293 cells were seeded in 10 cm culture dish (6×10^6 cells per dish) 24 prior to transfection. Cells were cotransfected with 5 μg pCATCH-DCSCRIPT or pCATCH (control) and 5μg pHA-nRα or pHA-n3 (control) by using Metafectene transfection reagent (Biontex, Martinsried/Planegg, Germany) according to the manufacturer’s protocol. Cells were lysed 24 hours after transfection, in immunoprecipitation assay buffer (50 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM Tris – HCl [pH 7.5], 5 mM EDTA, and 0.5% sodium-deoxycholate) containing the protease inhibitors 2 μg/mL leupeptin (Sigma Aldrich), 2 μg/mL aprotinin (Roche, Woerden, the Netherlands), and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cell lysates were used for immunoprecipitation (IP) of DC-SCRIPT using anti-DC-SCRIPT-coupled dynabeads (GE Healthcare, Hoevelaken, The Netherlands) according to the manufacturer’s protocol. Isotype-coupled-dynabeads were used as a control.

Western Blotting
Proteins were subjected to electrophoresis on an 8% 37.5:1 acryl/Bisacrylamide gel and transferred onto Protran nitrocellulose transfer membranes (Schleicher and Schuell) for 1 hour at 100 V at 4°C. Blots were blocked in 1%Elk/3%BSA in Phosphate-buffered saline with 0.01% Tween (PBST). DC-SCRIPT was detected with anti-DC-SCRIPT (R&D, 1,2 μg/ml) and IRDye 680CW donkey-anti-goat IgG (1:5000 Li-cor biosciences) as secondary antibody. GR was detected with a rat anti-HA (1:1000, 3F10, Roche) and IRDye 800CW goat-anti-rat IgG (1:5000 Li-cor biosciences), or Anti-Glucocorticoid Receptor antibody [3D5] (Abcam, 200 μg/μl, 1:80) and IRDye 680CW goat-anti-mouse IgG (1:5000 Li-cor biosciences) as secondary antibody. Blots were probed with a mouse-anti-β-actin (1:20.000, Roche Applied Science) or rabbit-anti-actin (Sigma Aldrich) and IRDye 680CW donkey-anti-mouse IgG or goat-anti-rabbit (1:5000 Li-cor biosciences) as secondary antibody, as loading control. Both actin and DC-SCRIPT or actin and GR were visualized on the same blot using 2 different secondary detection antibodies conjugated with fluorescent molecules detected at 800 and 680 nm. After staining, the membranes were scanned using the Odyssey™ Infrared Imaging system to visualize the labeled proteins.

Luciferase transcription assay
Hep3b cells were plated (6×10^4) in 24-wells plates 8 hours before transfection and transfected using the Calcium Phosphate precipitation method (Invitrogen). HEK293 cells were plated at (1×10^5) in 24-wells plates 24 hours before transfection and transfected using metafectene. Transfected cells were stimulated with 100 nM dexamethasone, 100 nM prednisolone or vehicle (0,1% EtOH) for 24 h. Cells were harvested 24 hours after stimulation and cell lysates were analyzed for luminescence according to manufacturer’s protocol (Dual-Luciferase® Reporter assay, Promega) using a Victor^3 luminometer (PerkinElmer). Relative light units (RLU) were calculated after correction for transfection efficiency based on the activity of the co-transfected pRL-SV40 (Promega). The data are expressed as the mean activity of at least four independent experiments +/-s.e.

DC-SCRIPT knock-down
Human moDCs day 4 were electroporated with a 23 nucleotide Custom ZNF366 siRNA termed siSC targeting the DC-SCRIPT gene at position 2349-2369 (Dharmacon, Lafayette, Colorado, US) or the irrelevant siRNA ON-TARGETplus Non-Targeting siRNA#1 (Dharmacon) termed control. Cells were washed twice in PBS and once in OptiMEM without phenol red (Invitrogen). A total of 10 μg of siRNA
was transferred to a 4-mm cuvette (Bio-Rad), and 10 × 10^6 DCs were added in 200 μL of OptiMEM and incubated for 3 minutes before being pulsed with an exponential decay pulse at 300 V, 150 μF in a GenePulser Xcell (Bio-Rad) as described previously (27). Immediately after electroporation, the cells were transferred to warm (37°C) DC culture medium without AA and supplemented with 1% ultraglutamine, 0.5% antibiotic-antimycotic, 10% (v/v) FCS, IL-4 (300 U/ml), and GM-CSF (450 U/ml). Day 6 (72 hours after transfection) DCs were stimulated with vehicle (0.1% EtOH) or 100 nM RU-486 (Sigma Aldrich), for 1 hour and subsequently with vehicle (0.1% EtOH) or 100 nM prednisolone for 24 hours. RNA was isolated with the Quick-RNA MiniPrep kit (ZymoReasearch). Total lysates were prepared 72 hours after transfection, lysing 50,000 cells in 50 μl 1% SDS lysis buffer containing 1% SDS and 62.5 mM TRIS pH 6.8 plus the protease inhibitors 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride.

**RNA isolation and quantitative PCR**

Total RNA was isolated from cells using an RNA isolation kit (Zymo research). RNA quantity and purity were determined on a NanoDrop spectrophotometer. Total RNA was DNase-I (amplification grade; Invitrogen) treated and cDNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). mRNA levels for the genes of interest were determined with a CFX96 sequence detection system (Bio-Rad, Veenendaal, The Netherlands) with SYBR Green (Roche, Woerden, The Netherlands) as the fluorophore and gene specific oligonucleotide primers. The primers for DC-SCRIPT and PBGD were described previously (20). Other used primers (forward, reverse): GR (5'-CCATTGTCAAGAGGGAAGGAAAC-3', 5'-ATGATTTCAAGCTACACATCTCGG-3'), GILZ (5'-AGAACCTCAATACCGACAAG-3', 5'-CATCAGATGATTCTTCAACCA-3'). Reaction mixtures and program conditions were used that were recommended by the manufacturer (Bio-rad). Quantitative PCR data were analyzed with CFX Manager V1.6.541.1028 software (Bio-rad) and checked for correct amplification and dissociation of the products. As a reference gene the housekeeping gene porphobilinogen deaminase (PBGD) was used. DC-SCRIPT and GILZ levels relative to PBGD were calculated according to the cycle threshold method, \(2^{-\Delta\Delta Ct \text{ DC-SCRIPT or GILZ} - \Delta \text{ Ct PBGD}}\) (28). Differences in

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**Part 3 - Nuclear Receptor Coregulators**

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**Figure 1. Co-immunoprecipitation of GR with DC-SCRIPT**

Association of DC-SCRIPT with the Glucocorticoid Receptor. Lysates prepared from HEK293 cells co-transfected with flag or flag-DC-SCRIPT and HA or HA-GR were subjected to immunoprecipitation using goat-IgG or goat-anti-DC-SCRIPT antibody-coated beads. Immunoblotting was performed on the IP proteins with goat-anti-DC-SCRIPT to detect DC-SCRIPT and rat-anti-HA to detect the Glucocorticoid Receptor. Data shown are from one of three experiments that produced the same result. * indicates background band.
mRNA expression were assessed using t tests. Two-sided P values less than .05 were a priori for samples to be considered statistically significant.

Results

**DC-SCRIPT co-immunoprecipitates GR**

DC-SCRIPT was previously shown to be present in protein complexes containing type I and/or type II NRs using co-immunoprecipitation (co-IP) assays (20). Whether DC-SCRIPT is also present in GR containing protein complexes is unknown. To investigate this, whole cell lysates were prepared from HEK293 cells co-transfected with expression vectors encoding DC-SCRIPT and GR or their controls. Immunoprecipitations (IPs) were performed on these lysates using goat-anti-DC-SCRIPT-coated beads or control goat-IgG-coated beads. Both the immunoprecipitated fractions and the total lysates were subjected to immunoblotting. The data show an effective IP of DC-SCRIPT using goat-anti-DC-SCRIPT antibody-coated beads, whereas unspecific binding to the control beads is minimal (Fig. 1). In addition, GR was effectively co-immunoprecipitated with goat-anti-DC-SCRIPT-coated beads from lysates of HEK-293 cells that were co-transfected with DC-SCRIPT and GR. No GR co-IP was observed when DC-SCRIPT was co-transfected with control HA only. These data demonstrate that in transfected HEK293 cells, DC-SCRIPT and GR can exist in the same protein complex. This occurs most likely through indirect interaction, as the co-IP of GR with DC-SCRIPT could only be demonstrated when using mild lysis conditions (data not shown).

**DC-SCRIPT represses GR function on MMTV**

Next, we investigated whether DC-SCRIPT affects transcriptional activity of GR by using luciferase reporter assays. Hep3B cells were co-transfected with DC-SCRIPT, GR or their controls and a reporter construct containing the MMTV promoter controlling luciferase expression (MMTV-luc). In the MMTV promoter, four hormone responsive elements (HRE) have been identified that bind ligand activated GR, thereby inducing transcription of the luciferase gene (29). Eighteen hours after transfection the cells were treated with vehicle, or the GR ligands dexamethasone (dex) or prednisolone (pred). Luciferase production was analyzed 24 h after stimulation. Hep3B cells lack endogenous GR expression, therefore only background luciferase activity was detected after GR-ligand stimulation in the absence of ectopically expressed GR. Cells that were co-transfected with the GR expression vector did show luciferase activity in a GR ligand dependent manner. Both dexamethasone and prednisolone were able to induce luciferase production (Fig 2A). Interestingly, increasing expression levels of DC-SCRIPT resulted in a dose- and ligand dependent repression of GR activity. Previously, DC-SCRIPT has been shown to directly interact with the global transcription co-repressor CtBP1 via its CtBP1 interaction motif (24). In addition, DC-SCRIPT harbors a LxxLL motif, a motif known to facilitate interactions with NRs (5). Next, we examined whether the CtBP1 and the NR binding (LxxLL) motifs within DC-SCRIPT were important for its repressive function on GR. We therefore mutated both CtBP1 domains or deleted the LxxLL motif. Both mutants were tested for protein expression levels (data not shown), which resembled the expression levels of wild-type DC-SCRIPT. Cellular localization was also expected to be similar, since deletion of the complete acidic domain does not alter its nuclear location (24). Figure 2B shows that both mutants are still able to strongly repress GR dependent luciferase production. Thus, DC-SCRIPT functions as a co-repressor of GR induced transcription on the MMTV promoter, independent of its CtBP1 binding- and LxxLL motif. Efficient transcription initiation via the MMTV promoter requires the presence of the most distal HRE site (HRE1), the regulatory element AA upstream of HRE1 (30) and binding of the transcription
factors NF-I and Oct1 (29, 31, 32). To assess the requirement of these regulatory elements for DC-SCRIPT mediated co-repression, we deleted these sequence elements from the MMTV-luc reporter. In the MMTVΔ1-luc and MMTVΔ2-luc reporters the AA element alone, or both the HRE1 and the AA element, were deleted. Figure 2. Effect of DC-SCRIPT on GR mediated transcription

Luciferase reporter assay for GR mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmid MMTV-luc, the expression plasmid for GR and increasing amounts of the expression plasmid for wild-type DC-SCRIPT (A) or with mutated CtBP- or deleted LxxLL binding sites (B). Cells were stimulated with vehicle, 100 nM dexamethasone or 100 nM prednisolone for 24 h. Luciferase activity is displayed relative to luciferase production upon dexamethasone stimulation in the presence of GR and absence of DC-SCRIPT. (C) Schematic representation of firefly luciferase reporters. Reporters consist of the MMTV promoter with 4 hormone responsive elements (HRE) in front of a TATA-box controlling luciferase gene expression. Three MMTV mutants were created from the wildtype (MMTVwt), deleting the upstream 3'-end including the AA domain (MMTVΔ1), the 3'-end and the most distal HRE (MMTVΔ2), or the binding sites for nuclear factor 1 (NF-I) and octamer transcription factor 1 (Oct1) (MMTVΔNF-I/Oct1). (D) Luciferase reporter assay for GR mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmids MMTVwt-luc, MMTVΔ1, MMTVΔ2 or MMTVΔNF-I/Oct1, the expression plasmid for GR and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (white bars) or 100 nM dexamethasone (black bars) for 24 h. Luciferase activity is displayed relative to luciferase production upon dexamethasone stimulation in the presence of MMTVwt and in the absence of DC-SCRIPT. Data from at least 3 independent experiments. Error bars correspond to +/- SEM.
element are deleted, respectively, whereas the MMTVΔNF-I/Oct1-luc reporter lacks the NF-I and Oct1 binding sites (33) (Fig. 2C). The wild-type MMTV-luc reporter was used as positive control. Deletion of the AA element or the NF-I/Oct1 binding sites reduced GR dependent luciferase expression compared to the wt reporter, which was even further decreased upon deletion of both AA and HRE1 (Fig 2D). On all MMTV reporters/mutants, DC-SCRIPT expression effectively repressed luciferase production. These data imply that that the repressive function of DC-SCRIPT does not depend on the HRE1 and the AA element, nor the NF-I and Oct1 binding sites.

**Figure 3. Effect of DC-SCRIPT on translational isoforms of GR**

(A) Translational isoforms of GR. One GR mRNA generates 9 isoforms by means of alternative translation initiation. Numbers indicate N- and C-terminal residue of each GR isoform. Different domains are indicated: the modulation domain contains the transactivation sequence, and is subject to phosphorylation and SUMOylation affecting protein interactions; the DNA binding domain (DBD); the hinge region and the ligand binding domain (LBD). Expression plasmids have been created for underscored isoforms. The western blot shows the GR-A, -B, -C and -D isoforms visualized with rat-anti-HA in GR-HA transfected HEK293 cells. (B) Luciferase reporter assay for GR-A, GR-C and GR-D mediated transcription. Hep3B cells were co-transfected with the firefly luciferase reporter plasmid MMTV-luc, expression plasmids for GR-A, GR-C or GR-D and increasing amounts of the expression plasmid for DC-SCRIPT. Cells were stimulated with vehicle (white bars) or 100 nM dexamethasone (black bars) for 24 h. Luciferase activity is displayed relative to luciferase production upon dexamethasone stimulation in the presence of GR-A and in the absence of DC-SCRIPT. Data from at least 3 independent experiments. Error bars correspond to +/- SEM.

