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der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Characterization of the vitamin D receptor complex at the epsilon germline promoter

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
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von

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To my mother

List of content

1. INTRODUCTION

1.1. Role of IgE in allergy

The incidence of allergic disease has increased substantially over the last years [1]. The key component of type-I-allergy is immunoglobulin (Ig) E directed against innocuous environmental antigens such as pollen, animal dander, and house dust mite. IgE represents an Ig isotype that is normally produced at very low levels. In fact, IgE abundance is 10^4-10^5 fold lower than that of IgG and IgA [2]. Therefore, IgE production is a tightly regulated process. Cross-linking of high-affinity IgE receptor (FcεRI)-bound IgE by multivalent allergens leads to a release of various mediators of hypersensitivity reactions by activated mast cells and basophiles [1]. Typical clinical appearances are allergic asthma, rhinoconjunctivitis, urticaria, food allergy and systemic life-threatening reactions known as anaphylaxis. The only causal therapy is specific immunotherapy. However, not all patients are responding towards that time-consuming and not inexpensive treatment.

1.1.1. Molecular regulation of human IgE production

There are nine antibody classes distinguished from each other by the constant regions (C) of their heavy (H) chains [3]. The variable (VDJ) region of the IgH chain is first linked to Cμ and Cδ. Upon antigen (Ag) and appropriate cytokine stimulation B cells change the antibody (Ab) CH region, enhancing the ability of the Ab to eliminate pathogens, while maintaining the same Ag-binding VDJ [4]. This process entitled isotype switching is regulated at the level germline gene transcription (GT) and class switch recombination (CSR) [3]. εGT is initiated at the Iε exon promoter and proceeds through the S region and the CH exons, resulting in the production of a sterile transcript named ε germline transcript (εGLT) (Figure 1) [5].

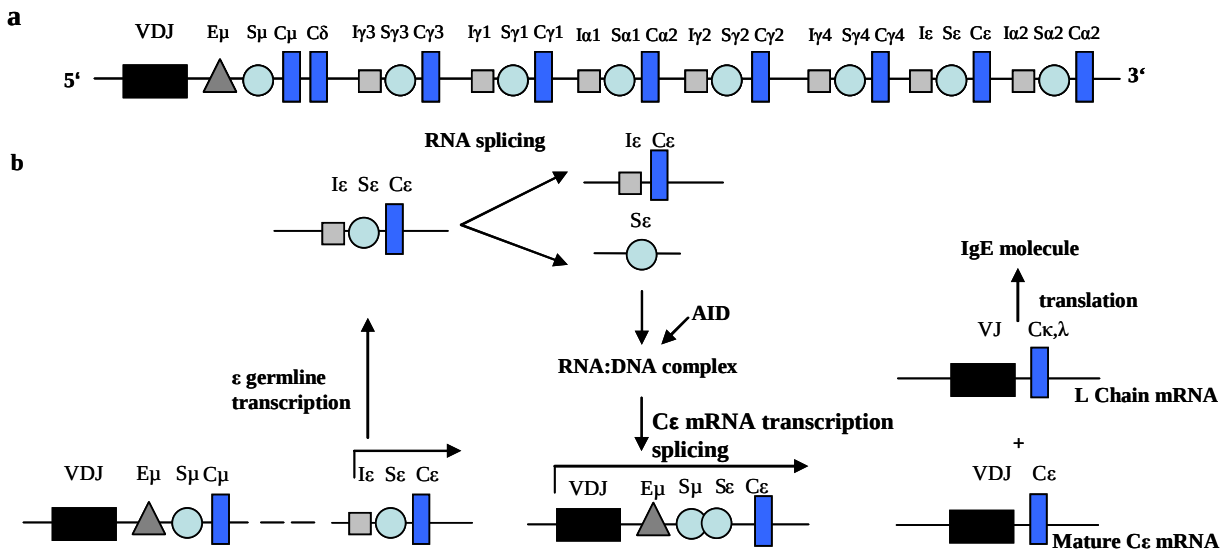

Figure 1. Schematic diagram of the molecular events in IgE isotype switching [6]. a) Genes of I, S and C regions in the IgM+ IgD+ B cell. b) CSR resulting in isotype switching to IgE.

CSR towards IgE and differentiation of IgE producing plasma cells require at least two independent signals [7]. The classical pathway involves IL-4 (or IL-13) and CD40L, and operates through simultaneous activation of the signal transducer and activator of transcription 6 (STAT6) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, respectively (Figure 2) [5, 6]. Various other cytokines influence IgE production by providing additional promoting or inhibiting signals, such as IL-21, IL-10, IL-6, IFNγ [5, 8-11].

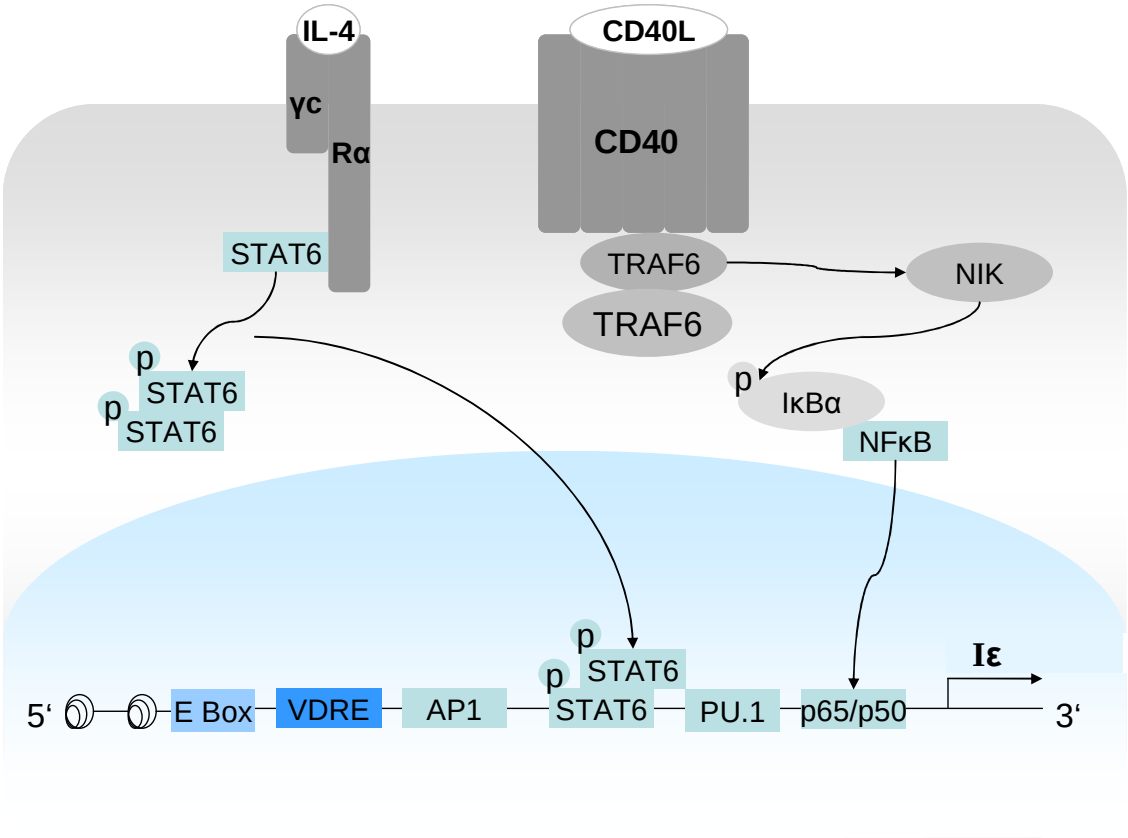

Figure 2. Induction of εGT by CD40 and IL-4R signalling pathways [12, 13]. Type I IL-4R complex is composed out of IL-4Rα and γc and binds exclusively to IL-4, which then activates the tyrosine kinases Janus kinase 1 and 3, and thereby STAT6. In B cells CD40 ligation mainly activates NF-κB by the rapid NFκB type 1 pathway via TNF receptor-associated factor 2, 3 and 6. Thereby NF-κB signalling is initiated through activity of Nik-related protein kinase leading to activation of the IkB kinase. The latter phosphorylates the inhibitor of κB (IκB) thereby promoting its ubiquitylation and proteasomal degradation. Consequently, liberated p50/p65 heterodimers translocate into the nucleus. IL-4R and CD40 signalling promote synergistically the transcription of εGLT by STAT6 and NF-κB, respectively.

1.1.2. Accessibility model

The recombination machinery responsible for DJ, VDJ recombination and CSR is available in the cell at the same time [14]. However, these processes take place subsequently . Furthermore, the cytokine-driven isotype switching directly correlates with the ability of the given cytokine to selectively induce GT [3]. Based on this, an “accessibility model” proposing that the local

unfolding of chromatin higher order structure initiates specific isotype switching has been described [3]. This model has been tested in mutant mice, in which the replacements of the germline promoters containing cytokine-response elements, selectively block GT and CSR to those specific loci [12-14].

It is well known that the activity level of individual genes can be modulated by local covalent modifications, in particular histone acetylation (HAc) [15]. It alters the net charge of nucleosomes and changes the folding properties of the chromatin fiber, promoting the DNA accessibility. Several reports showed that I and S regions of all isotypes with the exception of μ were hypoacetylated in resting B cells, and become exclusively hyperacetylated upon activation [15-17]. Acetylation levels of histone 3 levels in the S regions have been shown to influence CSR frequency, confirming the physiological importance of this modification [4, 17]. In contrast, a study by Chowdory et al. demonstrated that the CH genes undergo constitutive GT in the majority of human B cells, implying that these genes are in an ‘‘accessible’’ chromatin conformation [18, 19]. However, for the effective GT and activation induced deaminase (AID) action it is indispensable for the chromatin in I and S region to be in the open state (acetylated). Up to now, to our knowledge, it has not been elucidated whether deacetylation, as one of the major mechanisms of transcriptional control, can influence the IgE production.

1.2. Biology of calcitriol

Calcitriol (1α,25-dihydroxy vitamin D3, 1α,25-dihydroxy-cholecalciferol) is a well known regulator of skeletal development and calcium homeostasis [20]. Moreover, various additional impact of calcitriol on the immune system, cardiovascular system, and cancer prevention has been revealed during the last decade [22, 23].

1.2.1. Structure and metabolism of calcitriol

Two forms of vitamin D exist: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol, VD3) (Figure 3) [21]. The former is produced in a variety of plant materials and yeast whereas the latter, more relevant, is synthesized in the skin from 7-dehydrocholesterol, a process which depends on UVB (270–300 nm) or it can be alternatively acquired from the diet. It is then

converted in the liver to 25-dihydroxy VD3, which is the main circulating form of vitamin D. Finally, 25-dihydroxy VD3 is metabolized in the kidneys to 1α,25-dihydroxy VD3 (calcitriol), the physiologically most active VD3 metabolite [21, 22].

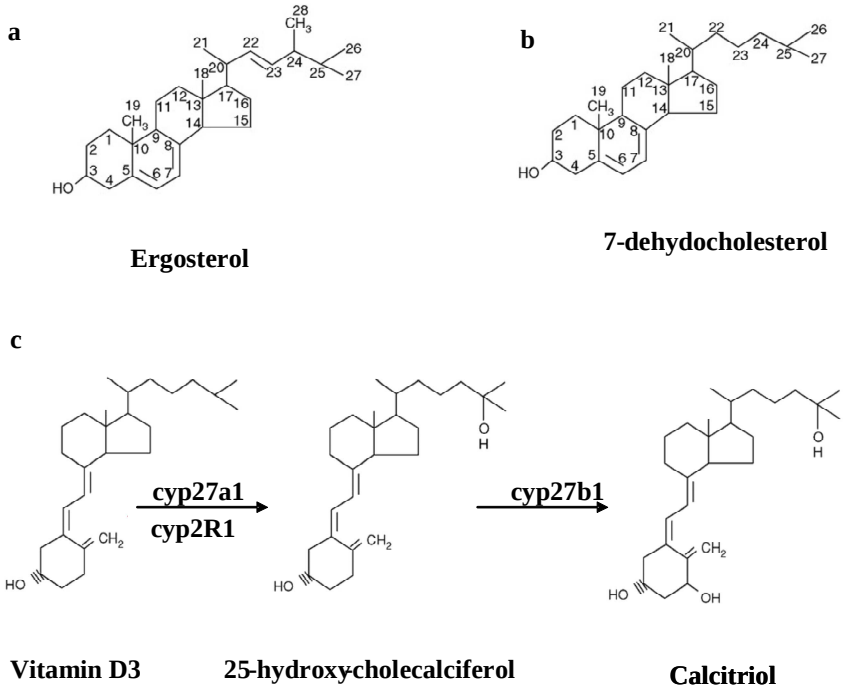

Figure 3. Vitamin D production and metabolism [21]. Vitamin D exists in two forms a) ergosterol and b) 7-dehydrocholesterol. c) In both cases vitamin D is produced from precursors by ultraviolet B radiation. However, vitamin D by itself is not biologically active and must first be converted to 25-hydroxy-cholecalciferol and then to 1α,25-dihydroxy-cholecalciferol.

In addition to being processed in the liver and the kidneys, VD3 can also be metabolized by cells of the immune system (Figure 4) [23]. Activated T cells and B cells express an enzyme 1α-hydroxilase (cyp27b1) which can only perform the final step of converting 25-hydroxy VD3 to calcitriol [24]. However, macrophages and some dendritic cells (DCs) express the two sets of enzymes (cyp27b1 and cyp27a1 trivially named 25-hydroxilase) necessary for conversion of VD3 into 25-hydroxy VD3 [23]. Finally, the enzyme 24-hydroxylase (cyp24a1), which is most abundant in the kidney and intestine, catabolises calcitriol to its inactive metabolite, calcitroic acid, which is then excreted in the bile [21].

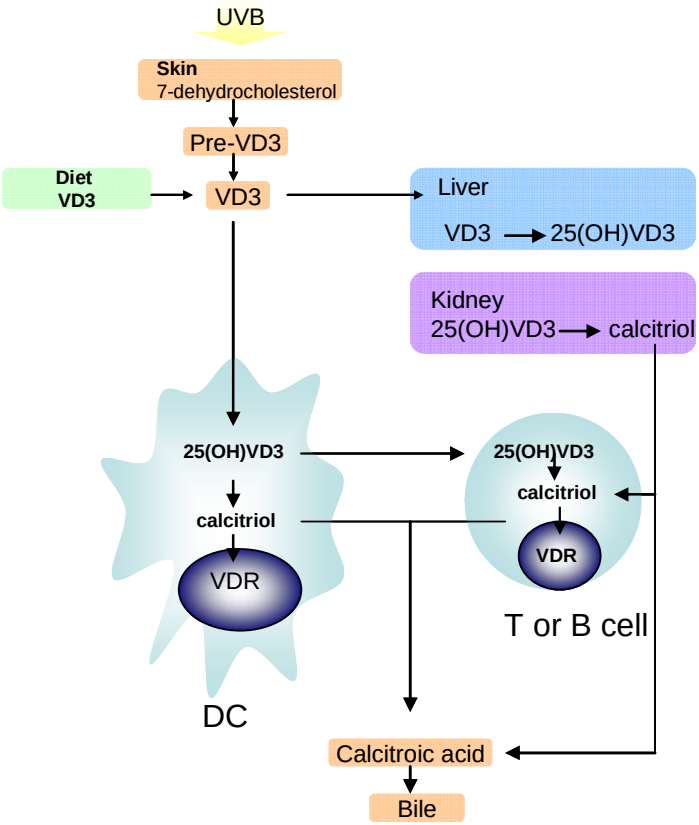

Figure 4. Overview of vitamin D metabolism in immune cells [23]. Cells of the immune system, including DCs, T and B cells express the enzymes cyp27a1 and/or cyp27b1, and therefore can also hydroxylate 25-hydroxy VD3 (25(OH)VD3) to calcitriol. Calcitriol acts on immune cells in an autocrine or paracrine manner by binding to the VDR. Cyp24a1 catabolises calcitriol to its inactive metabolite, calcitroic acid, which is then excreted in the bile. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology. Mora, J.R., M. Iwata, and U.H. von Andrian, Vitamin effects on the immune system: vitamins A and D take centre stage, copyright 2008.

1.2.2. Calcitriol-mediated signalling

1.2.2.1 The vitamin D receptor

Calcitriol signals through the vitamin D receptor (VDR), the only nuclear protein binding to it with high affinity and an endocrine member of the nuclear hormone receptor (NR) superfamily including retinoic acid, thyroid hormone, estradiol, progesterone, testosterone, cortisol and aldosterol [25]. In humans, the VDR protein consists of 427 amino acids, with a molecular mass of ~48 kDa. Members of the NR receptor superfamily all share a characteristic modular

organization consisting of a variable amino-terminal region, a highly conserved DNA binding domain (DBD), a nonconserved hinge domain, and a highly conserved carboxy-terminal ligand binding domain (LBD) (Figure 5a) [26]. The amino-terminal region in VDR is very short and has not been shown to modulate transcription. The core of DBD is composed of two zinc-finger motifs, each containing four highly conserved cysteine molecules which coordinate the binding of the zinc atom. Zinc atoms and cysteine residues are necessary for maintaining a three dimensional structure whose core is composed of two helices (helix I and II) oriented at approximately right angles to each other. Helix I is critical for the specific binding to the major groove of VDR-responsive element (VDRE) on a DNA and helix II is involved in receptor dimerization [27] (Figure 5b). The linker hinge domain is considered to be a carboxy-terminal extension of the DBD, as it often imparts additional dimerization and sequence specificity to the conserved DBD core region. The LBD is a globular multifunctional domain responsible for hormone binding, strong receptor dimerization and interaction with co-repressors and co-activators [27, 29].

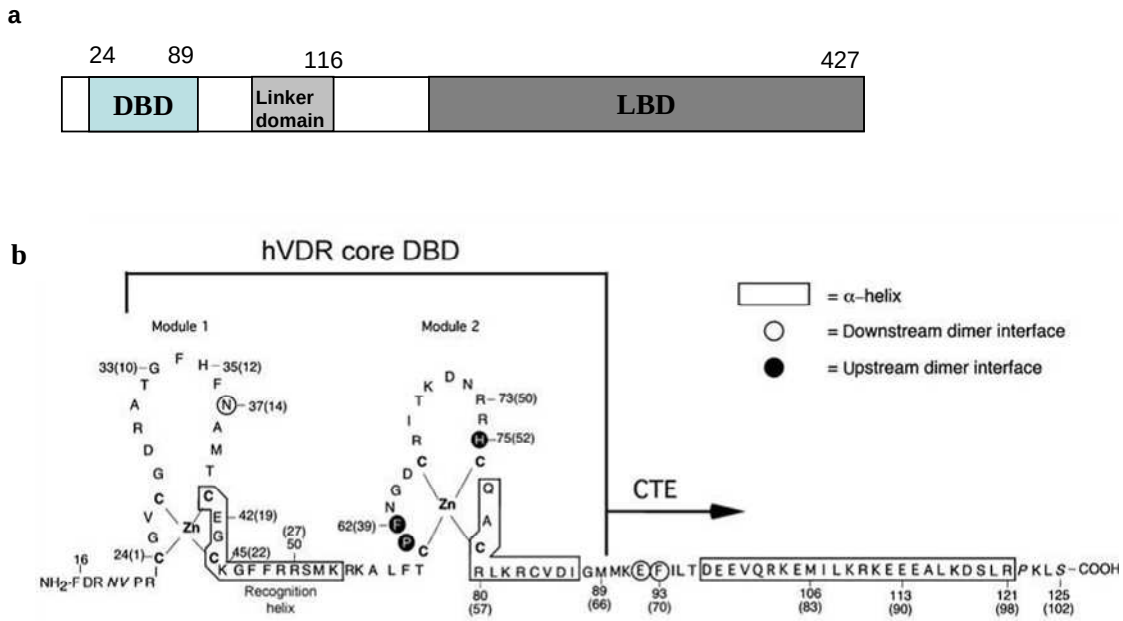

Figure 5. Structure of the VDR [26]. a) Domain organization of human VDR, DNA binding domain (DBD), ligand-binding domain (LBD). b) DBD organization and structure. Reprinted from Vitamins & Hormones, Vol.68, Paul L Shaffer and Daniel T Gewirth, Vitamin D Receptor–DNA Interactions, Pages No. 257-273, Copyright 2004, with permission from Elsevier.

1.2.2.2. VDR-response elements

An essential prerequisite for the direct modulation of transcription by VDR of the primary target genes is a location of specific VDR-binding site in their regulatory regions, which is referred to as a vitamin D response element (VDRE) [25, 26]. This is achieved, in most cases, through the specific binding of the DNA binding domain of the VDR to the major groove of a hexameric DNA sequence, referred to as core binding motif, with the consensus sequence RGKTSA (R=A or G, K=G or T, S=C or G) [26]. Typically, the VDR-RXR heterodimers bind to VDREs formed by a direct repeat (DR) of two hexameric core binding motifs with 3 intervening nucleotides (DR3-type), but also to DR4-type VDREs along with other members of the NR superfamily (Figure 6) [26, 27]. It should also be noted that effective VDR binding has also been observed on everted repeat (ER)-type VDREs with 6-9 spacing nucleotides (ER6, ER7, ER8, ER9) [25].