**DC-SCRIPT represses transcriptional activity of GR isoforms**

GR has been shown to be translated into 8 different isoforms using alternative translational start sites (Fig. 3A) (17). The GR expression vector includes all translational start sites, thus could express all GR isoforms. Indeed transfection of GR in HEK293 cells demonstrates expression of the full length GR-A, as well as the GR-B, -C, and, albeit at markedly lower levels, GR-D isoforms (Fig. 3A). To examine DC-SCRIPT function on different GR isoforms, we also cloned GR-C3 and GR-D3. Of note, the GR-C3 vector includes downstream ATGs, hence in addition to GR-C3, this vector could theoretically also express GR-D1, -D2, and -D3. However, only GR-C3 and GR-D1 could be detected (data not shown). The effect of DC-SCRIPT on transcription initiation of the reporter construct MMTV-luc by these isoforms was tested using luciferase transcription assays. GR isoforms expressed by both GR and GR-C3 expression vectors equally induced luciferase production in a ligand dependent manner. Cells expressing the GR-D3 isoform, however, were less potent in inducing luciferase expression, displaying a more than 4-fold reduction in luciferase production. Increasing expression levels of DC-SCRIPT resulted in a dose- and ligand dependent repression of the transcriptional activity of all GR isoforms (Fig. 3B). This indicates that the 1-336 amino acid N-terminal part of GR that was deleted in
the GR-D3 expression vector is not required for the effect of DC-SCRIPT on GR dependent transcription.

**DC-SCRIPT and GR expression in immature, mature and tolerogenic DCs**

Next, we investigated DC-SCRIPT and GR protein expression by western blotting and confocal laser scanning microscopy (CLSM) in immature- (iDCs), mature- (mDCs) and tolerogenic DCs (tolDCs). Immature and mature DCs were obtained by stimulating immature moDCs with vehicle or 24 hours of LPS, respectively. Tolerogenic DCs were generated from immature moDCs through 24 hour stimulation with dexamethasone and subsequent 24 hours with LPS. Western blot analysis of DC-SCRIPT and GR protein levels showed equal expression of both proteins in iDCs, mDCs and tolDCs (Fig. 4A). The translational isoforms GR-A, -B and -C, but not GR-D which lacks the epitope for the anti-GR antibody, could be detected in all conditions.

CLSM analysis revealed co-expression of DC-SCRIPT and GR protein in all three DC types tested. Expression of both proteins was observed predominantly in the nucleus, no major changes were detected between iDCs, mDCs and tolDCs (Fig. 4B). Co-localization between DC-SCRIPT and GR could be discerned but areas containing either GR or DC-SCRIPT were at least as abundant. As expected, nuclear expression of GR protein was increased upon 1 hour dexamethasone stimulation. In contrast, DC-SCRIPT localization was unaffected by dexamethasone treatment. Hence these data clearly demonstrate nuclear- and, to a lesser extent, cytoplasmic co-expression and partial co-localization of DC-SCRIPT and GR in moDCs.

**Knock-down of DC-SCRIPT enhances GILZ expression in moDCs**

To determine the physiological relevance of the interaction between DC-SCRIPT and GR, DC-SCRIPT expression was silenced in moDCs using siRNA electroporation. As DC-SCRIPT was demonstrated to repress GR mediated transcription on the MMTV promoter, we hypothesized a similar effect on the endogenous GR target GILZ in moDCs. DC-SCRIPT knock-down was therefore expected to enhance GR dependent transcription, which could be detected by an increase in GILZ expression.

Human moDCs were electroporated at day 4 with an DC-SCRIPT specific siRNA oligo (siSC) or an irrelevant control siRNA oligo. DC-SCRIPT protein expression was markedly reduced in siSC treated DCS 48- and 72 hours after electroporation (Fig. 5A), whereas GR expression remained unaltered (Fig. 5B). At day 6 moDCs were treated with vehicle or the GR antagonist RU-486 for 1 hour and subsequently stimulated with vehicle or the GR agonist prednisolone for 24 hours. After stimulation GILZ mRNA levels were measured. As expected, little or no GILZ mRNA expression was detected in vehicle treated control DCs. Stimulation with prednisolone resulted in an upregulation of GILZ expression levels in control DCs (Fig. 5C) which could be effectively blocked by inhibiting GR activation with the GR antagonist RU-486. Strikingly, vehicle treated siSC DCs already demonstrated a significant (p=.05) increase in GILZ expression compared to control DCs. This upregulation appears to be GR independent, as it is also detected in the presence of the GR antagonist RU-486. In the absence of the GR antagonist, treatment with prednisolone further increased GILZ expression in DC-SCRIPT knock down DC, with relative GILZ mRNA levels being significantly higher than in control siRNA treated DCs (p=.01). These data thus indicate DC-SCRIPT to function as co-repressor of GR mediated transcription of GILZ in DCs. Blocking GR activation with the GR antagonist abolished GILZ up-regulation in DC-SCRIPT knock-down DCs, confirming that this was indeed dependent on GR activation. These results indicate that DC-SCRIPT affects GR activity in DCs, and represses expression
of the endogenous GR target gene GILZ, a transcription factor that has been associated with the generation of tolerogenic DCs.

**Discussion**

The present study demonstrates that the NR co-regulator DC-SCRIPT modulates GR function in human moDCs. In cell-lines, DC-SCRIPT was present in GR containing protein complexes, and GR mediated transcription was found to be repressed by DC-SCRIPT. Human moDCs co-express DC-SCRIPT and GR in the nucleus and cytoplasm, and DC-SCRIPT silencing resulted in enhanced expression of the GR target gene GILZ, a transcription factor that is instrumental for the generation of tolDCs.

Co-presence of DC-SCRIPT and GR in protein complexes could be demonstrated in transfected HEK293 cells, however, we could not co-IP the endogenous complex from DCs. This could have several reasons. Human monocyte derived DCs express at least 20 different nuclear receptors (NRs)

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**Figure 4. DC-SCRIPT and GR expression in iDCs, mDCs and tolDCs**

(A) Proteins from iDC, mDC and tolDC lysates were subjected to immunoblotting with anti-DC-SCRIPT or anti-GR antibodies and anti-actin as loading control. (B) CLSM analysis of DC-SCRIPT and GR expression in iDCs, mDCs and tolDCs. DC-SCRIPT was visualized with goat-anti-DC-SCRIPT (green), GR with mouse-anti-GR (red) and the nucleus with DAPI (not in merge). Immature and mature DCs were stimulated with vehicle or 100 nM dexamethasone for 1 hour. Representative data from 1 out of 3 donors.
of which at least 6 (GR, PR, ER, RAR, VDR, PPAR) possibly contain DC-SCRIPT in their repressor or activator complexes (19-22). Therefore, enrichment of a specific NR via DC-SCRIPT IP may be extremely difficult. Further, endogenous DC-SCRIPT not present in protein complexes may possibly bind more efficiently to the beads, and is therefore more likely to be purified. In addition, DC-SCRIPT and GR likely interact indirectly. Hence, lysis buffers need to be mild in order to keep the complex intact, but at the same time sufficiently stringent to properly lyse the nucleus. Nonetheless, our co-IP findings in HEK293 cells are further supported by functional data that demonstrate the effect of DC-SCRIPT expression on GR-ligand mediated GR target gene expression, not only in HEK293 cells, but also in dendritic cells.

The present data describing the interaction between DC-SCRIPT and GR in cell-lines is in line with our previous findings, suggesting that DC-SCRIPT interacts with NRs via other proteins present in NR protein complexes. DC-SCRIPT has been shown to bind the transcription co-repressors RIP140 (receptor-interacting protein 140) and CtBP1, as well as histone de-acetylase (HDAC) 1, -3 and -6, all known to be present in NR protein complexes (5, 33, 34). The absence of a direct contact between DC-SCRIPT and GR is also consistent with the finding that deletion of the NR interaction motif LxxLL and the CtBP1 interaction motif in the acidic domain of DC-SCRIPT did not affect its repressive function on GR transcription. This implies that interaction with CtBP1 and the presence of the LxxLL motif is not required for the repressive effect of DC-SCRIPT. Interaction with NR complexes could, however, be facilitated via other, less defined NR interaction motifs (35-39). In addition to the LxxLL motif, DC-SCRIPT contains multiple putative NR interaction motifs that are possibly involved in the interaction with various NRs (data not shown).

Co-expression of DC-SCRIPT and GR in DCs was demonstrated with CLSM, albeit areas containing either DC-SCRIPT or GR were equally abundant. Ligand dependent GR translocation was observed in iDCs. However, in the absence of its ligand, GR was also detected in the nucleus of iDCs. The nuclear GR expression most likely reflects the expression of GRβ isoforms, known to have restricted nuclear localization (18). DC-SCRIPT expression was found to be mostly nuclear, in line with previous localization studies and its function as a transcriptional (co)factor (3, 34, 40).

Within DCs, DC-SCRIPT was shown to co-regulate GR function affecting expression of the well known GR target gene GILZ. Knock-down of DC-SCRIPT expression markedly increased GILZ expression upon GR activation, implicating co-repression of GR by DC-SCRIPT on the GILZ promoter in DCs. GILZ functions as an effector protein of GR activation in DCs, mediating the anti-inflammatory action of GR.
It has previously been shown that the immunosuppressive effect of GR can be reproduced in the absence of GR ligand by GILZ overexpression, whereas GILZ silencing abrogates the GR ligand effect (3). GILZ has been shown to interact with STAT, NF-κB, AP-1, 14-3-3, Raf-1 and Ras proteins, all of which lead to inhibition of inflammation (41). Hence, by regulating GR activity and GILZ expression, DC-SCRIPT may represent an important factor in DC biology, programming DCs towards immunity or tolerance. In line with this, we have recently demonstrated an important role for DC-SCRIPT in the repression of IL-10 secretion (40), an anti-inflammatory cytokine known to be produced in large amounts by tolDCs (42). In this study, DC-SCRIPT knock-down in immature moDCs significantly increased IL-10 expression levels upon TLR4 and -7/8 mediated maturation. The cell surface markers CD80, CD83, (40) and CD86 (data not shown), which show differential expression between mature DCs and tolDCs, were not affected by DC-SCRIPT knock-down, and resembled levels found on mature DCs. Nonetheless, elevated IL-10 levels subsequently impaired IL-12 secretion and T-cell proliferation, suggesting that DC-SCRIPT knock-down skews DCs towards tolerance.

In addition to GILZ, we also investigated the expression of other genes known to be induced by ligand dependent GR activation in other cells. However, identification of these other GR targets in DC and whether they are affected by DC-SCRIPT proved to be difficult (data not shown). For eight out of ten tested target genes no GR-ligand dependent upregulation in moDCs could be detected. This could be due to cell-type specific differences between DCs and other cells, like differences in the expression and function of the repressive GRα-D and GRβ isoforms. Increased expression upon GR activation was only detected for FKBP5 (51 kDa FK506-binding protein 5) and PTX3 (pentraxin 3), however, induction levels were low and DC-SCRIPT silencing did not affect expression of these genes (data not shown). One major reason could be the HRE sequence or the context of this sequence in the promoter of these genes. Meijsing et. al (2009) recently demonstrated that a single nucleotide change in the GRE sequence influences GR binding affinity and conformation, affecting co-factor recruitment and transcription activation (43). Similarly, the binding properties of DC-SCRIPT might differ depending on GR conformation and affect the binding of other co-factors. Furthermore, adjacent binding sites of other transcription factors can also affect GR mediated transcription and possibly DC-SCRIPT function (19). In line with this, we have preliminary data that suggests that DC-SCRIPT can also activate GR dependent transcription, depending on the promoter context (data not shown). As soon as a suitable DC-SCRIPT antibodies for Chromatin-ImmunoPrecipitation (ChIP) become available, it would be extremely interesting to perform ChIP-sequencing studies and expression arrays, to further investigate gene regulation specifically in DCs with respect to DC-SCRIPT and GR activation and expression.

Finally, besides GR, also other NRs are known to function in DC biology, affecting either differentiation, maturation, or both. The human NR superfamily contains 48 members, of which 20 NRs have been described to be expressed in monocyte derived DCs (44). Since we have recently indentified DC-SCRIPT as a NR co-regulator in breast- (20) and prostate carcinoma (22), affecting both type I and type II NR function, it is tempting to speculate about a role for DC-SCRIPT as NR co-regulator in DC biology. It would therefore be interesting to study target gene expression of various NRs after DC-SCRIPT knock-down in DCs.

Acknowledgments
This work was supported by The Netherlands Organisation for Scientific Research [918.66.615 to G.J.A].
Part 3 - Nuclear Receptor Coregulators

References

Chapter 5 - DC-SCRIPT regulates GR function


Chapter 6

DC-SCRIPT: AR and VDR regulator lost upon transformation of prostate epithelial cells

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Prostate (2012) 72 (16), 1708-1717
DC-SCRIPT: AR and VDR regulator lost upon transformation of prostate epithelial cells

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Abstract

Background Nuclear receptors (NR), including the Androgen Receptor (AR) and the Vitamin D Receptor (VDR) play an important role in prostate cancer etiology. We recently found that DC-SCRIPT is a prognostic marker in breast cancer and a unique NR coregulator differentially regulating different classes of NRs. Here we investigated the importance of DC-SCRIPT in prostate cancer.

Methods DC-SCRIPT mRNA expression was measured by qPCR. Immunohistochemistry was used to detect DC-SCRIPT protein expression. The functional effects of DC-SCRIPT on the transcriptional activity of AR and VDR were assessed by luciferase reporter assays and qPCR assays on well-known AR and VDR target genes.

Results DC-SCRIPT mRNA was readily detected in normal and malignant prostate tissue but could not be related to disease stage. DC-SCRIPT protein was found in morphologically normal prostate glands and in infiltrating immune cells. Strikingly, DC-SCRIPT protein expression was absent in malignant prostate epithelial tissue and prostate carcinoma cell lines. DC-SCRIPT protein expression appears to be lost prior to the basal cell marker HMW cytokeratin used in prostate carcinoma diagnostics. In addition, our data demonstrated that DC-SCRIPT repressed transcription mediated by wild-type and mutated AR while enhancing VDR mediated transcription. In addition, transient expression of DC-SCRIPT in prostate carcinoma cells strongly repressed carcinoma cell growth.

Conclusions DC-SCRIPT is a key regulator of nuclear receptors AR and VDR that play an opposite role in prostate cancer etiology and loss of DC-SCRIPT may be involved in the onset of prostate cancer.

Key words: DC-SCRIPT/ZNF366, prostate carcinoma, Androgen Receptor, Vitamin D3 Receptor

Introduction

Prostate cancer is the most frequently diagnosed cancer in the Western male population. Initially prostate cancer can be successfully treated with targeted therapies, including inhibiting the function of the Androgen Receptor (AR), a member of the type I nuclear receptor (NR) family. Deregulation of AR and/or AR coregulator expression or activity contributes to the transition to castration-resistant stage of the disease in patients undergoing endocrine therapy (1,2). In addition to androgens that bind to the type I NR AR, also vitamin D3 that binds to the type II NR VDR (Vitamin D3 Receptor) plays an important role in the etiology of prostate cancer. Functionally, the VDR ligand
1,25-(OH)2D3 (vitD3) has been shown to reduce the growth of several prostate cancer cell lines in vitro, as well as tumor growth in vivo (3-6).