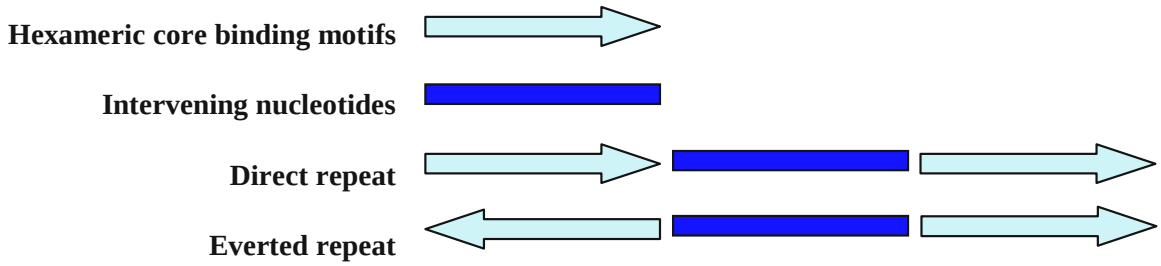

Figure 6. Schematic representation of the VDREs. Both binding motifs can be positioned in a palindrome, as direct or everted repeats.

1.2.3. Mechanisms of VDR-mediated transcriptional modulation

Transcriptional activation or repression by calcitriol can be mediated by VDR interaction with the specific protein families controlling transcription, by interaction with transcription factors or interference with their expression [28].

1.2.3.1 VDR interaction with transcriptional co-factors

Steroid NRs, such as vitamin D receptor (VDR), are transcription factors that can regulate gene activity by recruitment of co-regulators [29].

The VDR-mediated transactivation has been well described [25]. Usually, upon the ligand binding the association of a co-activator of the p160-family, such as SRC-1, TIF2 or RAC3, takes place. Some co-activators themselves show the histone acetyltransferase activity or form a complex with co-factors expressing such activity [25]. They can also cooperate with so called ATP-dependent chromatin remodelling factors, resulting in local chromatin relaxation [25, 30, 31]. Subsequently, ligand-activated NRs change rapidly from interacting with the co-activators of the p160-family to those of mediator complexes, which build a bridge to the basal transcriptional machinery [25].

The negative gene regulation can be divided in two categories ligand-independent and ligand-dependent repression, which can be further distinguished regarding the ligand features into antagonist- or agonist-driven one [29]. In case of the ligand-independent repression the non-ligated NR binds to the positive response elements within the target gene and recruits co-repressors. However, unbound steroid hormone NRs (like VDR) do not interact effectively with the co-repressors and therefore their silencing activity is controversially discussed [29]. Steroid NRs are the prototype for a second model, whereas interactions between co-repressors and receptor-bound antagonists as well as agonists were observed [29, 32, 33]. Many NRs require heterodimerization with RXR for high-affinity DNA binding, as this receptor appears to play an active role in co-repressor interaction [26].

Two major co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), have been shown to mediate repression associated with NRs [28]. Both proteins contain multiple repression domains. They are binding platforms for the various silencing enzymes including the histone deacetylases (HDACs), Swi3/Ada2/NCoR/TFIIID (histone binding sites) motifs and NR interaction domains (Figure 7) [28, 29]. HDAC3 is one of the four core factors in their complexes, however HDACs 1, 2, 4, 5 and 7 can also interact with SMRT or NCoR complexes [28].

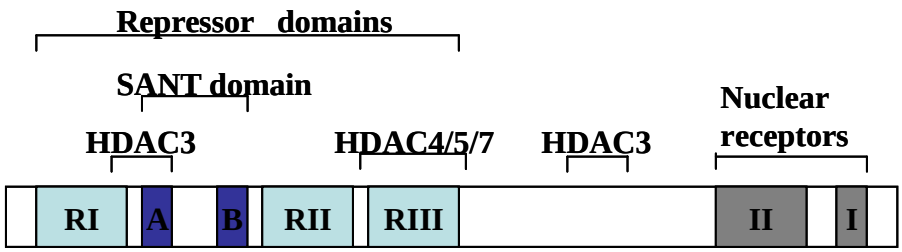

Figure 7. The domains of NCoR/SMRT [28]. Repression domains (RI, RII, RIII), Swi3/Ada2/NCoR/TFIIID (SANT) domains (A and B), as well as the interaction domains for HDACs, nuclear receptors interaction domains (I and II) are indicated.

1.2.3.2 Transcriptional regulation via chromatin modification

1.2.3.2.1 Histone acetylation and deacetylation

The distinctive patterns of gene expression require multiple levels of transcriptional control, which occurs through transcriptional regulators that bind specific DNA sequences [34]. This in turn controls the accessibility of DNA [34]. HAc is one of the most widespread modifications, which serves as a key modulator of chromatin structure. Acetylation of ε-amino groups of lysine residues within histone tails neutralizes their positive charge, thereby relaxing chromatin structure. Acetylated histones also serve as binding sites for bromodomain proteins, often acting as transcriptional activators. In contrast, histone deacetylation favours transcriptional repression by allowing for chromatin compaction. HAc is a dynamic process controlled by the antagonistic actions of two large families of enzymes - the histone acetyltransferases (HATs) and the HDACs [34, 35]. The balance between the actions of these enzymes serves as a key regulatory mechanism for gene expression [34].

Several transcription factors are known to have HAT activity themselves like members of the Src family [25]. On the other hand HDACs are part of larges co-repressor complexes around SMRT or NCoR [28].

The classical, Zn- dependent HDACs can be divided into following phylogenetic classes I, II, and IV [34]. HDAC class II are NAD-dependent. The HDACs class I are know to deacetylate all -acetlys at H4, H3, H2A, H2B, except H4K16 [30].

Ongoing investigations are elucidating the specific roles of the various HDACs and identify actual target lysine residues on histone tails.

1.3. Calcitriol and allergy

Recently substantially increased incidences of allergic diseases as well as vitamin D deficiency have been reported in developed countries [36-39]. Importantly, numerous studies revealed that vitamin D has a considerable impact on the immune system, and thereby allergic diseases, as reviewed in [22, 23, 38]. Initial direct evidence implicating vitamin D in allergy development came from human genetic association studies [38]. They could show that this relationship is highly complex and more studies investigating genes in the metabolic pathway and vitamin D level interactions are needed.

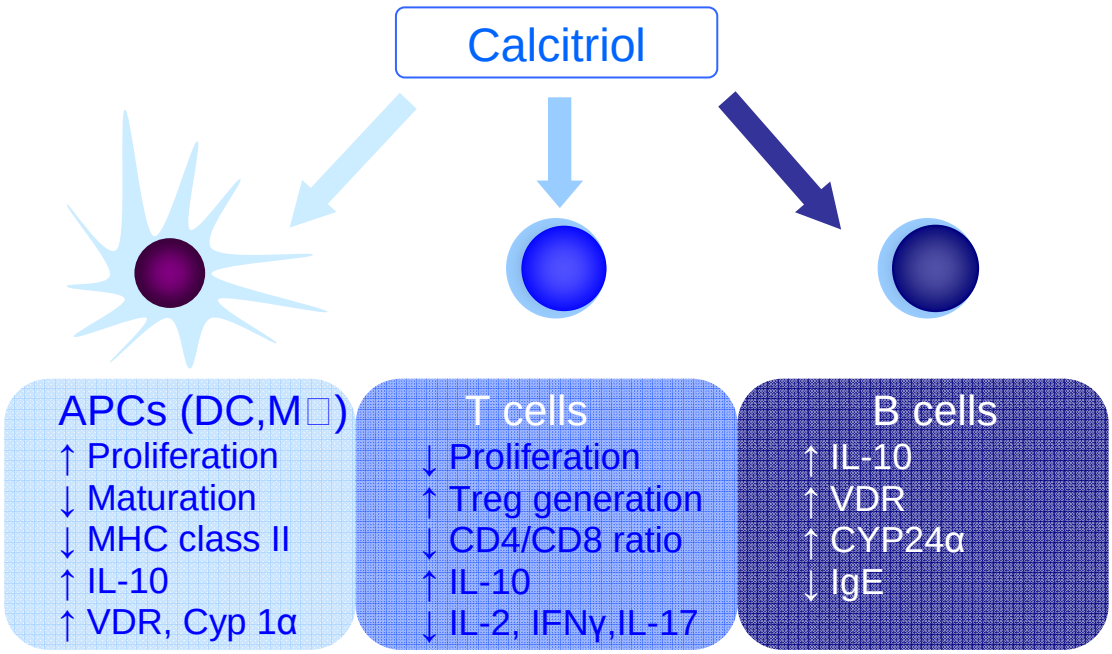

Figure 8. Mechanisms of calcitriol-mediated immunomodulation [23]. Calcitriol exerts its effects on several immune-cell types, including monocytes, T and B cells. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology. Mora, J.R., M. Iwata, and U.H. von Andrian, Vitamin effects on the immune system: vitamins A and D take centre stage, coryright 2008.

In vitro, calcitriol exerts potent effects on immune cells (Figure 8) [23]. VDR and vitamin D metabolic enzymes have been identified in cells of the immune system, such as activated T, B cells, and DCs [23, 24]. In the context of allergy, evidence exists that vitamin D may induce a shift from Th1- to Th2-immune response [22]. Reduced secretion of Th1 cytokines IL-2 and IFN-γ as well as an increase in the Th2 cytokine IL-4 have been observed after treatment with calcitriol [23]. In contrast, in human cord blood cells calcitriol not only inhibits IL-12–generated IFN-γ production, but also suppresses IL-4 and IL-13 [23]. The apparently contradictory effects may lie, in part, in the timing of exposure of the cells, but also in calcitriol-mediated induction of immunosuppressive Foxp3+ regulatory T (Treg) cells as well as inhibition proinflamatory Th17 cell-differentiation [20, 22, 23]. The activity of calcitriol on T cell-differentiation is further indirectly potentiated by inhibition of the differentiation, maturation and immunostimulatory capacity of DCs [22, 23].

Calcitriol exerts effects on B cells as well [23]. This can be achieved either by indirect action on other cells like Ag-presenting-cell function and/or T cells or be a direct action via VDR in B cells. Accordingly, previous report showed that calcitiol inhibits IgE production in human B cells [40], which plays a major role in type-I-allergic responses underlying development of allergic diseases [7]. Furthermore, activated B cells can convert 25-hydroxy VD3 to calcitriol [24], thereby increasing its concentration locally in the lymphoid microenvironment. This is not only enhancing its specific action, but also limiting potentially undesirable systemic effects, such as hypercalcaemia and increased bone resorption [23]. The clinical benefits for the treatment of allergic disease have been already observed for patients suffering from atopic dermatitis [41]. However further research is necessary for clarification of each and every immunomodulatory property of the vitamin D based treatment.

2. OBJECTIVE

The association between vitamin D deficiency and ‘allergy epidemic’ present during the last decades has been recently suggested [36, 38, 41]. Moreover, recent studies are revealing the role of calcitriol in immunomodulation and highlighting its possible impact on the treatment of allergic diseases [23]. Accordingly, previous data showed that calcitriol, the active form of vitamin D [40], inhibits εGT, a prerequisite for IgE production. However, neither the underlying mechanisms of calcitriol action nor the impact of the acetylation level on IgE production has been elucidated to our knowledge up now.

In order to unravel the mechanism behind the calcitriol-mediated IgE inhibition this work addressed the following questions:

1. At which level of IgE production (IgE secretion, plasma cell-differentiation or CSR) does calcitriol exert its inhibitory effects?
2. Which B cell subpopulation is responding to calcitriol?
3. Does calcitriol-mediated inhibition of IgE production involve a direct genomic action at the ε germline gene in B cells?
4. What is the composition of the VDR-recruited complex at the ε germline promoter?
5. What is the role on chromatin acetylation in εGT?

3. MATERIALS AND METHODS

3.1. Materials

Reagents, antibodies, equipment and software are listed in detail in the appendix section (page 57).

3.2. Methods

3.2.1. Donors and cells

The blood was obtained from healthy donors, defined as individuals not suffering from any acute or chronic disease. The study was approved by the ethics committee of the Charité-Universitätsmedizin Berlin and conducted according to the Declaration of Helsinki Principles.

3.2.1.1. Separation of CD19+ B cells

Human PBMC were isolated by density gradient centrifugation using ficoll hypaque isolation (d = 1.077 g/mL) at 300 g, for 20 min at the room temperature. B cells were purified by magnetic cell sorting using anti-CD19-coupled magnetic beads. Briefly, 2×10^8 PBMC were incubated with 400 µl buffer containing 0.2% bovine serum albumin (BSA), 2 mM EDTA in phosphate-buffered saline (PBS) and 100 µL CD19 multi-sort beads at 4°C. CD19+ B cells were separated by positive magnetic selection on the LS Columns. After the positive fraction was gained the bound beads was removed from the cells using a supplied release buffer for 15 min 4°C. The purity was > 99% B cells as assessed by flow cytometry.

3.2.1.2. Separation of CD23+ B cells

CD19+ B cells were cultured for 48 h with anti-CD40/IL-4 +/- calcitriol. Afterwards, cells were stained with anti-CD23 FITC, and subsequently labelled with anti-FITC microbeads. CD23+ cells were positively selected to > 90% purity by positive magnetic sorting on a LS Column.

Additionally, the purity and characterization of the CD23 enriched subpopulation was performed by flow cytometric analyses.

3.2.2.3. Human embryonic kidney fibroblast

For the promoter assay human embryonic kidney fibroblast cell line (HEK-293) was used.

3.2.2. Cell culture

The concentration of primary B cell suspension was adjusted to the 10^6/ml cells. The cultures were performed in the flat-bottom 48-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml).

HEK-293 cells were seeded into 24-well plates and grown for 48 h in the in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum.
Cultures were carried out at 37°C in humidified air and 5% CO_2.

3.2.3. Enzyme Linked Immuno Sorbent Assay

Enzyme Linked Immuno Sorbent Assay (ELISA) was used for quantification of the immunoglobulins. Cells were stimulated with anti-CD40/IL-4 +/- IL-21 +/- calcitriol for 10 days. For IgE detection clones HP6061 and HP6029 as well as matched Ab pairs for IgA, IgG and IgM ELISA (anti-IgA, anti-IgG and anti-IgM) were applied. Immunoplates were coated overnight at 4°C with the primary antibodies. After blocking of the unspecific binding by 2 % BSA/PBS supernatants and standards were incubated for 2 h. The second alkaline phosphatase conjugated anti-Ig Ab was added for 1.5 h. As the secondary mouse anti-human IgE Ab was biotinylated another hour of incubation with alkaline phosphatase conjugated streptavidin was performed. Following the final reaction with phosphatase substrate, plates were read in a microplate ELISA reader at 405 nm and the amount of immunoglobulin was calculated according to the standard curve. Diluted human serum of an atopic individual served as standard curve.

3.2.4. Enzyme Linked Immuno Spot Technique

The number of Ab secreting cells was determined by Enzyme Linked Immuno Spot Technique (ELISpot) technique. Therefore, B cells were stimulated with anti-CD40/IL-4 +/- IL-21 +/- calcitrol for 6 days. MultiScreen-High Protein Binding Immibilion-P Membrane plates were coated overnight with mouse anti-human IgE or with goat anti-human IgA or IgG. Unspecific binding was blocked by adding 2 % BSA/PBS. Serial dilutions of B cells from 6^{th} day cultures were incubated at 37°C overnight. The detection was performed with biotin conjugated mouse anti-human IgE or IgA or IgG. The reaction was developed with streptavidin-horseradish peroxidise and peroxidase substrate 33-amino-9-ethyl-carbazole/ n,n-dimethyl formamide. Ab secreting cells appeared as red spots and were counted with CTL ImmunoSpot® S4.

3.2.5. Flow cytometry

3.2.5.1. Flow cytometric analyses of CD23+ B cells

The purity and characterization of the CD23 enriched subpopulation was performed by flow cytometric analyses. Therefore, the CD23 enriched cells were stained with fluorochrome coupled anti-CD27, anti-IgM, anti-CD19. The dead cells were excluded using propidium iodide staining. The measurements were performed using flow- cytometer and the obtained data was analysed by FlowJo 7.2.2. software.

3.2.5.2. Flow cytometric analyses of IgE

B cells were stimulated with anti-CD40/IL-4 +/- IL-21 +/- calcitrol for 6 days. Additionally, B cells were re-stimulated for the last 6 h of culture with 10 ng/mL PMA. To accumulate the synthesised proteins intracellularly, 1 μg/mL brefeldin A was added 2 h prior to the end of the culture. After harvesting, cells were stained with fluorescence dye labelled monoclonal anti-human CD19, CD27 and CD38 for 15 minutes at 4°C in the dark. After washing, B cells were fixed with 2 % paraformaldehyde for 15 min at room temperature. The intracellular IgE staining was performed with anti-human IgE in 0.5 % saponin 1% BSA in PBS for 30 minutes at 4°C. The measurements and the analyses were performed as above.

3.2.7. RNA isolation

For analysis of εGLT, VDR and cyp24 mRNA expression 10^6 B cells were stimulated with anti-CD40/IL-4 +/- calcitriol for 2 days. RNA isolation of single cell suspensions was performed using RNA isolation kit Nucleospin RNA II according to manufacturer's guidelines. For RNA concentration, the visible dye labelled carrier Pellet Paint™ Co- Precipitant was used in combination with alcohol precipitation method.

3.2.8. cDNA synthesis

Synthesis of cDNA was performed with Taq Man Reverse Transcription Reagent according to manufacturer's instructions.

3.2.9. Quantitative real-time polymerase chain reaction

For the gene expression analysis as well as quantification of the precipitated DNA by chromatin immunoprecipitation assay real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed with the means of LightCycler-Fast Start Master SYBR Green I according to manufacturers' guidelines. The used primers were designed with the help of the internet databases UCSC and NCBI and created by Primer3 software (Table 1). The primers shown are applied at a hybridisation temperature of 65°C and at concentration of 500 nM. The PCR amplification progress can be detected simultaneously with intercalation of SYBR Green, which emits a strong fluorescent signal upon binding to double stranded DNA. Target gene levels were normalised to a reference gene as an endogenous control. Hypoxanthine guanine phosphoribosyltransferase (HPRT) was used to evaluate mRNA expression of target genes, whereas for quantification of the precipitated DNA after ChIP, input DNA was used.

The relative expression ratio (R) of a target gene is based on the real-time efficiency (E) of the primer pair, the CP deviation of an unknown sample versus a control and is expressed in comparison to a reference gene (ref) using the following formula $R = E_{ref}^{\Delta Cp\ ref} / E_{target}^{\Delta Cp\ target}$.

Table 1. Primers for quantitative real-time PCR.

	Sense primer	Anti-sense primer
HPRT	tggcttatatccaacacttcgtg	atcagactgaagagcattgtaatgacca
εGLT	gacgggccacaccatccacaggccaccaaatggacgac	caggacgactgtaagatcttcacg
cyp24	cgggtgtaccatttacaactcgg	ctcaacaggctcattgtctgtgg
VDR	acttgcatgaggaggagcat	aggtcggctagcttctggat
Iε	cttcatgtgctcgccttgag	ggaaagggagagggtactgg
Tprv6	tctccttccccaccagacactaaac	aagctatttgtgttcctgccgct
Tprv6 orf	tgatgtccaggccctgaacaagt	gctccggggcagcctccatcagc
VDREs	gatcacgcgtgcctccaggaaggccaccgagcccgagccc	gatcctcgagggaaagggagaggggtactgggcatcgacgc
VDREs-Iε	gatcacgcgtgcctccaggaaggccaccgagcccgagccc	gatcctcgagccgggtcgtccatttggtgcctgtggatgg
CYP27	gatcacgcgtgctgctaaccagaaagccccccagaggaga	gatcctcgagcccatcctcaggaattccccatcgggcagc

3.2.10. Protein isolation and Western blot

Western blot was used for comparison of the VDR expression between CD23+ and CD23- B cell fractions. Triton X-100 Lyses buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl, pH 8.0) containing a protease inhibitors was used for whole protein extract preparation. The amount of total protein in each sample was quantified by standard coomassie plus assay according to manufacturer's instructions. After separation of the denatured proteins by the length on a 12 % tris glycine sodium dodecyl sulfate (SDS) polyacrylamide gel, the proteins are transferred to the polyvinylidene difluoride membrane. After blocking with milk powder/PBS, the membrane was incubated with anti-human VDR at 4°C overnight. Horseradish peroxidase conjugated goat anti-rat was added for 1 hour at room temperature. The antigen detection was performed with the chemiluminescent detection system ECL Plus.