The activities of NRs such as AR and VDR are tightly regulated by coregulators. The balance of corepressors and coactivators determines NR transcriptional activity. Multiple studies have shown that in addition to alterations in NRs themselves, malfunction of coregulators of NRs are implicated in the development of cancer, including prostate cancer (7,8). Recently we have shown that DC-SCRIPT is an important coregulator of multiple NRs and is expressed by dendritic cells (DC) and breast ductal epithelial cells. DC-SCRIPT expression inhibited the activity of the type I NRs ER and PR, known for their pro-proliferative and anti-apoptotic activities in breast cancer cells. In contrast, the transcriptional activities of the type II NRs RAR/RXR and PPAR/RXR, known for their anti-proliferative and pro-apoptotic effects in breast cancer cells, were enhanced (9-11). Interestingly, DC-SCRIPT mRNA expression in breast cancer patients constituted a strong and independent prognostic marker for breast cancer (11,12). These data suggest that DC-SCRIPT acts as a tumor suppressor in breast cancer. Whether DC-SCRIPT also plays a role in other cancer types in which NRs play an important role, is currently unknown.

Herein we investigated DC-SCRIPT expression in normal and malignant prostate tissue and its relation with AR and VDR, two NR family members with opposite functions in prostate epithelial cells.

**Materials and Methods**

**Cells**

HEK293 cells were cultured in DMEM containing GlutaMAX (Invitrogen), 10% FCS (Greiner Bio-one), 1% nonessential-amino-acids (Invitrogen), and 0.5% antibiotic – antimycotic (aa) (Invitrogen). LNCaP and 22Rv1 prostate epithelial cancer cells were cultured in RPMI-1640 medium (Invitrogen), 10% FCS, and 0.5% aa.

**Co-immunoprecipitation assays**

LNCaP and 22Rv1 were transfected with pHA-DC-SCRIPT using metafectene (Biontex, Germany) according to the manufacturers’ protocol. Following transfection for 40 hrs, the cells were lysed in CHAPS buffer (1% CHAPS, 150mM NaCl, 10mM Tris-HCl pH 7.5 and 2 mM MgCl2 pH 7.5) containing the protease inhibitors leupeptin (Sigma), aprotinin (Roche) and PMSF (Sigma). Lysates were used for immunoprecipitation (IP) of DC-SCRIPT using anti-DC-SCRIPT-coupled dynabeads (GE Healthcare). Isotype-coupled-dyna-beads were used as a control.

Hek293 cells were transfected with pDC-SCRIPT-EYFP or pEYFP and HA-tagged VDR using metafectene. Following transfection for 24 hrs, the cells were lysed in RIPA buffer (50mM NaCl, 1% Triton, 0.1% SDS and 50mM Tris-HCl) containing the protease inhibitors leupeptin, aprotinin and PMSF. Lysates were used for IP of YFP-tagged proteins using anti-GFP coupled Protein G beads. Uncoupled beads were used as a control.

Proteins were subjected to electrophoresis and transferred onto Protan nitrocellulose transfer membranes (Schleicher and Schuell). DC-SCRIPT was detected with anti-DC-SCRIPT (R&D) and donkey anti-goat IRDye 800CW (Li-cor biosciences). AR was detected with anti-AR (N20, santa cruz) and IRDye 800CW Goat Anti-Rabbit IgG (Li-cor biosciences). Blots were probed with a mouse-anti-GFP (Roche Applied Science) and IRDye 800CW Goat Anti-Mouse IgG (Li-cor biosciences) to detect YFP-tagged proteins. HA-tagged VDR was detected with a rat anti-HA (3F10, Roche) and goat anti-rat alexa fluor 680 (Invitrogen). After staining, the membranes were scanned using the Odyssey™ Infrared Imaging system to visualize the labeled proteins.
Transcription assays
PSA85-luc was kindly provided by Prof. Dr. Trapman (Erasmus Medical Center, Rotterdam). pGL3-ARE-E1B-luc was kindly provided by Prof. Dr. Bernards (Netherlands Cancer Institute, Amsterdam). MMTV-luc was kindly provided by Prof. Dr. Stunnenberg (Nijmegen Centre for Molecular Life Sciences). The vitamin D3 reporters were obtained from SABioscience. pCATCH, pCATCH-DCSCRIPT, pEYFP-DC-SCRIPT and pEGFP-DC-SCRIPT have been described (9).
22Rv1 cells were transfected using metafectene. Transfected cells were stimulated with 1nM R1881 (sigma), 10nM DHT (sigma), 50nM vitD3 (sigma), 1µM AtRA (sigma), or vehicle for 24 hrs. Cells were lysed 40 hrs later and analyzed for luminescence according to manufacturer’s protocol (Dual Luciferase® Reporter assay, Promega) using a Victor® luminometer (PerkinElmer). Relative light units (RLU) were calculated after correction for transfection efficiency based on the activity of the co-transfected pRL-SV40 (Promega).

Target gene assays
For AR target gene experiments LNCaP cells were plated 3 days prior to transfection in RPMI, 10% charcoal-stripped FCS, ultraglutamine and aa. and transfected with pHA-DC-SCRIPT or pHA as a control using metafectene. Cells were stimulated with vehicle or R1881 for 24hrs. For VDR target gene experiments cells were plated in RMPI, 10% FCS, ultraglutamine and aa, 24 hrs prior to transfection. Cells were transfected with pHA-DC-SCRIPT or pHA as a control using metafectene. Cells were stimulated with vitD3 for 0 or 24hrs. RNA was isolated with the Quick-RNA MiniPrep kit (Zymoresearch).

cDNA synthesis and Quantitative PCR
Total RNA was extracted from tissue sections or cell cultures using Trizol reagent (Invitrogen). RNA quantity and purity were determined on a NanoDrop spectrophotometer. Two micrograms of total RNA was DNase-I-treated and cDNA was synthesized using random primers and SuperScript II-MMLV reverse transcriptase (Invitrogen). Upon quantification of the RT-reaction, 10 ng of cDNA was used for PCR analysis.

mRNA levels for the genes of interest were determined with a Bio-rad CFX96 (Bio-rad) with SYBR Green (Roche) as the fluorophore. Reaction mixes and program conditions were used that were recommended by the manufacturer (Bio-rad). Quantitative PCR data were analyzed with Bio-rad CFX manager (Biorad) and checked for correct amplification and dissociation of the products. As a reference gene TFRC was used (13). DC-SCRIPT levels relative to TFRC were calculated as: $2^{\Delta C_{t}} = 2^{(\Delta C_{t} \text{DC-SCRIPT} - \Delta C_{t} \text{TFRC})}$. Primer sequences are available on request.

Immunohistochemical studies
Snap-frozen prostate specimens were embedded in Oct embedding matrix (CellPath), sectioned at 5 µm intervals, placed on superfrrost slides, fixed and incubated with anti-DC-SCRIPT (R&  D Systems) and stained with biotinylated Horse-anti-Goat (Vector), ABC-AP (Vectastain) and fast red (Sigma) was used for signal development. HMW cytokeratin was stained with 34bE12 (Dako) and stained with biotinylated Horse-anti-mouse (Vector), ABC-AP and fast red. Isotype matched antibodies (gIgGs (R&D Systems) and mlgG1 (BD bioscience) respectively) were used as controls. Sections were counter-stained with hematoxylin to visualize the nucleus of the cells and analyzed on a Leica DMLB microscope.
Prostate cancer patient tissue specimens

The use of patient material was approved by the local ethical committee of the Radboud University Nijmegen Medical Centre. Based on the pathology findings and the case records, patients with low grade (Gleason score < 7), high grade (Gleason score ≥ 7), and castration-resistant prostate cancer were selected for this study. Normal prostate (NPr) and benign prostatic hyperplasia (BPH) tissue specimens were used as non-malignant controls. Upon radical prostatectomy or transurethral resection of the prostate, specimens were snap-frozen. Normal, BPH and tumor prostatic tissues were selected and processed by step sectioning.

Statistical analysis

Differences in TFRC-normalized DC-SCRIPT mRNA expression levels between normal prostate tissue and tumor tissue from the same patient and between target gene expression of VDR and AR in control and DC-SCRIPT transfected cells were assessed using paired t tests. Two-sided \( P \) values less than 0.05 were a priori considered to be statistically significant.

Figure 1. DC-SCRIPT mRNA and protein expression in prostate tissue samples

(a) DC-SCRIPT mRNA levels in healthy tissues (black bars) and corresponding prostate tumor (white bars) tissue. (b) DC-SCRIPT mRNA levels in 10 castration resistant prostate carcinoma, 10 low grade (<7) and 10 high grade (≥7) prostate tissue samples of prostate carcinoma patients. Immunohistochemistry staining of (c) DC-SCRIPT and 34βE12 on frozen morphologically normal prostate tissue (d) DC-SCRIPT and isotype control in frozen prostate tissue with well differentiated prostate carcinoma cells containing a region with tumor infiltrating leucocytes (e) DC-SCRIPT and 34βE12 on frozen prostate tissue with well differentiated prostate carcinoma cells.
Results

DC-SCRIPT mRNA and protein expression in prostate epithelium

The bifunctional NR coregulator DC-SCRIPT that is expressed in normal breast epithelial cells and at lower levels in their malignant counterparts represents a solid prognostic marker for breast carcinoma (11,12). Here we investigated whether DC-SCRIPT is expressed by normal and malignant prostate epithelial cells in which NRs also play an important role. DC-SCRIPT mRNA expression was determined in normal and malignant prostate tissue specimens from the same individual and in prostate carcinoma of different grades. The data revealed the presence of DC-SCRIPT mRNA in prostate tissue at levels comparable to those seen in breast cancer specimens, and a trend towards higher DC-SCRIPT expression in normal vs. tumor tissue that did not reach significance \( (P=0.1978) \) (Fig. 1a). The comparison of DC-SCRIPT mRNA levels in 10 low (Pca GG<7) grade patients, 10 high (Pca GG>=7) grade patients and 10 castration-resistant PCa patients did not reveal significant differences between the different subgroups (Fig. 1b). To confirm DC-SCRIPT expression at the protein level, frozen prostate cancer tissue sections were stained for DC-SCRIPT expression. The data showed DC-SCRIPT expression in the basal epithelial cell layer of morphologically normal prostate tissue (Fig. 1c). Little or no expression of DC-SCRIPT was observed in cancerous prostate epithelial cells of patients with poorly differentiated prostate carcinoma (n=5) or with castration resistant disease (n=4). As expected, DC-SCRIPT was expressed in cells with DC morphology in regions containing tumor infiltrating leukocytes (TIL) (Fig. 1d). Of note, TIL are abundant in prostate carcinoma tissue relative to healthy prostate tissue. These data demonstrate that DC-SCRIPT is expressed by normal prostate epithelial cells and imply that infiltrating DCs expressing DC-SCRIPT contribute to DC-SCRIPT mRNA expression observed in advanced prostate cancer tissue.

Subsequently, the expression of DC-SCRIPT was related to the expression of HMW (high molecular weight) cytokeratin (34βE12), which stains benign prostatic acini. Overall, DC-SCRIPT expression paralleled the expression of HMW cytokeratin in the basal prostate epithelial cells in healthy prostate tissue (Fig. 1e). HMW cytokeratin negative glands also did not express DC-SCRIPT and all glands that expressed DC-SCRIPT were also positive for HMW cytokeratin. More detailed analysis showed that

Figure 2. Association of DC-SCRIPT with AR in prostate carcinoma cell lines

Protein of cell lysates were immunoprecipitated (IP) with anti-DCSCRIPT antibody – or isotype control coupled beads. The IP-ed proteins were subjected to immunoblotting (WB) with anti-DCSCRIPT to detect IP-ed DC-SCRIPT or anti-AR to detect coimmunoprecipitated AR. Co-IPs were performed on (a) lysates from 22Rv1 cells cotransfected with DC-SCRIPT and with wt AR (b) lysates from 22Rv1 cells transfected with DC-SCRIPT and (c) lysates from LNCAP cells transfected with DC-SCRIPT.
DC-SCRIPT expression in the basal epithelial layer was lost in 32% (15 out of 36 cases of 19 pts) of the cases that uniformly (no focal disruptions) expressed HMW cytokeratin, while 53% (9/17 cases of 19 pts) of the cases with focal disruptions in HMW cytokeratin expression were completely DC-SCRIPT negative. Since focal disruption is a pre-requisite for tumor progression/invasion (14), the loss of DC-SCRIPT expression in glands with focal disruptions suggest that DC-SCRIPT expression is lost early upon malignant transformation. In line with this notion is the finding that no DC-SCRIPT mRNA expression is observed in all prostate cancer cell lines tested so far (DU145, LNCaP, DuCaP, DuCaP+R1881, 22Rv1 and LAPC-4, data not shown). Surprisingly, the hTERT-immortalized prostate basal epithelial cell line Ep156T (15) also does not express DC-SCRIPT, even when co-cultured with prostate-derived fibroblasts (data not shown). These results suggest that DC-SCRIPT expression is only present in non-dividing cells or cells present in complex organ structures providing the right microenvironment.

**DC-SCRIPT interacts with and represses AR**

As DC-SCRIPT is known to affect the function of multiple NRs (11), we investigated if DC-SCRIPT may also act as a coregulator for NRs important in prostate biology. Therefore, we first evaluated the interaction of DC-SCRIPT with AR by co-immunoprecipitation (co-IP) studies. For this, lysates were prepared from 22Rv1 prostate carcinoma cells co-transfected with cDNAs encoding DC-SCRIPT and wt AR. Immunoprecipitated DC-SCRIPT was readily able to co-IP wt AR, whereas no AR could be detected in the control IP (Fig. 2a). To further confirm this interaction, additional co-IP experiments were performed with DC-SCRIPT transfected 22Rv1 and LNCaP prostate cancer cell lines that endogenously expressed AR (Fig. 2b-c). Although weak, specific co-immunoprecipitation of endogenously expressed AR with DC-SCRIPT was observed in both 22Rv1 and LNCaP cells. These data thus show the co-presence of DC-SCRIPT in protein complexes containing AR.