3.2.11. Chromatin immunoprecipitation

CD23+ B cells (10^6 cells) were fixed using 1% formaldehyde for 10 minutes (at room temperature), treated with 2 M glycin and lysed in a 1% SDS, 10 mM EDTA, 50 mM Tris and protease complete without EDTA solution (pH 8.1). DNA was sheared by sonication in the dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, including protease complete, pH 8.1). Sample was precleared on the magnetic µ column after incubation with salmon sperm and Protein A microbeads for 30 minutes. The precleared sample was incubated with the appropriate Ab against VDR, RXRα, RXRβ, RARα, RARβ, HDAC1, HDAC2, HDAC3, SMRT or NCoR for 4 h. Subsequently Protein A microbeads were added for 1 h. The samples was loaded on the magnetic µ columns and washed with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.1), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.1), LiCl buffer (0.25 M LiCl, 1% nonidet-P-40,1% deoxycholate, 1 mM EDTA,10 mM Tris-HCl, pH 8.1) and TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA pH 8.0). The elution of the precipitated DNA was performed using preheated 1% SDS in bicarbonate buffer. The defixation was carried out with 300 mM NaCl at 65°C overnight. The DNA was purified with NucleoSpin extract II kit according to the manufacturer's instructions. The amount of precipitated DNA was quantified using qRT-PCR.

3.2.12. Cloning procedures

3.2.12.1. Reporter gene constructs

The appropriate regions of the ε germline promoter or cyp27b1 promoter were obtained through amplification of B cell genomic DNA. All reporter plasmids were prepared by cloning the mentioned DNA fragments into SV40-pGL3-luciferase vectors. SV40 promoter is driving the firefly *luciferase* gene (LUC) in a pGL3-derived Luciferase Reporter Vector. For the region of the ε germline promoter containing vitamin d responsive elements (VDREs) the fragment -417 to -178 bp from the transcription start (TSS) was used. For the whole ε germline promoter containing VDREs (VDREs-Iε), the sequence -417 to +231 bp from the TSS were used. For the chosen Cyp27b1 region (cyp27) the fragment -776 to -350 bp from the TSS were used. The promoter regions were amplified from genomic DNA using PCR with a proof reading

polymerase. Primer sequnces containing the restriction enzyme sites MluI and XhoI are found in table 1. After restriction enzyme digestion the inserts were ligated into the MluI and XhoI sites of the SV40-pGL3-luciferase vector. The ligation was performed for 20 minutes on ice using an inser:vector ratio of 1:1, T4 ligase and supplied buffer. All constructs were verified by sequencing after plasmid-DNA isolation from *Escherichia coli* (E.coli).

3.2.12.2. Transformation of chemo-competent bacteria

E. coli bacteria were thawed on ice and incubated with plasmid DNA on ice for 10 minutes, followed by heat-shock at 42°C for 40 seconds. The cells were then put on ice for 2 minutes and incubated with pre-warmed SOC medium (SOB-Medium, 20 mM Glucose) for 30 minutes under slow continuous rotation. After centrifugation the pellet was resuspended and incubated on the LB-agar containing ampicillin over night at 37°C. The chosen grown colonies were then expanded in the liquid cultures LB-medium supplemented with ampicillin.

3.2.12.3. Plasmid-DNA isolation from E.coli

For analytic purposes, such as restriction enzyme digestion and sequencing, the plasmid-DNA was isolated using NucleoleoSpin Plasmid Kit, whereas for purification of the large amounts of plasmid-DNA (from 50 ml cultures) Nucleobond Ax Kit was used according to the manufacturer's instructions.

3.2.12.4. Calcium-phosphate based transfection of HEK-293 cells

HEK-293 cells were seeded into 24-well plates and grown for 48 h in in Dulbecco's modified Eagle's medium (Invitrogen, Darmstadt, Germany) supplemented with 10% foetal calf serum.
The solution of 2 M calcium chloride, 50 µg pRL-TK Vector (renilla expression vector), 12.5 µg pmscv-GFP (VDR expression plasmid) and 50 µg of either cyp27b1-SV40-pGL3-luciferase or Iε-SV40-pGLT-luciferase or VDREs-SV40-pGLT-luciferase or SV40-pGL3-luciferase were added carefully under shaking to the buffered saline solution. After 20 minutes of incubation 50 µl were added to each well for further at for 4 h at 37°C. The transfection was stopped by washing with PBS and addition of fresh medium supplemented +/-calcitriol (1 µM). After 24 h

of stimulation the cells were treated with lysing buffer for 10 minutes. The luciferase activity was analysed using the Luciferase Reporter-Gene Assay. The expressed luciferase catalyses its substrate, a reaction accompanied by light emission which is detected by the Photomultiplier in the Lumat LB9501 Berthold. The emitted light is proportional to the transcription activity of the promoter. The activities of firefly luciferase reporter were normalised to renilla luciferase (pRL-TK-LUC) and shown as relative luciferase activity.

3.2.13. Statistical analysis

Wilcoxon test for non-parametrical, paired data using GraphPad Prism Software was carried out to analyse the statistical significance of within-group changes. P-value < 0.05 was considered to be statistically significant.

4. RESULTS

4.1. Impact of calcitriol on IgE culture system

4.1.1. Calcitriol reduces IgE production

IgE production relies on CSR towards IgE and differentiation of IgE plasma cells, which require at least two independent signals [7]. While CSR to IgE is very potently induced by anti-CD40/IL-4, the initiation of a distinct genetic programme responsible for plasma differentiation remains modest [5]. To investigate the impact of calcitriol on IgE secretion, purified B cells were incubated with anti-CD40/IL-4 +/- calcitriol (1 µM) for 10 days. IgE production from unstimulated B cells was quite modest (<0.70 ng/ml). Upon anti-CD40/IL-4 stimulation IgE increased in supernatants up to 4.72+/-0.92 ng/ml, whereas calcitriol addition reduced IgE levels by 62% down to 1.32+/-0.33 ng/ml (Figure 9).

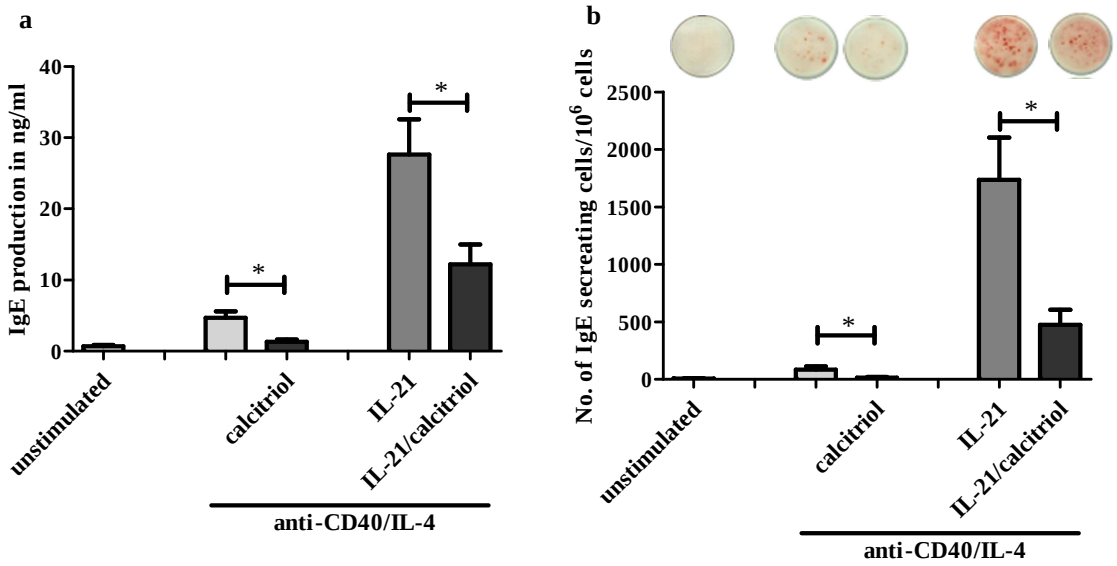

Figure 9. Calcitriol inhibits IgE production and generation of IgE secreting cells. a) B cells were stimulated with anti-CD40/IL-4 +/- IL-21 +/- calcitriol for 10 days. IgE was determined in supernatants by ELISA. b) B cells were stimulated with anti-CD40/IL-4+/- IL-21 +/- calcitriol for 6 days. Number of Ab secreting cells was determined by ELISpot. The wells show single values from one donor. Data are shown as mean +/- SEM. N = 4-5. * $P < 0.05$.

Next, we were interested to investigate whether calcitriol also has an impact on B cells co-stimulated by anti-CD40/IL-4/IL-21, i.e. cells ideally stimulated to produce large amounts of

IgE. Recently described cytokine IL-21 is a potent enhancer of IgE production and secretion [11, 42]. However, it requires anti-CD40 and/or IL-4 co-stimulation, since it is not able to initiate the CSR to IgE alone [10, 11, 43-46]. Therefore for gaining the optimal system for IgE production IL-21 was included in the culture. As expected, IL-21 exerted a strong IgE-promoting effect when combined with anti-CD40/IL-4 (27.64+/-4.95 ng/ml) (Figure 9a). Under these conditions calcitriol was also able to decrease IgE secretion by 56%, precisely down to 12.20+/-2.80 ng/ml (Figure 9a).

Furthermore, IgA and IgG production were examined to delineate whether calcitriol acts in an isotype specific manner. As calcitriol treatment did not significantly alter IgA and IgG production, at least under these IgE-optimized culture conditions (data not shown), calcitriol has a selective effect on IgE production in a Th2-primed environment.

4.1.2. Calcitriol impairs the generation of IgE plasma cells

To verify whether the enhanced amount of IgE in supernatants was due to impaired Ab secretion or the inhibited generation of IgE secreting cells, the IgE plasma differentiation by ELISpot assay and flow-cytometry was analysed.

For that purpose B cells were stimulated with anti-CD40/IL-4 +/- IL-21 +/- calcitriol and the numbers of IgE secreting cells were determined by ELISpot assay after 7 days of culture. The chosen timepoint was determined in the previous experiments regarding the kinetic of Ab secretion and plasma cell differentiation (data not shown).

As depicted in Figure 9b, calcitriol treatment of anti-CD40/IL-4 stimulated B cell cultures caused a decrease in numbers of IgE secreting cells by 83%, exactly from 84.33 +/-27.56 down to 14.50+/- 4.49 per 10^6 B cells. In case of IL-21 co-stimulated B cells calcitriol significantly reduced the number of IgE secreting cells by 73% as well, precisely from 1736.00+/-367.70 down to 473.30+/-132.20 per 10^6 cells. In contrast, calcitriol had no impact on the number of IgA or IgG secreting cells under these specific culture conditions (data not shown).

Next, the ELISpot findings were confirmed by flow cytometric analyses of the intracellular IgE staining. B cells stimulated as above were stained for surface markers of plasma cells (CD19,

CD27 and CD38) [47] and for intracellular IgE. Calcitriol decreased the numbers of CD19+ CD27++ CD38+ IgE+ cells differentiated under anti-CD40/IL-4/IL-21 culture conditions (Figure 10). The same effect was observed when cells were stimulated with anti-CD40/IL-4 +/- calcitriol (data not shown).