Next, we assessed the impact of DC-SCRIPT expression on the transcriptional activity of AR in luciferase reporter assays. Therefore 22Rv1 endogenously expressing the AR mutant T877A (16) were transfected with an androgen responsive MMTV-reporter construct. Upon provision of the AR-ligands DHT or R1881, they produced luciferase as expected. Introduction of DC-SCRIPT revealed a dose-dependent repression of ligand-dependent AR mediated luciferase production (Fig. 3a). In addition, using another AR reporter construct, pGL3-ARE, we confirmed that DC-SCRIPT represses AR-mediated transcription in an AR ligand- and dose-dependent manner (Fig. 3a). Moreover, DC-SCRIPT specifically repressed luciferase production from a reporter construct, PSA85luc (17), containing a genomic fragment of the AR responsive PSA-promoter (Fig. 3a). These data indicate that DC-SCRIPT specifically represses transcription mediated by endogenously expressed AR on 3 distinct promoters containing androgen response elements in a dose and ligand dependent manner. In addition, we show that DC-SCRIPT can repress ectopically expressed wildtype AR in Hep3b cells (data not shown). Finally, we investigated if DC-SCRIPT could also repress endogenous genomic AR targets in prostate cancer cells. Therefore, DC-SCRIPT was transiently transfected into the AR positive prostate cell line LNCaP and the expression of the AR target genes TMPRSS2 and SPDEF was monitored. Upon stimulation of control transfected cells with the AR agonist R1881 the expression of TMPRSS2 and SPDEF is clearly upregulated (Fig. 3b). R1881 stimulation of LNCaP cells transfected with DC-SCRIPT (10-20% transfection efficiency) resulted in significantly lower expression of both AR target genes. These data thus demonstrate that DC-SCRIPT can modulate the function of AR on genomic targets in prostate carcinoma cells.
In contrast to the growth promoting role of AR, the type II NR VDR plays an important anti-proliferative and pro-apoptotic role in prostate cancer. As we previously showed that DC-SCRIPT modulates type II NRs in addition to type I NRs, we investigated whether DC-SCRIPT is able to affect the function of VDR. Therefore, we performed co-IP experiments using lysates prepared from HEK293 cells cotransfected with constructs encoding YFP-tagged DC-SCRIPT or YFP- (control) and HA-tagged VDR. Although YFP–DC-SCRIPT and the control protein YFP were both effectively immunoprecipitated with anti-GFP antibody–coupled beads, only in the YFP–DC-SCRIPT IP the HA-tagged VDR could be detected (Fig. 4a), indicating a specific interaction between DC-SCRIPT and VDR.

To determine the effect of DC-SCRIPT on the transcriptional activity of VDR, luciferase reporter assays were performed in 22rv1 cells. 22rv1 cells transfected with a VDRE luciferase reporter construct show luciferase activity upon stimulation with the VDR/RXRα ligand vitD3 (1,25-(OH)2D3), consistent with the expression of endogenous VDR and RXR. Strikingly and in contrast to the repressive effect on the type I steroid receptor AR, co-expression of increasing amounts of DC-SCRIPT resulted in a dose-dependent increase in luciferase activity upon addition of vitD3 (Fig. 4b). Removal of the VDR/RXR response elements in the reporter construct completely abolished the luciferase production, further demonstrating that the activating function of DC-SCRIPT is mediated via binding
of VDR/RXR to its response elements (data not shown). To demonstrate that DC-SCRIPT enhances expression mediated by VDR/RXR rather than RXR homodimers, cells were treated with the RAR/RXR ligand AtRA. As shown in Fig. 4b, DC-SCRIPT could only enhance luciferase production upon stimulation with vitD3 and not with AtRA or R1881. Moreover, we show that DC-SCRIPT expression in LNCaP cells enhances the expression of vitD3 induced Cyp24A1 expression, the strongest known endogenous genomic target gene of VDR (Fig. 4c). These data indicate that DC-SCRIPT can activate VDR mediated transcription in prostate epithelial cells.

DC-SCRIPT expression inhibits prostate carcinoma cell growth
Prostate tumor growth is highly dependent on androgens, whereas vitD3 is mainly involved in the inhibition of prostate cell growth. Our current findings combined with our previous report (11) that DC-SCRIPT can repress the primarily proliferative type I NR mediated transcription and can activate the primarily antiproliferative type II mediated transcription implies that DC-SCRIPT may also be involved in modulating the growth response of prostate carcinoma cells to hormones and vitamins. To test this, we transiently transfected DC-SCRIPT in 22Rv1 cells and monitored their cell growth. As shown in Fig. 5, both C-terminally GFP tagged DC-SCRIPT and N-terminally YFP tagged DC-SCRIPT expression significantly represses cell growth compared to the YFP control transfected cells.

Discussion
Here, we investigated the importance of DC-SCRIPT in prostate carcinoma biology. DC-SCRIPT mRNA expression was readily detected in normal and malignant prostate tissue but could not be related to differentiation grade. DC-SCRIPT protein expression was found in the basal epithelial cells of morphologically normal prostate glands, and was absent in malignant prostate epithelial tissue and prostate carcinoma cell lines. DC-SCRIPT protein expression appears to be lost prior to the basal cell marker HMW cytokeratin in

Figure 4. Association and functional effects of DC-SCRIPT on VDR function.
(a) Lysates from Hek293 cells cotransfected with YFP-DC-SCRIPT and with VDR-HA were immunoprecipitated (IP) with Anti-GFP to IP YFP-tagged DC-SCRIPT or the control protein YFP. The IP-ed proteins were subjected to immunoblotting (WB) with anti-GFP to detect IP-ed YFP-tagged DC-SCRIPT or GFP and anti-HA to detect coimmunoprecipitated AR. (b) 22Rv1 cells were transfected with the reporter plasmid VDRE-luc and increasing amounts of DC-SCRIPT expression plasmid. The cells were treated for 24 hrs with 0 nM vitD3 (white bars), 1nM R1881 (light grey bars), 1µM AtRA (dark grey bars) or 50 nM vitD3 (black bars). Data are expressed as the mean values of at least four independent experiments +/- SD. (c) LNCaP cells were transiently transfected with a control or DC-SCRIPT expression vector and subsequently stimulated for 0 or 24 hrs with vitD3. Cyp24A1 mRNA levels are indicated relative to TFRC mRNA. Data are expressed as the mean values of three independent experiments +/- SEM.
focally disrupted glands. Moreover, DC-SCRIPT repressed transcription mediated by AR while enhancing VDR mediated transcription in prostate epithelial cells. Finally, transient expression of DC-SCRIPT in prostate carcinoma cells showed that DC-SCRIPT strongly represses cell growth.

Prostate cancers are exquisitely dependent on androgens for development, growth and survival (18-21). Therefore, suppressing AR activity by androgen deprivation and the use of AR antagonists are at the forefront of therapy regimens in prostate carcinoma for years (21-23). Deregulation of coregulators of AR has often been associated with prostate cancer biology (24,25) and demonstrate that also coregulators of AR play an important role in prostate carcinoma biology.

DC-SCRIPT was previously shown to interact with several proteins known to be present in large multiprotein complexes modulating NR function (9,11,26,27). Here, we demonstrate that DC-SCRIPT is present in a protein complex together with AR and is able to repress the activity of WT and mutated AR in luciferase reporter assays. Moreover, upon stimulation with androgens DC-SCRIPT repressed the expression of several endogenous targets of AR. DC-SCRIPT expression in human prostate tissue was mostly confined to the basal epithelial cells. Although AR mRNA and protein expression has been identified in the basal cells (28,29), AR activity in these cells has often been described to be low. AR expression and activity are much more pronounced in the luminal cell layer in prostate glands (28). The finding that DC-SCRIPT is much more abundant in the basal cells relative to the luminal cells might therefore be part of the explanation for the low level of AR activity in the basal cells. The observation that DC-SCRIPT regulates AR function and prostate cell growth, both of which need to be tightly controlled in normal prostate physiology, is indicative for a crucial role of DC-SCRIPT in prostate biology. The exact molecular mechanism by which DC-SCRIPT regulates AR activity in basal epithelial cells is currently unknown. So far, the lack of suitable anti-DC-SCRIPT antibodies has hampered ChIP experiments to elucidate the molecular basis.

Because DC-SCRIPT can modulate AR function in prostate epithelial cells we hypothesized that DC-SCRIPT may play a role in prostate cancer. Our data revealed the presence of DC-SCRIPT mRNA in prostate cancer tissue and corresponding healthy tissue. However, DC-SCRIPT mRNA expression could not be related to disease stages. This may be explained by the fact that mRNA from the non-microdissected tissue is not solely derived from tumor cells, but also from adjacent normal tissue, adipose tissue, blood vessels and immune infiltrates, the latter of which is known to express DC-SCRIPT. Immunohistochemistry showed that DC-SCRIPT protein is expressed in the basal epithelial layer of morphologically normal prostate glands, but also in infiltrating DCs. In addition we found that DC-SCRIPT protein expression parallels \( \beta e 12 \) expression. \( \beta e 12 \) is normally used for the detection of basal cells of the prostatic acini and demonstration of this HMW cytokeratin in the basal cells of

![Growth of transfected cells](image-url)

**Part 3 - Nuclear Receptor Coregulators**

**Figure 5. Effect of DC-SCRIPT expression on cell growth of 22Rv1 prostate carcinoma cells**

To assess the effect of DC-SCRIPT expression on cell growth, 22Rv1 cells were transfected with YFP, YFP-DC-SCRIPT or DC-SCRIPT-GFP. Cells were counted 24 hrs and 72 hrs after transfection and analyzed for YFP expression by means of FACS. Thereafter, the number of transfected cells was calculated. Number of cells plotted in the graph are relative to the number of YFP transfected cells. Data are expressed as the mean of 3 experiments +/- SD.
prostatic acini is indicative of benignity (30,31). Our detailed analysis showed that the loss of DC-SCRIPT expression in basal epithelial cells precedes the loss of HMW cytokeratin and that DC-SCRIPT expression is lost early upon malignant transformation. The basal cell population is the sole source of several tumor suppressors (14,32,33), together with the finding that DC-SCRIPT expression in prostate carcinoma cell lines inhibits their cell growth, it suggests that DC-SCRIPT may function as a tumor suppressor in prostate epithelial cells.

Recently we have demonstrated that DC-SCRIPT plays an important role in breast cancer via modulating NR activity. DC-SCRIPT mRNA expression in breast cancer biopsies positively correlated with clinical outcome. Overexpression of DC-SCRIPT inhibited the activity of the type I NRs, ER and PR. In contrast, the transcriptional activities of the type II NRs, RAR/RXR and PPAR/RXR, were enhanced (11,12). Functional crosstalk of type I and II NRs in breast carcinoma, especially between ER and RAR has been known for years, but the molecular mechanism has only recently been elucidated (34-36). ER has been reported to exhibit mainly pro-proliferative and anti-apoptotic activities in breast cancer cells (37-39), whereas RAR mainly has anti-proliferative and pro-apoptotic effects (37,40). For prostate cancer a similar functional link between AR and VDR has been described. In contrast to AR, VDR is associated with anti-proliferative and differentiating actions on prostate cells (5,6,41). Moreover, the growth inhibiting effects of VDR in prostate cells are most pronounced in the presence of androgens (42-45), further supporting the crosstalk between AR and VDR.

Altogether, our data indicate that the reciprocal effect of DC-SCRIPT on type I and II NRs found in breast carcinoma, now can be extended to prostate carcinoma. As prostate epithelial cells do not only express the NRs AR and VDR, we hypothesize that the growth-inhibitory effects of DC-SCRIPT are not solely mediated via these NRs, but that it is rather a collective effect of DC-SCRIPT on multiple type I and II NRs expressed in these cells.

DC-SCRIPT appears to be a key regulator of the NRs AR and VDR, which exhibit opposite functions in prostate cancer cells. In conclusion, DC-SCRIPT represents a potential novel marker for NR-dependent tumors.

Acknowledgments

We thank Prof. Stunnenberg (Department of Molecular Biology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands), Prof. Trapman (Erasmus Medical Center, Rotterdam) and Prof. Bernards (Netherlands Cancer Institute, Amsterdam) for providing plasmids. This work was supported by The Netherlands Organisation for Scientific Research [918.66.615 to G.J.A].

References


Chapter 6 - DC-SCRIPT in prostate cancer


Part 4

Discussion & Summary
Chapter 7

Summarizing Discussion

Partially adapted from:
In a physiological context cells are not alone; they communicate with surrounding cells and interact with extracellular matrix. Cell derived signaling molecules and the nature of the ECM offer a highly dynamic setting for cells. Within their local microenvironment they experience complex mixtures of compounds and substrates ranging from growth factors and metabolic products over immune relevant cytokines and chemokines to **hormones and vitamins**. Hormones and vitamins manipulate the function of cells through downstream signaling via specific transcription factors, nuclear receptors (NR). The ligand activated NRs are expressed by virtually every cell of the body. NRs repress and induce gene transcription within a protein complex comprising corepressor and coactivator molecules. These multiprotein complexes control cellular function and are implicated in a variety of physiological processes like development, homeostasis, cell cycle and immunity. Coherently, NRs are associated with a multiplicity of pathologic conditions including diabetes, chronic inflammatory diseases and cancer. In this thesis, we investigated the role of NRs in dendritic cell (DC) and cancer biology. We show that both NRs and the versatile NR coregulator DC-SCRIPT critically influence the cellular functions of different DC subsets and prostate tumor cells. Therefore, we conclude that NRs and their ligands, hormones and vitamins, are orchestrators of DCs and cancer. This chapter evaluates our results and discusses prospective implications.

**Hormones and vitamins orchestrate DC function**

**Dendritic cell heterogeneity – also a matter of residence**

DCs are pivotal decision makers in the balance between tolerance and immunity. Upon activation through pattern recognition receptor (PRR) signaling, they undergo maturation specific transcriptional changes that subsequently allow for the activation of effector cells, including T cells and B cells. DCs comprise a very heterogeneous family; they can generally be divided into conventional DCs (cDC) and plasmacytoid DCs (pDC). cDCs represent the classical DC subset; they are fast in antigen uptake and can efficiently present antigen to naïve T cells. DCs are highly capable to adapt to their microenvironment. In order to sense their surroundings and to react accurately, DCs express a huge variety of different receptors, like PRRs, cytokine receptors, chemokine receptors and NRs. We have shown that pDCs and cDCs from different lymphoid tissues, like spleen, bone marrow (BM), skin draining lymph nodes (SLN) and mesenteric lymph nodes (MLN) express the same repertoire of NRs (**chapter 2**). Moreover, mRNA levels of the different NRs within one DC subset were quite stable when compared between the organs. Different studies have shown that DCs from distinct tissues display characteristic functional responses towards pathogenic stimuli. Proietto et al. demonstrated that CD8α⁺ cDCs, CD8α⁻ cDCs and pDCs from the spleen markedly differed in their production of cytokines/chemokines and in their ability to prime naïve T cells compared to their respective counterparts in the thymus. Remarkably, all three subsets from the thymus showed enhanced secretion of CCL-17, CCL-21, CCL-22 and CCL-25. As the individual subsets from both tissues expressed comparable levels of TLRs, it is likely that the difference in functional responses can be allocated to tissue specific microenvironmental factors, like hormones and vitamins. cDCs and pDCs from spleen and thymus are likely to express the same NR repertoire. The distinct composition of a given microenvironment will therefore potentially determine the immune response of the DCs rather than the cells’ receptor expression profile. The concept that the heterogeneity within a given DC subset is a matter of residency is further supported by the distinct functional characteristics of DCs in mucosal organs like the gut and skin. Vitamin A (VitA) is now well known to be necessary for optimal cDC function in MLNs and in the microenvironment of the gut. Epithelial cells of the gut
and stromal cells of the local MLNs secrete the VitA metabolite retinoic acid (RA) and thereby imprint the resident CD103⁺ intestinal DCs with their unique tolerogenic properties. RA conditioned CD103+ DCs then produce RA themselves, a pivotal factor for the attraction of gut-homing regulatory T cells (Treg). The signaling of RA through the retinoid acid receptor (RAR) is therefore pivotal for the homeostasis of the tolerogenic microenvironment in the gut.

Similar to the gut, the skin is a part of the first line of defense and also harbors various DCs, including the epidermal Langerhans cells (LC) and dermal DCs (DDC). Sunlight induced vitamin D (VitD) is a key environmental factor in the skin, responsible for the imprinting of skin resident DCs to promote epidermotropism of T cells. DCs in the skin express the VitD metabolizing enzymes Cyp27A1 and Cyp27B1 and actively convert VitD precursors to the active ligand calcitriol. The DC secreted calcitriol then binds to the VitD receptor (VDR) in the T cells and induces the expression of skin homing chemokine receptor CCR10, enabling the T cells to migrate towards the keratinocyte excreted CCL27.

In conclusion, DC heterogeneity is not only a matter of distinct subsets but also of tissue residence. Microenvironmental factors, like hormones and vitamins, play key roles in the orchestration of DC mediated immune responses in tissues like the gut and skin. Moreover, DCs themselves metabolize and secrete hormones and vitamins, i.e. VitA and VitD, to further coordinate T cell responses.

Nuclear receptors in dendritic cell subsets

pDCs comprise a unique DC subset, they are well known for their rapid and vast production of interferon alpha (IFNα). Yet, like cDCs, activated pDCs are efficient in antigen uptake, processing and presentation. Despite the appreciated effects of NR ligand stimulation on cDCs, very little is known regarding the expression and function of NRs in pDCs. In chapter 2 we compared the NR expression profile of cDCs and pDCs. Our data show that both DC subsets expressed the same NR repertoire, in vitro and ex vivo. The mRNA expression levels of individual receptors, however, differed between cDCs and pDCs, especially upon maturation with toll like receptor (TLR) ligands. These data indicate that NR expression in DCs is regulated in a subset specific manner and that NR ligand stimulation might differentially affect the function of cDCs and pDCs.

The heterogeneous DC family extends beyond cDCs and pDCs. In human blood, cDCs can be further sub-divided into BDCA-1 (CD1c), BDCA-3 and CD16 expressing DCs. These three cDC subsets share the ability to produce IL-12 upon recognition of pathogen associated molecular patterns (PAMP). Yet, they distinctly differ in their expression of PRRs, production of cytokines/chemokines and T cell stimulating capacity. In the murine system, at least three cDC subsets are distinguished in the spleen: CD8α⁺/CD4⁺, CD8α⁻/CD4⁺ and double negative CD8α⁻/CD4⁻ cDCs. These three murine cDC subsets each express a characteristic PRR repertoire and show distinct responses towards pathogenic stimuli. In addition to these migratory DCs, human and murine organs harbor tissue-resident DC subsets, like langerhans cells in the skin or CD103⁺ cDCs in the gut lamina propria. Little is known regarding the NR expression between the different cDC subsets. Given that murine cDCs (in general) and pDCs express an identical NR repertoire (chapter 2); it is tempting to speculate that the individual cDC subsets share the same NR profile as well. Resting DCs will therefore be shaped by the microenvironment of the tissue in which they reside. The expression dynamics of the NRs, however, are likely to differ between cDC subsets upon maturation, as seen for cDCs and pDCs. Inflammatory conditions induce distinct transcriptional responses in DCs to ensure that the cells can react appropriately and elicit efficient immune responses. We show that cDCs and pDCs regulate NR
expression levels upon maturation with TLR ligands. Therefore, it would be interesting to compare the NR expression profile of the different cDC subsets in steady state and following PRR stimulation.

**Hormones and vitamins orchestrate dendritic cell activation**

The outcome of an immune response largely depends on the integration of pathogen associated signals and microenvironmental cues by DCs. PRR signaling in DCs controls the expression of co-stimulatory and co-inhibitory molecules like members of the B7 family, CD86 and PD-L1. Moreover, activation of PRRs leads to the expression of pro-inflammatory and anti-inflammatory cytokines, like IL-12 and IL-10. Hormones and vitamins in the local microenvironment are well known to steer the PRR mediated maturation of DCs by binding to their respective receptors. NR signaling can consequently either enhance or dampen the activation state of DCs. The retinoid acid receptor (RAR) and the liver X receptor (LXR) for example have been shown to amplify secretion of the pro-inflammatory cytokines IL-12, IL-6 and TNFα and expression of the co-stimulatory molecule CD86 on cDCs. The glucocorticoid receptor (GR), the peroxisome proliferator-activated receptor (PPAR) and VDR on the other hand have been shown to inhibit the secretion of the pro-inflammatory cytokines IL-12 and TNFα, whereas they enhance the secretion of the anti-inflammatory cytokine IL-10 by cDCs. NR ligands, i.e. hormones and vitamins, therefore partly steer the balance between pro-inflammatory and tolerogenic DCs (see chapter 1, Figure 3).

**Vitamin D renders DCs tolerogenic**

Tolerogenic DCs are characterized by the decreased expression of pro-inflammatory cytokines and surface receptors, the increased secretion of anti-inflammatory cytokines and surface receptors and consequently the impaired capacity to prime T cells. As aforementioned, different NRs and their ligands have been shown to induce a tolerogenic phenotype in cDCs, i.e. corticosteroids (GR), lipids (PPAR) and VitD (VDR). The latter has been extensively studied with respect to its inhibitory effect on DCs. Various studies have shown that the addition of VitD during the differentiation process of human monocytes towards DCs (moDC) in vitro renders moDCs tolerogenic. VitD treated cells express decreased levels of the co-stimulatory molecules CD40, CD80 and CD86 and increased levels of the inhibitory receptors ILT-3 and PD-L1. Moreover, they secrete lower amounts of the pro-inflammatory cytokine IL-12 and higher amounts of the anti-inflammatory cytokine IL-10. Consequently, VitD treated DCs are less potent in the induction of T cell responses and activate the generation of Tregs. Furthermore, Penna and colleagues demonstrated that the production of IL-12 and activation of T helper 1 responses of human blood derived BDCA1+ cDC is also inhibited by VitD, whereas pDCs were not affected by VitD. In line with these data we showed that VitD did not affect the secretion of IFNα, IL-6 and TNFα, or the expression of CD80, CD86, MHC-II and ILT3 in human pDCs (chapter 3). However, we clearly showed that VitD impaired the capacity of murine and human pDCs to induce T cell proliferation and secretion of IFNγ, thereby extending the tolerogenic effect of VitD from moDCs and cDCs to another unique DC subset, pDCs.

The unaffected expression of cytokines and surface receptors of VitD treated pDCs and cDCs seems to be paradoxical to the clear impairment of T cell activation. However, the priming of T cells by DCs largely depends on three signals: 1) MHC-T cell receptor binding, 2) engagement of co-stimulatory receptors and 3) cytokine signaling. The outcome of the immune response is steered by the balance of all three factors. Downstream signaling of these pathways generally leads to activation of NfkB. Studies have shown that VitD treatment enhances the protein levels of the NfkB-inhibitor IkBα in human macrophages thereby leading to diminished NfkB transcriptional activity. In our experimental
settings we could not detect any significant differences in the protein levels of IκBα and its phosphorylation in VitD treated murine and human pDCs (unpublished data). Yet, the NfκB pathway is highly sensitive to stimulation kinetics and includes a large variety of proteins; therefore, involvement of NfκB cannot be excluded at this stage. However, pDCs comprise a very scarce population in human blood (appr. 0.5%); therefore it will be challenging to delineate elaborate kinetics on the different NfκB components in this DC subset.

The activation of DCs via PRR ligands is mediated by complex signaling cascades involving multiple adaptor molecules and downstream transcription factors (TF). Most TLRs signal via the adaptor protein MyD88 (myeloid differentiation primary response gene 88) which further transduces signals culminating in the activation of the TFs NfκB and members of the interferon-regulatory factor family (IRF) with subsequent initiation of pro-inflammatory gene transcription. Interestingly, we found a significantly increased expression of IRF4 in VitD treated murine and human pDCs (unpublished data). IRF4 is a known inhibitory factor that competes with IRF5 for the binding sites on MyD88 and TRAF6 thereby blocking downstream gene transcription. Upregulation of IRF4 might therefore represent a mechanism of the tolerogenic effect of VitD treatment on pDCs. Experiments with murine IRF4 knockout pDCs or the application of human siRNA targeting IRF4 could verify this hypothesis.

**Nuclear receptor coregulators and inflammation**

NRs are dependent on and form large multi-protein complexes together with coregulatory molecules to repress or activate transcription. These molecules are therefore divided into corepressors and coactivators. As NRs play key roles in the regulation of immune responses, it is not surprising that their coregulators are involved in the control of immunity and tolerance as well. Recent studies provide increasing evidence that corepressors actively inhibit inflammatory gene transcription in the absence of PAMPs. In macrophages, the nuclear receptor corepressor 1 (NCoR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) have been shown to sequester inflammatory gene transcription controlled by NfκB and AP-1, a mechanism referred to as corepressor dependent checkpoints. In steady state, NCoR1 is bound to the transcription factor c-jun on AP-1 target sites while SMRT tethers the ETS transcription factor TEL and the p50 subunit of NfκB on their respective binding sites. Upon maturation of macrophages NCoR and SMRT are released and transcription of pro-inflammatory genes is initiated. The clearance of NCoR is triggered by LPS mediated signaling through TLR4 or TLR2 and subsequent activation of p65/IKKe kinase or calmodulin kinase (CaMKII), respectively. Repression by SMRT is inactivated by signaling through TLR4 or the interferon γ (IFNγ) receptor and a mitogen-activated protein kinase (MEKK) cascade. This inflammation dependent clearance of NCoR and SMRT has been shown to be inhibited by ligand induced activation of PPARγ and LXR, a process referred to as transrepression. The binding of ligand to PPARγ induces the sumoylation of its ligand binding domain and consequently the tethering of PPARγ to the inflammatory promoter bound NCoR/HDAC complex. This ligand dependent binding inhibits the ubiquitylation/19S proteosome machinery and thereby degradation of NCoR. As a result, inflammatory gene transcription remains repressed. Interestingly, transrepression by ligand activated LXR is very similar to the mechanisms utilized by PPARγ involving the sumoylation of LXR. Transrepression mediated by PPARγ and LXR thus represents a mechanism for the integration of inflammatory and anti-inflammatory signals that play essential roles in immunity and homeostasis. To date, it is unknown whether these NR corepressor dependent checkpoints of immune activation
also play a role in DCs. Moreover, the role of NR coregulators in general remains to be investigated in DCs.

The nuclear receptor coregulator DC-SCRIPT orchestrates dendritic cell activation

We have shown that the NR coregulator DC-SCRIPT has an important function in the immunobiology of DCs (chapters 4 and 5). DC-SCRIPT is a versatile NR coregulator that is specifically expressed by DCs within the immune system. It is an early hallmark of DC differentiation from human monocytes. In chapter 4 we show that expression of DC-SCRIPT in moDCs is dependent on IL-4 and that mRNA and protein levels remain present during the complete life cycle of the cells. Moreover, DC-SCRIPT protein is also expressed ex vivo in blood circulating BDCA1+ cDCs, pDCs and in vivo in DC-SIGN+ cDCs of human tonsils; thus DC-SCRIPT is a universal DC marker. In order to better understand the function of DC-SCRIPT in DCs we silenced its expression in moDCs with targeted siRNAs. Knockdown of DC-SCRIPT results in enhanced expression of the anti-inflammatory cytokine IL-10 and subsequent IL-10 dependent repression of the pro-inflammatory cytokine IL-12. Furthermore, silenced DC-SCRIPT expression in moDCs leads to enhanced expression of the GR target gene GILZ (chapter 5). GILZ is a known GR target gene and has been shown to be sufficient for the induction of tolerogenic DCs even in the absence of GR activation. Moreover, silencing of GILZ prevents tolerance. Our experiments showed that silencing of DC-SCRIPT in moDCs results in increased, GR dependent GILZ expression, suggesting that DC-SCRIPT represses GR transcriptional activity on the GILZ promoter. Accordingly, DC-SCRIPT inhibited GR dependent transcription in reporter assays. As DC-SCRIPT is a NR coregulator it is tempting to speculate about a putative IL-10 transrepression mechanism similar to the role of NCoR and SMRT in macrophages (see “Nuclear Receptor coregulators and inflammation”). In steady state, DC-SCRIPT may be bound to the IL-10 promoter and repress transcription while recognition of microbial stimuli may induce clearance of DC-SCRIPT and expression of IL-10. This hypothesis is supported by the fact that DC-SCRIPT has been shown to interact with the histone deacetylases HDAC-1, -3 and -6; proteins which allow for tight DNA wrapping and thereby block transcription (Figure 1). Binding of DC-SCRIPT to the promoter might be direct or through interaction with other important transcription factors like NfκB or AP-1. IL-10 is classically defined as an inhibitory cytokine induced by negative regulators of immune responses. Corinti et al. have shown that moDCs secrete significant amounts of IL-10 after stimulation with the TLR4 ligand LPS, a suggested autocrine, immune regulatory mechanism to prevent sustained inflammatory responses. The immune suppressive function of IL-10 has been demonstrated in vivo as IL-10 deficient mice have decreased bacterial loads after infection with mycobacterium tuberculosis, suggesting an enhanced inflammatory response in the absence of IL-10. In line with these data, blocking of the IL-10 receptor during vaccination against tuberculosis in mice significantly enhanced antigen specific IFN-γ and IL-17A responses and thereby protection against re-infection. However, clinical trials studying the administration of IL-10 in inflammatory conditions like endotoxemia and psoriasis paradoxically showed that IL-10 treatment exacerbated inflammation suggesting a pro-inflammatory role of IL-10. Moreover, small amounts of IL-10 are thought to be necessary for the production of pro-inflammatory cytokines like IL-12. Taken together, the immune modulatory functions of IL-10 extend beyond immunosuppression towards more complex pleiotropic effects. The underlying mechanisms of the seemingly conflicting inhibitory and stimulatory properties of IL-10 remain to be elucidated but are likely to be cell-type and pathogen specific. The role of DC-SCRIPT in this process is currently unknown (Figure 1). Preliminary data suggest that DC-SCRIPT affects multiple different processes in moDCs, including the
phosphorylation status of crucial signaling molecules (J.N. Søndergaard, M. Ansems, G. Adema unpublished data). These pathways may be involved in the induction of IL-10 in the absence of DC-SCRIPT; however, the exact molecular interactions are still to be investigated. Current experiments include chromatin immunoprecipitation assays (CHIP) with DC-SCRIPT in DCs to further elucidate the mechanism of IL-10 repression by DC-SCRIPT and to gain more insight into putative additional target genes.