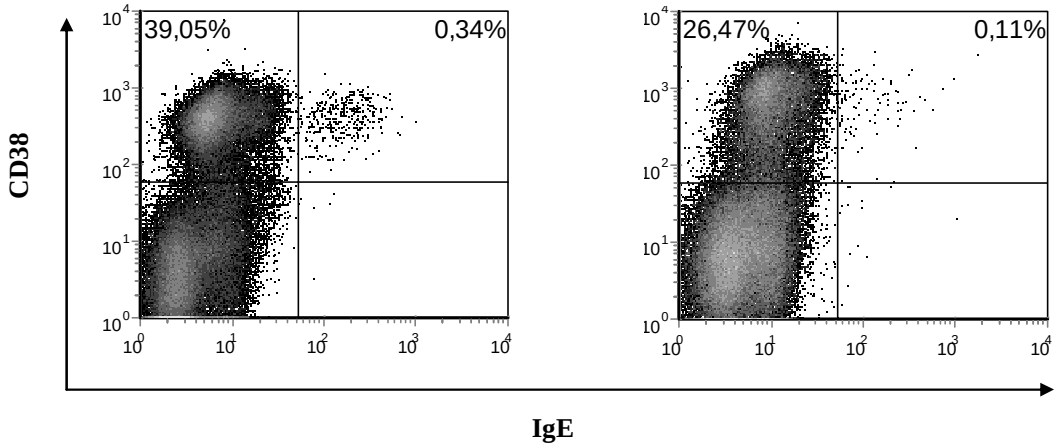

Figure 10. Calcitriol inhibits differentiation of IgE plasma cells. B cells were stimulated with anti-CD40/IL-4/IL-21 +/- calcitriol for 6 days. Flow cytometric analyses of the surface markers CD19, CD27 and CD38 and intracellular IgE were performed. Cells shown in upper right panel were all CD27++. Data show one representative out of three donors.

The inhibition of IgE secretion as well as differentiation of IgE plasma cells were in the same range. This implies that calcitriol is targeting either the plasma cell differentiation or preceding events such as CSR to IgE.

4.1.3. Calcitriol inhibits ε germline transcript expression in CD23+ human B cells

εGLT expression is a prerequisite for switching towards IgE and consequent IgE production [5]. In order to delineate the effect of calcitriol on CSR to IgE isotype its impact on εGT was investigated. Since IL-21 does not increase εGLT expression [46], only anti-CD40/IL-4, known to efficiently induce εGLT [5], were used for further experiments.

For gaining a more selected population for investigation of mechanistic action of calcitriol, an identification of the calcitriol-responding cells was necessary. As anti-CD40/IL-4 are well known to induce VDR [48] and CD23 expression [49] in B cells, it was primarily examined

whether calcitriol inhibits εGLT expression in these cells. For that purpose B cells were stimulated with anti-CD40/IL-4 +/- calcitriol for 48 h and subsequently CD23+ subpopulation was enriched (Figure 11).

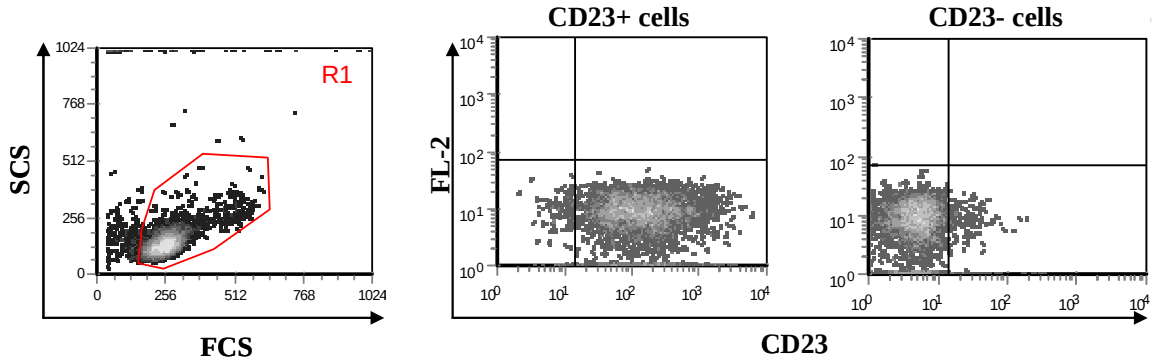

Figure 11. CD23+ and CD23- B cells after enrichment. B cells were stimulated with anti-CD40/IL-4 for 48 h. CD23+ cells were separated using positive magnetic separation. Surface expression of CD23 was detected by flow cytometry. Dot blots are gated on viable B cells. Data show one representative out of six.

To verify whether CD23+ cells possess the ability to switch to IgE and to respond to calcitriol, qRT-PCR analyses of εGLT, VDR and cyp24 mRNA were performed. The data show that εGLT expression is robustly induced upon anti-CD40/IL-4 stimulation in CD23+ B cells and significantly reduced in additional presence of calcitriol (Figure 12a). The results demonstrate that these cells up-regulate cyp24, a confirmed vitamin D responder gene [50], chosen as positive control (Figure 12b). Furthermore, the investigation of VDR expression revealed 4-fold higher expression in purified CD23+ compared to CD23- B cells (Figure 12c), implying that CD23+ cells represent indeed a calcitriol-responding B cell subpopulation.

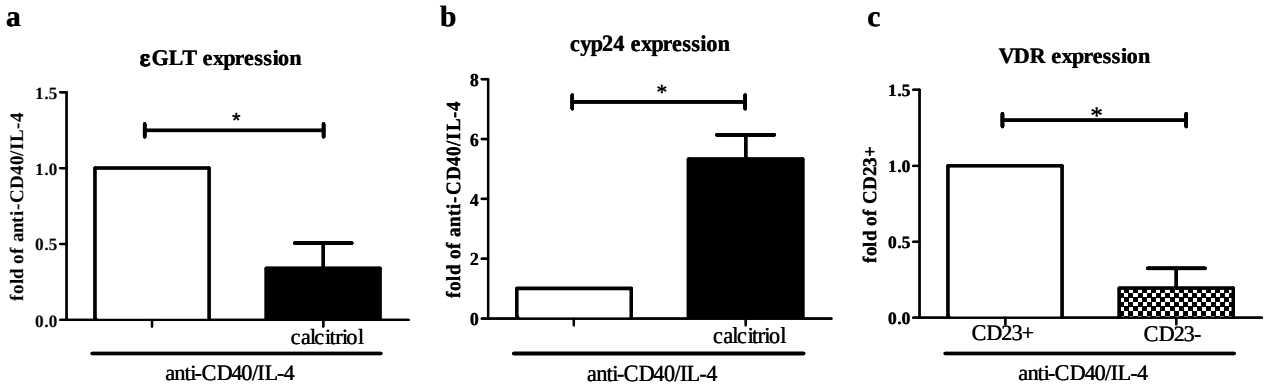

Figure 12. Calcitriol inhibits εGLT expression in CD23+ B cells. a) and b) B cells were stimulated with anti-CD40/IL-4 +/- calcitriol for 48 h. After purification of CD23+ cells εGLT and cyp24 expression were determined by qRT-PCR. N=6. c) CD23+ and CD23- cell subsets were purified after 48 h of anti-CD40/IL-4 stimulation. VDR mRNA expression was analysed by qRT-PCR. Data are shown relative to HPRT expression as mean +/- SEM. N=5; * $p<0.05$.

4.2. Characterisation of the VDR complex at the ε germline promoter

4.2.1. Identification of putative VDR binding sites

As calcitriol inhibits εGLT transcript expression, it was aimed to investigate whether ε germline gene represents a primary VDR target. VDR, which is a transcription factor, binds directly to VDREs within the control regions of its primary target genes [25]. This implies that there must be at least one VDRE within the regulatory region of the gene. *In silico* analyses using MatInspector (www.genomatix.de) and Patch (www.gene-regulation.com) revealed several putative VDREs in the Iε region. The four putative VDREs are in close proximity to the transcription start (TSS) of the ε germline gene and were selected for further analysis (Figure 13). One mismatch per motif was allowed and the list of motifs especially for pairs DR3 and DR4 type was filtered, since they may allow efficient VDR:RXR heterodimer [25].

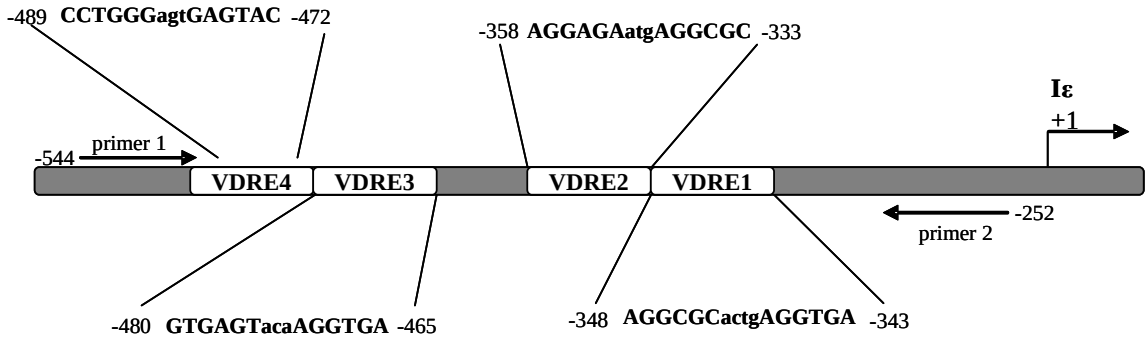

Figure 13. Schematic diagram of the ε germline promoter with sequences of putative VDREs and primer positions for the PCR analyses.

4.2.2. VDR:RXRα heterodimer binds at the promoter of the ε germline gene

The binding of VDR at Iε promoter was analysed using the ChIP assay. As mentioned previously, calcitriol-responding cells, CD23+ B cells separated after B cell culture with anti-CD40+IL-4 +/- calcitriol were used for this purpose. Calcitriol was additionally added to the culture for 180 minutes. The DNA precipitated with anti-VDR Ab was quantified by qRT-PCR amplifying the target sequence of ε germline promoter (Iε), *trpv6* promoter (tprv6; positive control) and the open reading frame within *trpv6* sequence (orf; negative control). The data show that VDR upon calcitriol binds specifically to the Iε and trpv6 region, but not to the orf control segment (Figure 14a).