In conclusion, our data provide evidence that the NR coregulator DC-SCRIPT plays a key role in the immunobiology of DCs, regulating the expression of the pleiotropic cytokine IL-10.

**Hormones and vitamins orchestrate cancer**

A substantial part of carcinomas is associated with the endocrine system, as hormones and vitamins are important factors in the homeostasis of cell proliferation. Hormone dependent cancers typically evolve from glandular tissues like liver, pancreas, ovaries, testicles, prostate and breast. The latter has been particularly well studied with respect to the role of NRs during its development and progression. In breast cancer, the type I NR estrogen receptor (ER) and the type II NR RAR exert opposing effects on apoptosis, cell cycle regulation and growth factor signaling. Typically, estrogens are referred to as pro-tumorigenic, displaying proliferative and anti-apoptotic effects via ER activation, whereas RAR stimulation by retinoids is considered anti-tumorigenic, repressing cell growth while inducing differentiation and/or apoptosis. These observations indicate that balancing the activity of NR is of major importance in keeping a healthy cell phenotype. The effects of a particular NR ligand on a cell will thus not only depend on its own concentration, the expression levels of its receptor and its coregulators, but also on the presence or absence of other ligands, other NR and their coregulators. Indeed, NR coregulators, like the coactivators SRC-1 (steroid receptor coactivator 1) and AIB1 (amplified in breast cancer-1 also known as SRC3) and NR corepressors (NCoRs) play a key role in regulating the cell’s response to NR ligands and have been associated with breast cancer. They play a central role in NR crosstalk, customizing the effect of NRs to each
Recently, we showed that the versatile NR coregulator DC-SCRIPT is expressed in breast epithelial cells and that DC-SCRIPT is an independent prognostic marker.\(^55,56\) DC-SCRIPT was shown to interact with multiple NRs, including ER and RAR; it represses transcription mediated by ER and surprisingly enhances transcription mediated by RAR. Interestingly, exogenous DC-SCRIPT in the breast cancer cell line MCF-7 adversely regulates transcription mediated by multiple NR at the same time. In the presence of multiple NR ligands, DC-SCRIPT is able, in a single cell, to repress transcription mediated by the type I NR PR and activate transcription mediated by the type II receptor RAR.\(^55\) To our knowledge, there are currently no known coregulators that have such a distinct effect on type I and type II mediated transcription. So far, studies investigating crosstalk between ER and RAR have mostly been performed in MCF-7 cells. As this cell line and all other cell lines tested so far are essentially negative for DC-SCRIPT, in contrast to breast epithelial cells, the effect of DC-SCRIPT on the estrogen and RA signaling pathways in breast cancer cells remains to be investigated. This may shed novel insight into the crosstalk between ER and RAR.

In chapter 6 we further extend the role of DC-SCRIPT in hormone dependent carcinomas from breast cancer to prostate cancer. DC-SCRIPT is expressed in morphologically normal prostate epithelial cells and similar to breast cancer, expression is rapidly lost upon malignant transformation of these cells. Moreover, DC-SCRIPT expression is also absent in the prostate carcinoma cell lines 22RV1 and LNCaP. In luciferase based reporter assays DC-SCRIPT represses transcription mediated by the type I NR AR (androgen receptor), whereas it activates transcription mediated by the type II NR VDR. In line with these data, exogenous expression of DC-SCRIPT in 22RV1 and LNCaP cells leads to reduced expression of the AR target genes TMPRSS2 and SPDEF and increased expression of the VDR target gene Cyp24A1. AR and VDR play opposing roles in the etiology of prostate cancer with AR promoting and VDR inhibiting cancer progression.\(^57-60\) The opposing effect of DC-SCRIPT on these prostate cancer associated NRs suggests that DC-SCRIPT counter regulates their effects on cell cycle, promoting apoptosis and repressing cell growth. Indeed, exogenous expression of DC-SCRIPT in 22RV1 and LNCaP \textit{in vitro} inhibits cell proliferation.

Our data show that the NR coregulator DC-SCRIPT plays an important role in the etiology of breast and prostate cancer. Moreover, it is tempting to speculate about a possible function of DC-SCRIPT in additional hormone dependent cancers like ovarian carcinoma. Its role in breast and prostate carcinoma is likely to be mediated by its opposing effects on the transcriptional activity of type I and type II NRs. Whether this hypothesis holds true is yet to be determined and objective to current studies. CHIP based assays will shed more light on the NR target genes of DC-SCRIPT but may also provide insight into putative direct targets in epithelial cells.

**Hormones and vitamins – therapeutic applications**

Hormones, vitamins and their receptors are implicated in and orchestrate a wide variety of physiological processes like homeostasis, cell growth, development and immunity. Pathological conditions within these processes are consequently also associated with NRs. The ligand dependent activity of NRs designates them as ideal targets for pharmaceutical intervention. Among the most prevailing synthetic NR agonists and antagonist used in the clinic are tamoxifen for the ER (targeted in breast cancer), casodex for the AR (targeted in prostate cancer) and dexamethasone for the GR (targeted in inflammatory diseases).\(^61\) Dexamethasone and other pharmaceutical glucocorticoids are used in the clinic for their anti-inflammatory effects mediated by binding of the GR. Systemic treatment with GR agonists is applied for a wide variety of inflammatory disorders including rheumatoid arthritis, asthma, psoriasis and inflammatory bowel disease. Moreover, glucocorticoids
are used to prevent graft rejection in transplantation settings. The underlying mechanisms of the immune suppressive function of these treatment modalities include the inhibition of pro-inflammatory cytokine production and enhancement of anti-inflammatory cytokine expression. Target cells within the immune system thereby range from T- and B cells, over granulocytes to DCs.

**Hormones and vitamins targeting NRs in dendritic cells**

The key role of DCs within the immune system designates these cells as strategic targets of immunotherapy. In chapter 3 we show that both murine and human pDCs are inhibited by VitD signaling through the VDR and suggest that impaired pDC responses may be an underlying mechanism of efficient VitD treatment in psoriasis (Figure 2). Interestingly, in addition to psoriasis, pDCs have been implicated in the onset and progression of systemic lupus erythematosus (SLE). Autoantigen immune complexes released by apoptotic cells in SLE patients activate pDCs which in turn induce activation of cDCs subsequently leading to the priming of autoreactive T- and B cells. Similar to psoriasis, SLE is also associated with VitD deficiency suggesting a role of reduced anti-inflammatory signaling via the VDR in the pathogenesis of SLE. A recent clinical trial with VitD supplementation in SLE patients indeed showed reduced inflammatory marker expression. Therefore, it is tempting to speculate about a putative role for VitD mediated inhibition of pDCs and cDCs in patients with SLE.

![Hypothetical model of the suppressive effect of vitamin D in inflammatory skin diseases.](image)

**Figure 2. Hypothetical model of the suppressive effect of vitamin D in inflammatory skin diseases.** Plasmacytoid dendritic cells (pDC) are implicated in the onset of different inflammatory skin diseases like psoriasis and SLE. Topical application of vitamin D (VitD) analogs is an effective treatment of against skin inflammation. This effect might be mediated by the VitD induced inhibition of pDCs and subsequent impairment of T cell proliferation and secretion of IFNγ.

About 15 years ago, DCs have first been exploited for anti-cancer immunotherapy by Ralph Steinman. Studies then and nowadays mostly make use of *in vitro* generated moDCs that are loaded with tumor associated antigens in the form of mRNA or peptide. The primed moDCs are subsequently activated and administered to the patient where they are thought to induce the activation of tumor specific cytotoxic T cells, amongst other immune cells, and thereby an antitumor
immune response. DC vaccines have been proven to be non-toxic and effective but clinical responses still hold short. Different strategies are exploited to improve DC based anti-cancer vaccines aiming to induce the most optimal immunogenic phenotype of DCs. Hormones and vitamins orchestrate the function and phenotype of DCs and therefore NRs may represent useful targets for the improvement of DC-based vaccines. Recently, the NR4A orphan receptor NOR1 has been shown to induce apoptosis in DCs and silencing of NOR1 significantly inhibited activation-induced cell death of DCs. Moreover, knockdown of NOR1 enhanced the antitumor efficacy of DC vaccines in mice. Interestingly, we have preliminary data that Nur77, another NR4A orphan receptor, inhibits the immune response of pDCs and cDCs. The combined silencing of NOR1 and Nur77 could therefore result in a hyperactive, immunogenic DC with enhanced survival. These two NR4A receptors therefore represent suitable targets to improve DC-based vaccines.

Concluding remarks

In summary, this thesis extends the current knowledge on how hormones and vitamins orchestrate DCs and cancer. We show that different DC subsets and prostate cancer are functionally affected by NRs, their ligands and the NR coregulator DC-SCRIPT. The targeted modification of NRs and their coregulators may represent a suitable approach to improve DC-based vaccination strategies for malignant diseases. Further research is essential to provide a more comprehensive understanding of different hormones and vitamins in DC and cancer biology and thus a rationale basis for therapeutic applications. The important role of hormones and vitamins as orchestrators of DCs and cancer explains the plasticity of cells and highlights that cells should not be seen individually but rather as a part of their given microenvironment.

References


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Part 4 - Discussion & Summary


Chapter 8

Nederlandse Samenvatting
&
Deutsche Zusammenfassung
Nederlandse Samenvatting

Het menselijk lichaam is opgebouwd uit miljarden kleine bouwstenen die we met het blote oog niet kunnen zien, de zogenaamde cellen. Al deze cellen kunnen verschillende functies hebben en vormen onze organen, zo hebben we speciale hersencellen, huidcellen, hartcellen, maagcellen etc. In een orgaan staan al deze cellen met elkaar in contact om te waarborgen dat een orgaan goed functioneert. Om goed met elkaar te kunnen communiceren scheiden cellen verschillende ‘boodschapper’ stofjes uit die er voor zorgen dat de informatie efficiënt van de ene cel naar de andere cel komt. Een voorbeeld van deze belangrijke chemische boodschappers in ons lichaam zijn hormonen en vitamines.

Hormonen en Vitamines
In ons lichaam worden hormonen geproduceerd door gespecialiseerde organen, de zogenaamde endocriene klieren. Voorbeelden hiervan zijn de geslachtsorganen, de alvleesklier, de hypofyse en de schildklier. Elk orgaan zorgt voor de productie van een bepaald repertoire aan hormonen. Hormonen reguleren belangrijke processen in ons lichaam, zoals lichamelijke groei, voortplanting, metabolisme en immunologische reacties. Verstoringen in de hormonale balans kunnen derhalve leiden tot ziekten zoals osteoporose, onvruchtbaarheid, diabetes en auto-immuun syndromen.

“Eet je groente op!”, is een zin die we in onze jeugd bijna allemaal dagelijks hebben gehoord en is sterk gekoppeld met de vraag “Waarom?”. Vitamines kunnen worden gedefinieerd als essentiële voedingsstoffen, dat wil zeggen stoffen die we in ons lichaam zelf niet in voldoende mate kunnen produceren, welke wij constant door middel van voedsel tot ons moeten nemen. Met uitzondering van vitamine B12, die alleen in dierlijke producten voorkomt, komen alle andere 13 vitamines voor in onze dagelijkse portie groenten. Vitamines zijn voor ons lichaam essentiële voedingsstoffen en een tekort aan vitamines kunnen resulteren in pathologische effecten zoals bijvoorbeeld blindheid en osteoporose.

Nucleaire Receptoren
Cellen zijn uitgerust met zogenaamde nucleaire receptoren (NR) welke instaat zijn om hormonen en vitamines te herkennen en te binden. Echter deze NRs herkennen niet alleen hormonen en vitamines, maar zijn ook instaat om vetten en andere stofwisselingsproducten te binden. In het menselijk lichaam kunnen we wel 50 verschillende typen NRs onderscheiden, en elk van deze receptoren wordt geactiveerd door een specifieke stof, het ligand. Zo bindt de oestrogeen receptor het vrouwelijk hormoon oestrogeen, en wordt door zonlicht geactiveerd vitamine D door de Vitamine D receptor gebonden. Over het algemeen kunnen NRs worden ingedeeld in twee hoofdcategorieën op basis van hun werkingsmechanisme: 1) Type I nucleaire receptoren en 2) type II nucleaire receptoren. Bekende voorbeelden van type I NRs zijn de oestrogeen receptor (ER), de progesteron receptor (PR), de androgeen receptor (AR, bindt testosteron) en de glucocorticoid receptor (GR, bindt cortisol). Type II nucleaire receptoren werken samen met de retinoïc X receptor (RXR) om hun functie uit te kunnen oefenen. Voorbeelden van type II nucleaire receptoren zijn de vitamine D receptor (VDR), all-trans-retinoic acid receptor (RAR) en peroxisoom proliferator-geactiveerde receptor (PPAR, bindt vetzuren). Een derde categorie omvat de zogenaamde ‘orphan’ receptoren waarvoor geen ligand bestaat of welke nog niet is gevonden. Als NRs hun liganden binden, leidt dit tot de productie en secretie van bepaalde eiwitten door cellen wat er voor zorgt dat cellen adequate reageren. De activiteit van NRs is afhankelijk van specifieke eiwitten, de zogenaamde
coregulatoren. Op dit moment zijn er meer dan 300 coregulatoren bekend en beschreven. Sommige van deze coregulatoren kunnen de functie van NRs activeren (co-activatoren), terwijl anderen coregulatoren de functie van NRs remmen (co-repressoren). Een speciale coregulator is het eiwit DC-SCRIPT (dendritic cell specific transcript). DC-SCRIPT is instaat om enerzijds de functie van type I NRs te remmen terwijl het tegelijkertijd de functie van type II NRs kan activeren.

Kort samengevat, vitamines, hormonen en hun bijbehorende NRs en coregulatoren reguleren veel belangrijke processen in ons lichaam en worden daarom geassocieerd met verschillende klinische syndromen. In dit proefschrift wordt dieper ingegaan op enkele aspecten van vitamines en hormonen en welke rol ze spelen in het immuunsysteem en kanker.