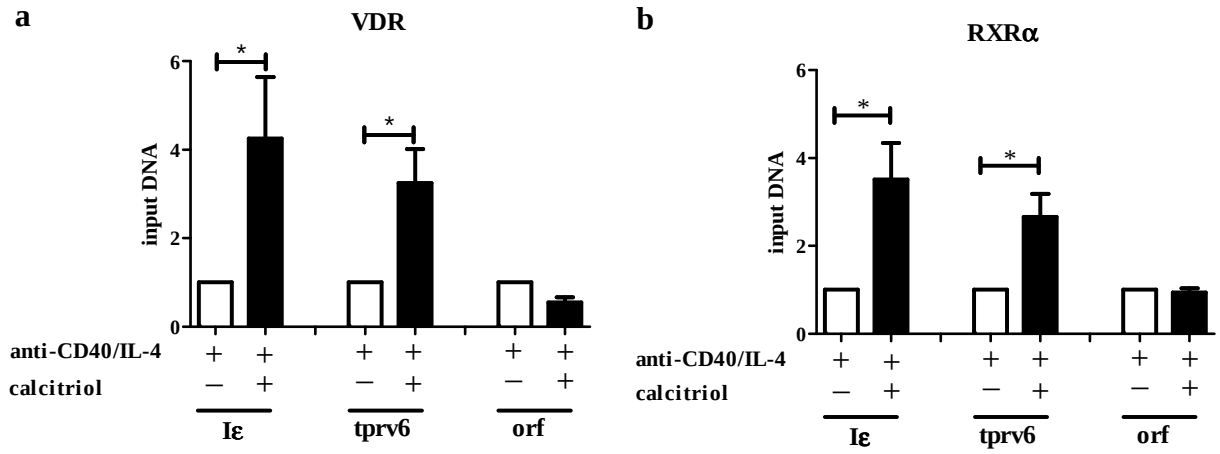

Figure 14. VDR:RXRα heterodimer binds to the Iε promoter. CD23+ B cells treated with anti-CD40/IL-4+/- calcitriol were used for VDR or RXRα ChIP. Iε, trpv6 and orf region were amplified using qRT-PCR. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM; n=6; *p<0.05.

It was previously described that VDR usually acts as a heterodimer [26], therefore it was aimed to investigate whether this is also the case for the Iε region. For the ChIP assay CD23+ B cells were used. DNA was precipitated using Ab against RXRα, RXRβ, RARα, RARβ and analysed by qRT-PCR. The data reveal that VDR binds as a heterodimer with RXRα to the Iε (Figure 14b). On the contrary, RXRβ, RARα and RARβ were not recruited within the Iε upon calcitriol stimulation (data not shown).

The kinetics of receptor association to the Iε region was examined next. The complex binding was assessed in 30-minute intervals up to 180 minutes of calcitriol stimulation. The data show cyclic binding of VDR and RXRα to the Iε promoter (Figure 15).

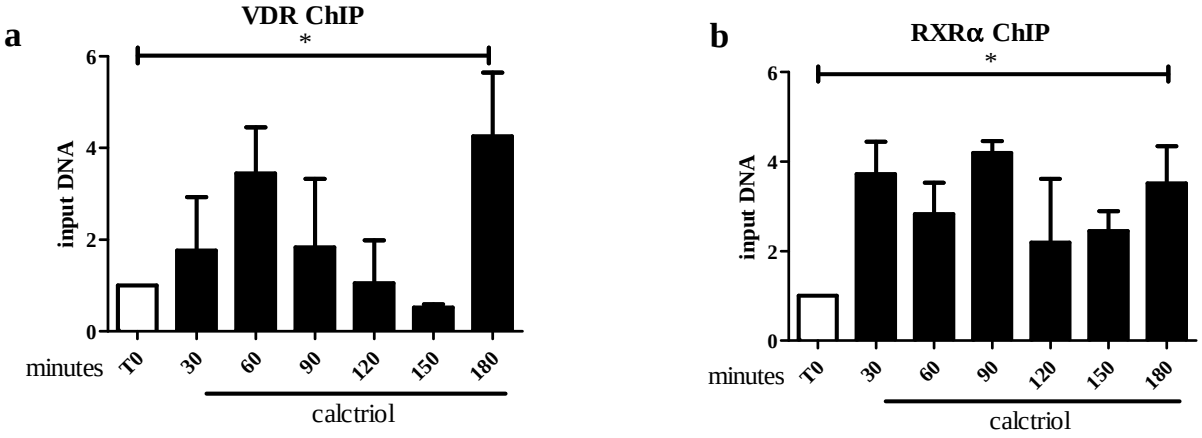

Figure 15. Kinetic of the VDR:RXRα heterodimer binding to the Iε promoter. CD23+ cells were enriched after B cell stimulation with anti-CD40/IL-4 +/- calcitriol for 48 h. Calcitriol was additionally added at the time indicated. ChIP using Ab against a) VDR, b) RXRα were performed, followed by qRT-PCR amplification of the Iε. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM. N= 3-6; * $p<0.05$.

4.2.3. VDR-complex recruits the transcriptional co-factors SMRT, HDAC1 and HDAC3

Previous reports indicated that NRs interact with two major co-factors, SMRT and NCoR, to repress gene expression [28]. It was aimed next to identify the composition of the VDR complex at the Iε region. Therefore the ChIP assays using Abs against SMRT, NCoR, HDAC1, HDAC2

and HDAC3 were performed. The data revealed that SMRT, HDAC1 and HDA3 are being recruited to the Iε region upon calcitriol stimulation in CD23+ B cells (Figure 16).

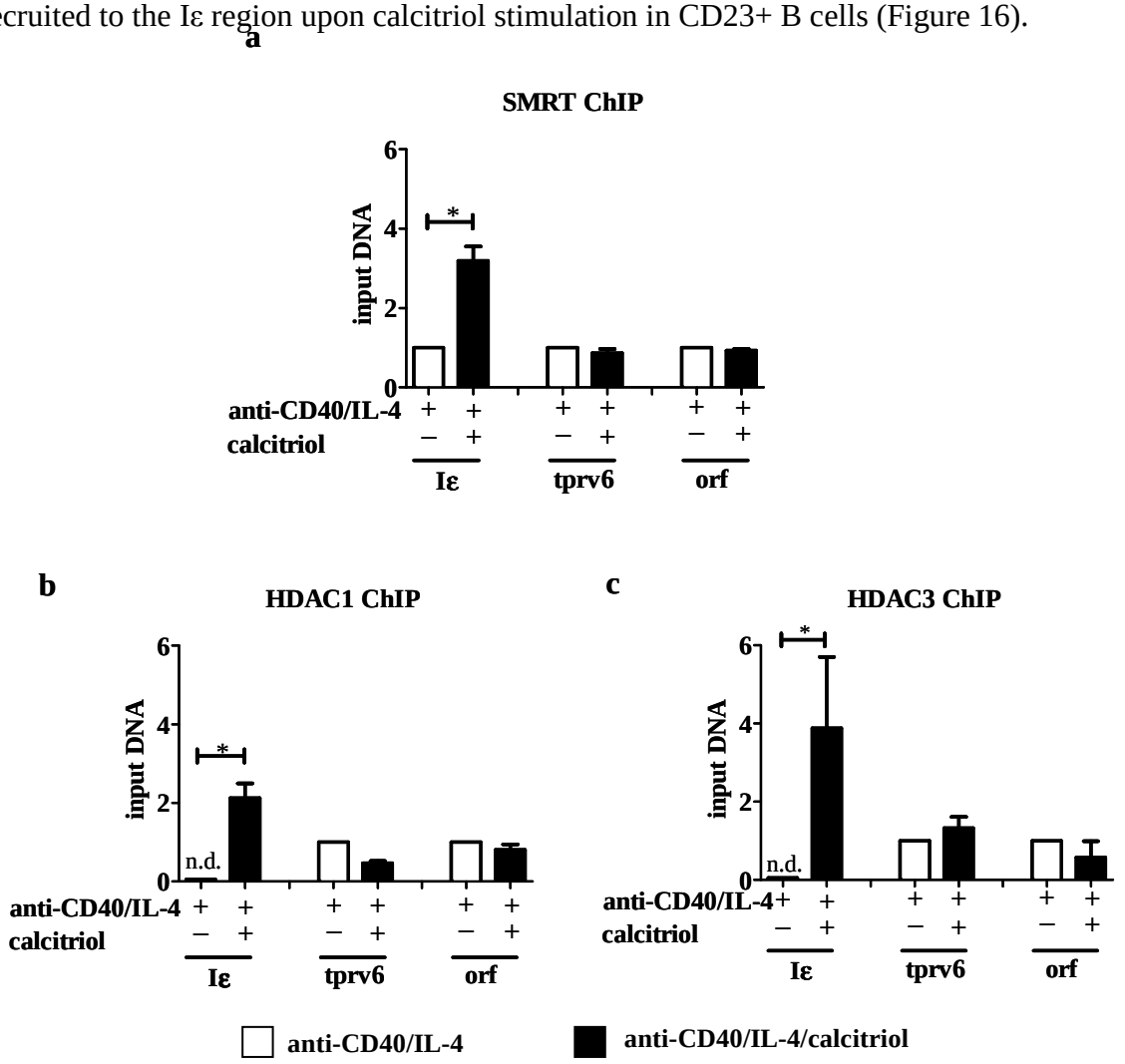

Figure 16. VDR recruits SMRT, HDAC1 and HDAC3 to the Iε region. B cells were stimulated with anti-CD40/IL-4+/- calcitriol for 48 h. Calcitiol was additionally added for a) 90 minutes, b) and c) 180 minutes. CD23+ cells were isolated and ChIP was performed using Ab against a) SMRT, b) HDAC1 and c) HDAC3. Amplification of the Iε, trpv6 promoter region (positive control) and trpv6 open reading frame (orf; negative control) was analysed by qRT-PCR. Data are shown as the relative amount of Ab-bound DNA to unprecipitated DNA as mean +/- SEM; n=6. N.d. stands for not detectable.

Subsequently, the kinetics of the co-factor-enrolment in 30-minute intervals over 180 minutes of calcitriol stimulation was analysed. Data show that SMRT is engaged previous to HDAC1 and HDAC3 to the Iε region (Figure 17)

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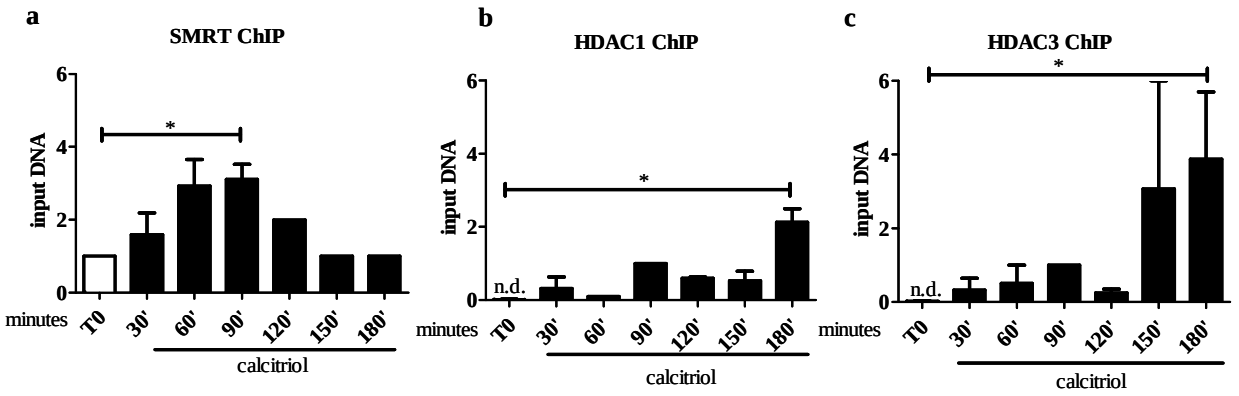

Figure 17. Kinetic of the VDR complex formation in the Iε region. CD23+ B cells were stimulated with calcitriol for the time indicated and a) SMRT, b) HDAC1 and c) HDAC3 ChIP were performed. Iε was analysed by qRT-PCR. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM; n=3-6; * $p<0.05$; N.d. stands for not detectable.