 Het Immuunsysteem
Ons immuunsysteem beschermt ons dagelijks tegen allerlei verschillende ziekteverwekkers van buitenaf, zoals bacteriën, virussen en parasieten. Bovendien verschaf ons immuunsysteem, in de meeste gevallen, ook bescherming tegen veranderde lichaamseigen cellen, zoals kankercellen, terwijl gezonde normale cellen niet worden dood gemaakt. Deze efficiënte immunologische afweer tegen ziekteverwekkers en gevaarlijke cellen enerzijds en de tolerantie voor gezonde lichaamseigen cellen anderzijds is een complex en fijn afgestemd systeem. Het immuunsysteem bestaat uit verschillende gespecialiseerde cellen en factoren die met elkaar communiceren en op een gecoördineerde wijze informatie uitwisselen. In het algemeen kunnen twee mechanismen van het immuunsysteem worden onderscheiden: 1) de aangeboren en 2) de verworven immuuniteit. In eerste instantie worden bacteriën en virussen geconfronteerd met de mechanische en fysiologische barrières van het immuunsysteem. Onze huid en de slijmvliezen van bijvoorbeeld de ogen, mond en neus kunnen de invasie van bacteriën en virussen voorkomen. Mocht een pathogeen toch binnenvallen, dan stuiten ze op toxische antimicrobiële stoffen en de aanval van de witte bloedcellen van het aangeboren immuunsysteem, zoals granulocyten, mestcellen, macrofagen en dendritische cellen. Deze bescherming van het aangeboren immuunsysteem is zeer snel en valt pathogenen op een niet-specifieke wijze aan. Het verworven (adaptieve) immuunsysteem is vele malen langzamer dan het aangeboren immuunsysteem, maar heeft als grote voordeel dat het pathogene-specifiek is. De witte bloedcellen van het adaptieve immuunsysteem, T en B cellen, herkennen specifieke eiwitten die op het oppervlak van ziekteverwekkers voorkomen en reageren hierop. Voordat T en B cellen pathogenen kunnen herkennen, moeten ze worden geactiveerd en getraind door zogenaamde antigeen presenterende cellen. Deze cellen hebben de capaciteit om pathogenen op te nemen, om ze vervolgens intern te verteren en in kleine stukjes te verwerken en daarna deze stukjes te presenteren op hun celoppervlak aan de T en B cellen. Na activatie door antigeen presenterende cellen zijn B cellen instaat om pathogene-specifieke antilichamen uit te scheiden die zorgen voor een betere pathogene herkenning en zijn T cellen instaat om virus-geïnfecteerde cellen of tumorcellen te doden. De familie van de meest professionele antigeen presenterende cellen zijn dendritische cellen (DC).

Hormonen & Vitamines in de context met dendritische cellen
DCs zijn witte bloedcellen die in ons lichaam patrouilleren en voortdurend op zoek zijn naar ziekteverwekkers. DCs zijn te vinden in ons bloed en ook in organen waar zij in een immature staat verblijven. In deze immature staat zijn DCs zeer efficiënt in het opsporen en registreren van ziekteverwekkers. Tijdens een infectie worden DCs geactiveerd door specifieke structuren die zich op het oppervlak van pathogenen bevinden. Na activatie zullen de DCs vanuit de organen naar de...
lymfeknopen migreren waar ze vervolgens zorgen voor het instrueren en activeren van T en B cellen. De DC familie is zeer heterogeen en bestaat uit verschillende cellen met allemaal hun eigen specifieke eigenschappen. De twee belangrijkste subsets zijn de klassieke DCs (cDC) en de plasmacytoïde DCs (pDC). cDCs zijn uitgerust met vele moleculen om snel en goed pathogenen in hun omgeving op te nemen en te verwerken, terwijl pDCs voornamelijk bekend staan om hun snelle productie van grote hoeveelheden van het antivirale eiwit interferon alfa (IFNα). DCs beschikken over een grote verscheidenheid aan receptoren, waaronder NRs, die ze gebruiken om hun omgeving te herkennen en om zich aan hun omgeving aan te passen. Het is bekend dat hormonen en vitamines, door te binden aan NRs, de functies van DCs kunnen reguleren. Zo is vitamine A zeer belangrijk in onze darmen om de functie van de lokale DCs te remmen, zodat de darmbacteriën, die belangrijk voor ons zijn, niet worden aangetast. Vergelijkbaar met de functie van vitamine A in de darm, speelt door de zon geïnduceerde vitamine D een belangrijke rol in de huid. Ook vitamine D onderdrukt de activatie van DCs en voorkomt daarmee de vernietiging van belangrijke bacteriën op en in de huid. Hoewel het lichaam de natuurlijke eigenschappen van hormonen en vitamines al gebruikt om de functie van DCs te controleren, kunnen we uiteraard in de geneeskunde ook gebruik maken van deze eigenschappen. Een voorbeeld hiervan is cortisol, een anti-inflammatoire stof, die de glucocorticoïde receptor bindt en hierdoor de activiteit van vele immuun cellen (waaronder DCs) verhinderd. Hoewel we redelijk veel weten wat de invloed is van vitamines en hormonen op klassieke DCs, weten we relatief weinig over hun effect op pDCs. In Deel II van dit proefschrift hebben we daarom verschillende aspecten van de functie van NRs in pDCs onderzocht.

In hoofdstuk 2, hebben we de aanwezigheid van 28 verschillende NRs in cDCs en pDCs vergeleken. Onze gegevens tonen aan dat cDCs en pDCs in vitro, dat wil zeggen buiten het lichaam in het laboratorium, en geïsoleerd uit lymfoïde organen hetzelfde repertoire NRs vertonen. Echter, er is wel een verschil in de hoeveelheid van individuele NRs in cDCs of pDCs, en deze verschillen worden vooral duidelijk in geactiveerde cellen.

In hoofdstuk 3 onderzoeken we de effecten van vitamine D op pDCs. Het is bekend dat vitamine D de functie van het immuunsysteem kan remmen en in het bijzonder cDCs (zie hierboven). Daarom worden vitamine D bevattende zalven vooral toegepast voor de behandeling van auto-immuunziekten zoals systemische lupus erythematoses en psoriasis. Echter, het exacte werkingsmechanisme van deze therapie is tot op heden niet bekend. Voor verschillende auto-immuunziekten, zoals systemische lupus erythematoses, is beschreven dat pDCs een belangrijke rol spelen in de ontwikkeling en progressie van deze auto-immuunziekten. Wij laten zien dat pDCs die behandeld zijn met vitamine D verminderde functies hebben en niet goed instaat zijn om T cellen te activeren (Hoofdstuk 3). Voor de behandeling van immunologische huidziekten zouden we kunnen profiteren van de beschreven remmende eigenschappen van pDCs door patiënten te behandelen met vitamine D preparaten. Zoals eerder beschreven is de activiteit van NRs afhankelijk van coregulator eiwitten. De meeste coregulatoren hebben de capaciteit om NRs zowel te kunnen remmen als te activeren. In Deel III van dit proefschrift werd onderzoek gedaan naar de coregulator DC-SCRIPT, een speciaal eiwit dat zowel negatieve als positieve regulerende functies heeft. In de hoofdstukken 4 en 5, hebben we de rol van DC-SCRIPT in DCs onderzocht. Onze gegevens tonen aan dat DC-SCRIPT in cDCs en pDCs de productie van immuun activerende moleculen genaamd cytokines beïnvloedt. DC-SCRIPT remt de productie van de immuun onderdrukkende cytokine IL-10, waardoor het tegelijkertijd de productie van de immuun stimulerende cytokine IL-12 bevordert (Hoofdstuk 4). Als gevolg lijkt het dat DC-SCRIPT de immuun stimulerende eigenschappen van DCs versterkt. In hoofdstuk 5 laten we zien dat DC-SCRIPT
de werking van de glucocorticoid receptor (GR) remt. Verder laten we zien dat in de afwezigheid van DC-SCRIPT het GR geactiveerde molecuul ‘Gilz’ in grotere hoeveelheden in DCs aanwezig is. GR is bekend om zijn remmende effecten op DCs, en DC-SCRIPT lijkt dus de immuunsysteem onderdrukkende eigenschappen van DCs te remmen. Samengevat, de resultaten beschreven in de hoofdstukken 2 tot 5 geven nieuwe inzichten en benadrukken de belangrijke effecten van hormonen, vitamines, NRs en hun coregulatoren op de functie van DCs en daarmee op immunologische reacties.

**Hormonen & Vitamines in de context van kanker**

Kanker betekend in de geneeskunde het ontstaan van nieuwe kwaadaardige weefsels. Door verschillende mechanismen kunnen in het genetisch materiaal (DNA) vastgelegde kenmerken van een gezonde cel veranderen (muteren), wat tot gevolg kan hebben dat een cel ongecontroleerd gaat delen en dochtercellen gaat vormen wat uiteindelijk leidt tot de formatie van een kwaadaardige weefselmassa (gezwel). Het is bekend dat tumoren verschillende eigenschappen van hun gezonde moedercellen overnemen. Dit concept is vooral duidelijk bij tumoren van klierweefsels, zoals bij borstkanker en prostaatkanker. De gezonde ontwikkeling van de borst is afhankelijk van de vrouwelijke hormonen oestrogeen en progesteron. Gedurende transformatie behouden borsttumoren deze eigenschappen en is ook hun groei grotendeels afhankelijk van deze hormonen en daarmee ook van de aanwezigheid van de oestrogeen en progesteron receptor (ER en PR). Andere NRs, zoals PPAR en RAR kunnen daarentegen de groei van tumorcellen remmen. Wederom is het ook hier mogelijk om deze kennis toe te passen voor het ontwikkelen van nieuwe therapeutische behandelingen. Zo bestaat de mogelijkheid al om patiënten met hormoonafhankelijke tumoren te behandelen met het geneesmiddel tamoxifen. Tamoxifen is een anti-oestrogeen. Dat wil zeggen dat het de werking van het vrouwelijke geslachtshormoon oestrogeen tegengaat.

Recent is aangetoond dat de NR coregulator DC-SCRIPT een belangrijke rol speelt in de ontwikkeling van borstkanker. Hoewel het DC-SCRIPT eiwit in gezonde cellen in de borst tot expressie komt, is het vele malen lager te vinden in borst tumorcellen. Interessant genoeg remt DC-SCRIPT de functie van de groeijezinkende receptoren ER en PR, en activeert het de functie van de groeiwerkingende receptoren PPAR en RAR. Bovendien correleert de aanwezigheid van grote hoeveelheden DC-SCRIPT met een betere prognose van borstkankerpatiënten. Net als de ontwikkeling van borstkanker is de gezonde ontwikkeling van de prostaat en ook de groei van kwaadaardige prostaat tumor op soortgelijke wijze afhankelijk van het mannelijke hormoon testosteron wat weer bindt aan de nucleaire androgeen receptor (AR). Daarentegen is het voor de vitamine D receptor (VDR) beschreven dat het juist de groei van tumorcellen remt. Prostaatkankerpatiënten ontvangen geneesmiddelen die ofwel de testosteron niveaus verminderen of AR direct remmen. In hoofdstuk 6, gaan we dieper in op de rol van DC-SCRIPT in tumoren en hebben we onderzocht wat de functie van DC-SCRIPT in prostaatkanker. We tonen aan dat in gezonde prostaat cellen het eiwit DC-SCRIPT aanwezig is, terwijl in prostaat tumorcellen DC-SCRIPT niet meer aantoonbaar is. Bovendien blijkt uit onze resultaten dat DC-SCRIPT de groeiwhassuilerende werking van AR remt en de functie van de groeiwerkingende VDR activeert. Samengevat is het dus waarschijnlijk dat DC-SCRIPT een belangrijke rol speelt in de ontwikkeling van prostaatkanker.

In hoofdstuk 7, beschrijf en bediscussieer ik de resultaten die in dit proefschrift zijn beschreven en implementeer en combineer ik de betekenis van deze resultaten in de bredere context omtrent het effect van vitamines, hormonen en hun bijbehorende NRs in DCs en kanker.
Perspectief
De in dit proefschrift beschreven resultaten geven nieuwe inzichten over de effecten die hormonen en vitamines kunnen hebben op dendritische cellen en kanker cellen. We zien dat de functie van verschillende soorten dendritische cellen en de functie van prostaattumoren wordt beïnvloed door hormonen, vitamines, NRs en de NR coregulator DC-SCRIPT. De specifieke modificatie van NRs en hun coregulatoren kan bijdragen tot de verbetering van bestaande en het ontwikkeling van nieuwe innovatieve vormen van immuuntherapie. Echter, naar mijn mening zijn er ook nog vele deuren die geopend dienen te worden om de exacte rol van NRs te begrijpen in gezondheid en ziekte.
Deutsche Zusammenfassung


Hormone und Vitamine

Nukleäre Rezeptoren
Part 4 - Discussion & Summary


Das Immunsystem


Hormone, Vitamine und Dendritische Zellen im Kontext

DCs sind weiße Blutzellen, die unseren Körperständig patrouillieren und nach Krankheitserregern suchen. Sie sind in unserem Blut und in unseren Organen zu finden, wo sie in unreifem Zustand verkehren. Unreife DCs sind sehr effizient in der Erkennung und Aufnahme von Keimen. Während einer Infektion werden DCs durch spezifische Strukturen auf der Oberfläche von Erregern aktiviert. Hierdurch können die DCs aus den Organen in unsere Lymphknoten migrieren, wo sie T- und B-Zellen aktivieren. Die DC-Familie besteht aus verschiedenen Kategorien von Zellen. Die zwei wichtigsten Sorten sind einerseits die klassischen DCs (cDC) und andererseits die plasmazytoiden DCs (pDC). Beide Sorten haben spezielle Funktionen; cDCs sind sehr schnell in der Aufnahme von Erregern und pDCs sind bekannt für ihre schnelle Produktion von großen Mengen des antiviralen Interferon Alpha (IFNα). DCs verfügen über eine Vielzahl von Rezeptoren, um ihre Umgebung zu erkennen und sich entsprechend anzupassen, mitunter haben sie auch Nukleäre Rezeptoren. Es ist bekannt, dass Hormone und Vitamine die Funktion von DCs steuern können. So ist das Vitamin A in unserem Darm
wichtig, um die Funktion der örtlichen DCs zu hemmen, so dass die für uns wichtigen Darmbakterien nicht angegriffen werden. Ähnlich zu der Funktion von Vitamin A im Darm bekleidet das Sonnenlicht-induzierte Vitamin D eine wichtige Rolle in der Haut. Auch Vitamin D unterdrückt die Aktivierung von DCs und verhindert somit die Zerstörung von wichtigen Hautbakterien. Neben diesen natürlichen Funktionen, werden die prägenden Eigenschaften von Vitaminen und Hormonen auf DCs auch in der Medizin ausgeschöpft. Das entzündungshemmende Kortisol ist ein Stoff, der den Glukokortikoid Receptor bindet und neben anderen Immunzellen auch DCs in ihrer Aktivität hindert. Obwohl wir viel über den Einfluss von Vitaminen, Hormonen und ihren NRs auf klassische DCs wissen, ist hinsichtlich pDCs noch wenig bekannt. In Teil II der vorliegenden Dissertation haben wir darum Aspekte der Funktion von NRs in pDCs erforscht.

In Kapitel 2 verglichen wir die Anwesenheit von 28 verschiedenen NRs in cDCs und pDCs. Unsere Daten belegen, dass cDCs und pDCs in vitro, d. h. außerhalb des Körpers im Labor, und isoliert aus lymphatischen Organen dasselbe NR Repertoire aufweisen. Die Menge von individuellen NRs in cDCs oder pDCs ist jedoch unterschiedlich; diese Unterschiede sind besonders deutlich in aktivierten Zellen.

In Kapitel 3 untersuchen wir die Effekte von Vitamin D auf pDCs. Es ist bekannt, dass Vitamin D die Funktion des Immunsystems und insbesondere von cDCs inhibieren kann. Aus diesem Grund werden Vitamin D-haltige Salben zur Behandlung von Autoimmunkrankheiten, wie Schuppenflechte und Schmetterlingsflechte, eingesetzt. Die exakten Wirkmechanismen dieser Therapie sind jedoch nicht bekannt. pDCs sind in entscheidendem Maße beteiligt an der Entwicklung und dem Verlauf der genannten Autoimmunerscheinungen. Wir demonstrieren, dass Vitamin D behandelte pDCs verminderte Funktionen haben und T-Zellen somit nicht vollständig aktivieren können (Kapitel 3). Das Vermögen der repressiven Eigenschaften von pDCs in therapeutischen Anwendungen könnte somit von der klinischen Verfügbarkeit von Vitamin D Präparaten zur Behandlung von immunologischen Hautkrankheiten profitieren.

Wie zuvor beschrieben, ist die Aktivität von NRs abhängig von koregulatorischen Proteinen. Die meisten Koregulatoren können NRs entweder hemmen oder aktivieren. Teil III dieser Arbeit untersucht DC-SCRIPT, ein besonderes Protein, welches sowohl die negativen als auch die positiven regulatorischen Funktionen erfüllen kann. In Kapitel 4 und 5 haben wir die Rolle von DC-SCRIPT in DCs ermittelt. Unsere Daten belegen, dass DC-SCRIPT in cDCs und pDCs anwesend ist und die Produktion von immunaktiven Molekülen, den sogenannten Zytokinen, beeinflusst. DC-SCRIPT hemmt die Produktion des immunrepressiven Zytokins IL-10 und fördert damit die Produktion des immunstimulierenden Zytokins IL-12 (Kapitel 4). Demzufolge scheint DC-SCRIPT die immunstimulierenden Eigenschaften von DCs zu verstärken. In Kapitel 5 zeigen wir, dass DC-SCRIPT die Funktion des Glukokortikoid Receptors (GR) hemmt. Übereinstimmend zeigen wir, dass in Abwesenheit von DC-SCRIPT das GR aktivierte Molekül GILZ in größerer Menge in DCs anwesend ist. GR ist bekannt für seine repressiven Effekte auf DCs, und DC-SCRIPT scheint somit die immunrepressiven Eigenschaften von DCs zu unterdrücken. Zusammenfassend geben unsere Daten aus den Kapiteln 2 bis 5 neue Einsichten und stellen die bedeutenden Effekte von Hormonen, Vitaminen ihren NRs und Koregulatoren auf die Funktion von DCs und somit auf immunologische Abwehrreaktionen nachdrücklich heraus.
Hormone, Vitamine und Krebs im Kontext


In Kapitel 7 fasse ich die erworbenen Erkenntnisse dieser Dissertation zusammen und erörtere die Bedeutung unserer Daten im Kontext des aktuellen Forschungsstandes.

Perspektive

Chapter 9

Acknowledgements
Curriculum Vitae
List of Publications
Acknowledgements

The final chapter – time to say “Thank You”.

The most important part of a thesis? Probably. The most read part of a thesis? Definitely! It is a matter of fact that quality outstands quantity. Moreover, it is definitely more challenging to put things in fewer rather than (too) many words. “Thank you”: two words that already say it all, so I only want to add a few more. I hope that each of you already knows what I am thankful for because I can’t even mention all of you and all the little things that make me smile 😊

Beste Gosse, vier jaar promotietijd vergen een goede interactie tussen promotor en PhD student. Je hebt me veel vrijheid gelaten in mijn keuzes rondom mijn project, maar ook de nodige stimulaties gegeven. Ik denk graag terug aan onze discussies. Bedankt voor je altijd open deur en oor!

Lieve Annemiek en Marleen, als PhD studente heb je soms (vrouwelijke) hulp nodig in de vorm van co-promotoeren. Gelukkig is Gosse de vrouwen thuis al gewend, want ik denk dat we het hem soms niet makkelijk hebben gemaakt. Bedankt voor al jullie input, snelle reacties en steun!

Lieve (Ex)-bewoners van het Aquarium: Maaike, Marleen, Saartje, Anna, Dada, Bas, Jonas en Melissa (jij hoort er ook gewoon bij ;-) ). Het is erg special om elke dag met een lach naar je werk te mogen gaan, bedankt! Lieve SCRIPT-meisjes, alleen teamwork leid tot goede resultaten. Ik heb ervan genoten urenlang samen tumoren te bewerken (Marleen, Saartje, Maaike) en de record voor Elisa’s per dag altijd weer opnieuw te breken (Maaike). Het was erg gezellig! Maaike, mijn paranimpje, jou naam staat niet voor niets op alle publicaties in dit boekje, bedankt. We houden contact! Ania, our aged coffee deal is still on 😊 Jonas, I will miss the Danish radio at work. Melissa, mijn tweede paranimpje, je hebt me vaak gered met grote muis experimenten, bedankt. Ik hoop we gaan nog veel samen shoppen en koffieleuten, en gelukkig is er what’s app.

My students: Shuo and Lisanne. You both worked very hard and believe me, I learned just as much from you, as the other way around. I very much enjoyed working with you, thanks!

Paul en Michiel, keep on smiling 😊 Stanley, sommige algemene PBS flessen zijn toch echt schoon ;-) . Alie, bedankt voor je hulp in nood van reuze experimenten. All the rest of the TILers, you just are too many 😊, thank you for almost 6 fun years filled with cookies, parties and laughter at work. It’s good that I leave Jurjen behind to have an excuse for future visits to the lab.

Dear friends: Bubs, Joost, Christina, Luis, Sonja, Friedel, Matthi, Qian, Sabri and Marce. Sharing food, drinks, games, stories (serious and fun) is what makes life so colorful, thank you! It’s comforting to have good friends around the world, even though we don’t always have enough chances to see or talk to each other.

Lieve bewoners van de Canalstreet 31 t/m 37. Ik had nooit gedacht, dat ik van het communeleven zou genieten ;-) Het is erg fijn om te weten, dat er altijd iemand is, bedankt!

Liebe Mama, Papa, Anne und Spiro. Man sagt, dass man sich seine Familie nicht aussuchen kann. Ich würde euch immer wieder nehmen! Danke für euren Rückhalt, ein zu Hause, gute Gespräche, die verrückten Aktionen und einfach alles!

Oma Gisela, der letzte Mohikaner. Ich bin sehr glücklich, dass du diese Zeilen noch lesen darfst. Du bist das beste Beispiel dafür, dass man auch im Alter niemals auslernnt.

Leave Heit, Mem, Richtsje, Patrick en Happy. Net iderien hat it lok in twadde famylje te meie ha! Ik fiel my thús by jimme, tige tanke.
Lieve Jurjen. Alleen voor jou zou ik een pagina kunnen vullen, en juist daroom hou ik het heel kort. Jij bent alles wat ik niet ben en ik ben alles wat jij niet bent. Dat maakt ons beiden gek, op de meest mooie en speciale manier die er is! Ik hou van jou.

Nina

“Begin at the beginning and go on till you come to the end: then stop.”
(Lewis Carroll, Alice in Wonderland)
Curriculum Vitae (Nederlands)

Nina Karthaus werd geboren op 6 Juni 1984 te Arnsberg, Duitsland. Na het behalen van haar Abitur diploma in 2004 aan het Graf-Gottfried-Gymnasium te Arnsberg, is zij de studie Biologie met zwaartepunt genetica, cel biologie en fysiologie gaan volgen aan de Universität Bielefeld. In haar bachelorstage, onder begeleiding van dr. Brigitte Dreiseikelmann op de afdeling Gentechnologie en Microbiologie, onderzocht zij de functie van de c1-repressor in de temperente bacteriofaag ØO18P. Hiermee behaalde zij in 2007 haar Bachelor diploma. In september van hetzelfde jaar vertrok ze naar Nijmegen om het Top Master programma ‘Molecular Mechanisms of Disease’ aan de Radboud Universiteit te volgen; een moleculaire studie met focus op translationeel onderzoek. Tijdens deze studie heeft zij een jaar stage gelopen op de afdeling Tumor Immunologie van het Radboud Universitair Medisch Centrum onder begeleiding van professor dr. Gosse J. Adema waar ze onderzoek deed naar de interactie van nucleaire receptoren met het eiwit DC-SCRIPT in dendritische cellen. Een tweede stage van acht maanden werd uitgevoerd op de afdeling Pathology and Immunology van de Washington University School of Medicine in St Louis, Verenigde Staten van Amerika. Onder begeleiding van professor dr. Marco Colonna deed zij onderzoek naar de inhiberende receptor TREML6 op immuun cellen. In september 2009 is Nina cum laude afgestudeerd.

Onder begeleiding van professor dr. Gosse J. Adema begon ze aansluitend op de afdeling Tumor Immunologie als onderzoeker in opleiding te werken aan haar door de Radboud Universiteit Nijmegen gesubsidieerd, persoonlijke onderzoeksplan. De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift. Vanaf december 2013 is ze werkzaam op het laboratorium Klinische Chemie en Hematologie in het Jeroen Bosch ziekenhuis te s’-Hertogenbosch, waar zij onder begeleiding van professor dr. Ron Kusters de opleiding tot klinisch chemicus volgt.
Curriculum Vitae (Deutsch)


Unter der Leitung von Prof. Dr. Gosse J. Adema begann sie anschließend als Doktorandin am Institut für Tumor Immunologie, wo sie an einem von der Radboud Universität finanzierten, individuellen Forschungsvorhaben arbeitete. Die Ergebnisse dieser wissenschaftlichen Arbeit werden in der vorliegenden Dissertation beschrieben. Seit Dezember 2013 arbeitet sie im Labor für Klinische Chemie und Hämatologie im Jeroen Bosch Krankenhaus in s’-Herzogenbosch, wo sie unter der Leitung von Prof. Dr. Ron Kusters die Weiterbildung zur Klinischen Chemikerin absolviert.
Curriculum Vitae (English)

Nina Karthaus was born on June 6th, 1984 in Arnsberg, Germany. After obtaining her Abitur diploma at the Graf-Gottfried-Gymnasium in Arnsberg in 2004, she started her bachelor studies at the University of Bielefeld with the focus on genetics, cell biology and physiology. Her bachelor thesis, performed at the department of Gene technology and Microbiology under the supervision of Dr. Brigitte Dreiseikelmann, investigated the function of the c1-repressor in the temperent bacteriophage ØO18P. After graduating from her bachelor in 2007, she moved to Nijmegen to start the top master’s program ‘Molecular Mechanisms of Disease’ at the Radboud University; a molecular program with a strong focus on translational research. During these studies she performed a yearlong internship at the department of Tumor Immunology, Radboud University Medical Centre, under the supervision of Professor Dr. Gosse J. Adema, where she investigated the interaction between nuclear receptors and the protein DC-SCRIPT in dendritic cells. She carried out a second internship of 8 months at the department of Pathology and Immunology at the Washington University School of Medicine in St. Louis, United States of America. Under the supervision of Professor Dr. Marco Colonna she studied the inhibitory receptor TREML6 on immune cells. In September 2009, Nina received her master’s degree cum laude.

Subsequently, she obtained a personal research grant from the Radboud University Nijmegen and started working as a PhD student at the department of Tumor Immunology under the supervision of Professor Dr. Gosse J. Adema. The results of her PhD training are described in this thesis. In December 2013 she moved to the Laboratory for Clinical Chemistry and Hematology at the Jeroen Bosch hospital in ’s-Hertogenbosch, where she trains to be a clinical chemist under the supervision of Professor Dr. Ron Kusters.
Scientific Publications

   **Nuclear Receptor Nur77 is a negative regulator of conventional and plasmacytoid dendritic cell activation**
   Manuscript in preparation

2. Nina Karthaus, Marleen Ansems, Rebecca A.M. Blom, Gosse J. Adema
   **Regulation of tolerogenic immune responses by the Vitamine D Receptor**
   Manuscript in preparation

   **Vitamin D controls murine and human plasmacytoid dendritic cell function**
   Journal of Investigative Dermatology, In press

   **Nuclear receptor expression patterns in murine plasmacytoid and conventional dendritic cells**

5. Saartje Hontelez, Nina Karthaus, Maaike W. Looman, Marleen Ansems, Gosse J. Adema
   **DC-SCRIPT regulates glucocorticoid receptor function and expression of its target GILZ in dendritic cells**
   Journal of Immunology, 2013 Apr 1;190(7):3172-3179

6. Nina Karthaus, Ruurd Torensma, Jurjen Tel
   **Deciphering the message broadcasted by tumor infiltrating dendritic cells**
   American Journal of Pathology, 2012 Sep;191(3):733-42

7. Saartje Hontelez*, Marleen Ansems*, Nina Karthaus, Maaike W. Looman, Vassilis Triantis, Gosse J. Adema
   **Dendritic cell-specific transcript: dendritic cell marker and regulator of TLR-induced cytokine production**
   Journal of Immunology, 2012 Jul 1;189(1):138-145

8. Marleen Ansems, Nina Karthaus, Saartje Hontelez, Tilly Aalders, Maaike W. Looman, Gerald W. Verhaegh, Joost A. Schalken, Gosse J. Adema
   **DC-SCRIPT: AR and VDR regulator lost upon transformation of prostate epithelial cells**
   Prostate, 2012 Dec 1;72(16):1708-1717

9. Marleen Ansems, Saartje Hontelez, Nina Karthaus, Paul N. Span, Gosse J. Adema
   **Crosstalk and DC-SCRIPT: expanding nuclear receptor modulation**
   Biochimica et Biophysica Acta - Reviews on Cancer, 2010 Dec;1806(2):193-199

    **DC-SCRIPT: nuclear receptor modulation and prognostic significance in primary breast cancer**
    Journal of the National Cancer Institute, 2010 Jan 6;102(1):54-68

* authors contributed equally
## TRAINING ACTIVITIES

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## TEACHING ACTIVITIES

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| Supervision of assay student (1) | 2012 | 5.6  |
| 2012 | 0.7  |

**TOTAL** 35.775

<sup>*</sup> = oral presentation, <sup>##</sup> = poster