In contrast, NCoR and HDAC2 were not engaged by VDR at the Iε region (Figure 18).

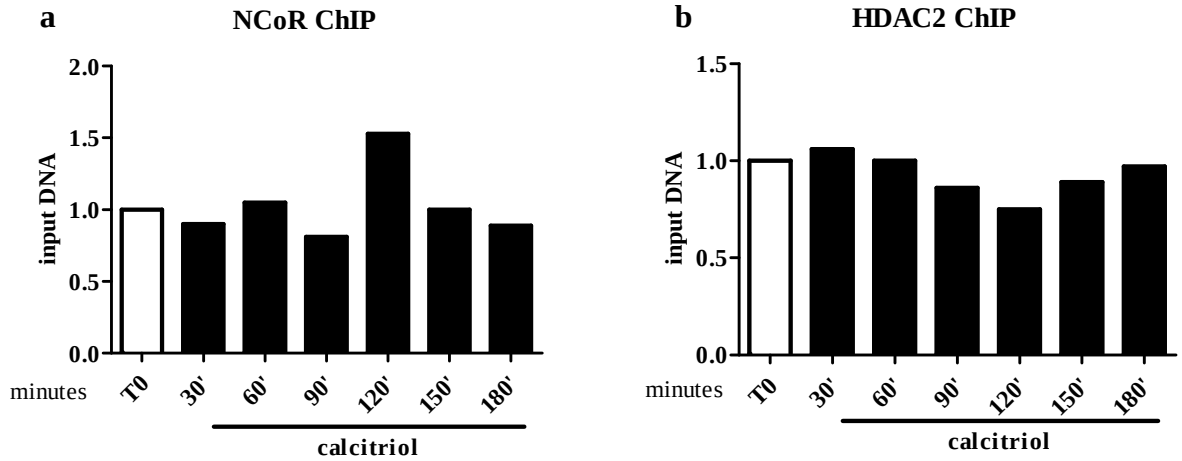

Figure 18. NCoR and HDAC2 are not recruited by calcitriol to the Iε promoter. CD23+ B cells were stimulated with calcitriol for the time indicated and a) NCor and b) HDAC2 were performed. Iε was analysed by qRT-PCR. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM; n=3-6.

The binding profile of the transcription factors at Iε suggests that VDR:RXRα is initially binding to the DNA and followed by an engagement of the SMRT co-repressor, which in turn recruit HDAC1 and HDAC3 enzymes.

4.3. Functional impact of the VDR-DNA complex on the ε germline gene activity

4.3.1. VDR-mediated posttranslational histone modifications

In order to prove the function of the calcitriol-mediated HDAC recruitment, the HAc pattern of the Iε region was analysed. For that purpose CD23+ B cells stimulated with /without calcitriol underwent ChIP analyses using Ab against AcH 3 and 4. Upon calcitriol stimulation decreased acetylation of histone 3 (after 180 minutes) and histone 4 (after 120 minutes) were revealed. This suggests that calcitriol indeed modulates the gene accessibility of ε germline gene (Figure 19a and Figure 19b). As tprv6 expression is upregulated upon calcitriol stimulation [51], the increased acetylation of this locus was verified (Figure 19a and Figure 19b).

It was previously shown that the acetylation of the S region plays a role in AID targeting [52], therefore it was also investigated whether the calcitriol mediated deacetylation spreads into the S region. The decreased deacetylation of histone 3 and 4 was observed at Sε upon calcitiol treatment (Figure 19). This is implying that the CSR to IgE might be further inhibited by this mechanism as well.

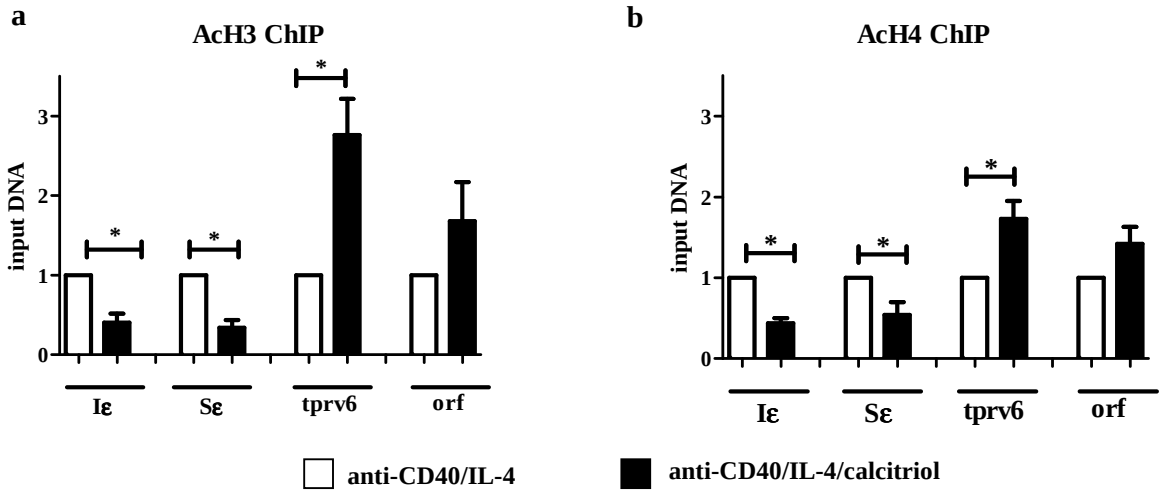

Figure 19. Calcitriol induced changes in acetylation pattern at the Iε and Sε region. CD23+ B cells were stimulated with calcitriol and a) AcH3 or b) AcH4 ChIP was performed, followed by amplification of the Iε, Sε tprv6, orf. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM; n=6; * $p<0.05$.

Chromatin acetylation and deacetylation are dynamic processes [34]. Therefore, the acetylation pattern upon calcitriol addition at early time points in 30-minute intervals up to 180 minutes, as well as later time points (6 h, 12 h and 24 h) was followed. Data show that calcitriol-mediated deacetylation at histone 3 is initiated after 120 minutes and proceeds until 180 minutes. This is a transient effect as the level of histone 3 acetylation is normalised after 24 h (Figure 20a). On the other hand, the deacetlyation of histone 4 is faster and remains at low level after 24 h of calcitriol stimulation (Figure 20b), suggesting a longer lasting effect of calcitriol.

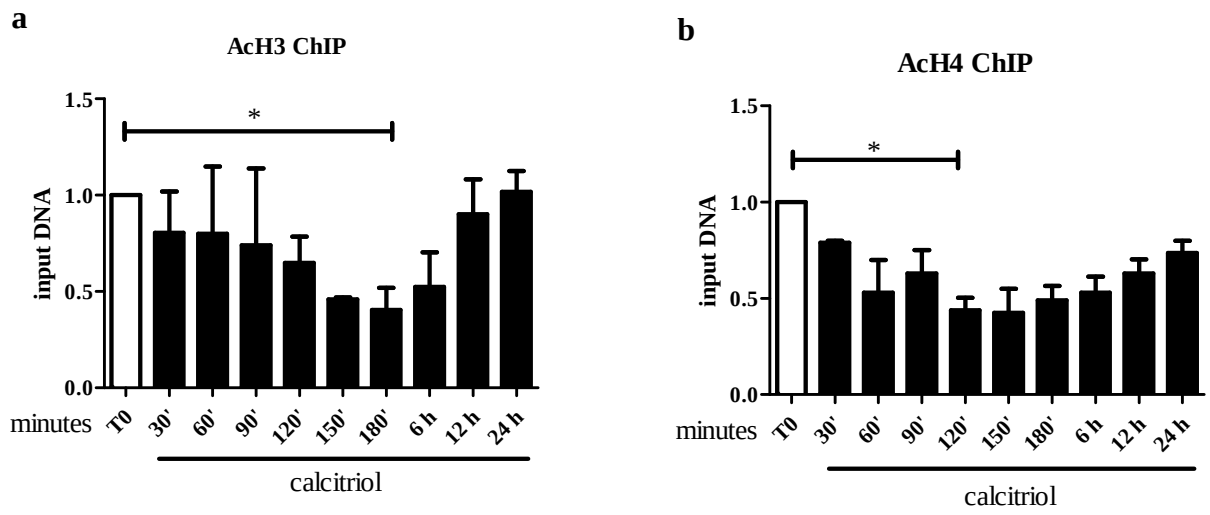

Figure 20. Kinetic of the calcitriol-mediated histone deacetylation at Iε. CD23+ cells were enriched after B cell stimulation with anti-CD40/IL-4+/- calcitriol for 48 h. Calcitriol was additionally added at the time indicated. ChIP using Ab against a) AcH3 and b) AcH4 were performed, followed by amplification of the Iε. Data are shown as the relative amount of Ab-bound DNA to unprecipitated as mean +/- SEM. N= 3-6; * $p<0.05$.

4.3.2. Functional relevance of the acetylation pattern of the ε germline gene promoter

To prove the functional activity of the VDR complex it was interfered with this complex by using SMRT and/or HDAC inhibitors. SMRT, HDAC1 and HDAC3 are known to be selectively inhibited by arsenic-(III)-oxide (As_2O_3), MS-275 and apicidin, respectively [53, 54]. The mentioned inhibitors were added together with calcitriol to the culture of CD23+ enriched cells. In their presence calcitriol-driven deacetylation of Iε region was reduced (Figure 21), suggesting that HDAC1 and HDAC3 both contribute to the local change of deacetylation of histone 3 and 4. However, the effect of HDAC3 is more robust. To rule out possible toxicity of the used

inhibitors the flow cytometric analyses of propidium-iodide stained cells was performed and verified that the concentrations used had no impact on the cell survival (data not shown).

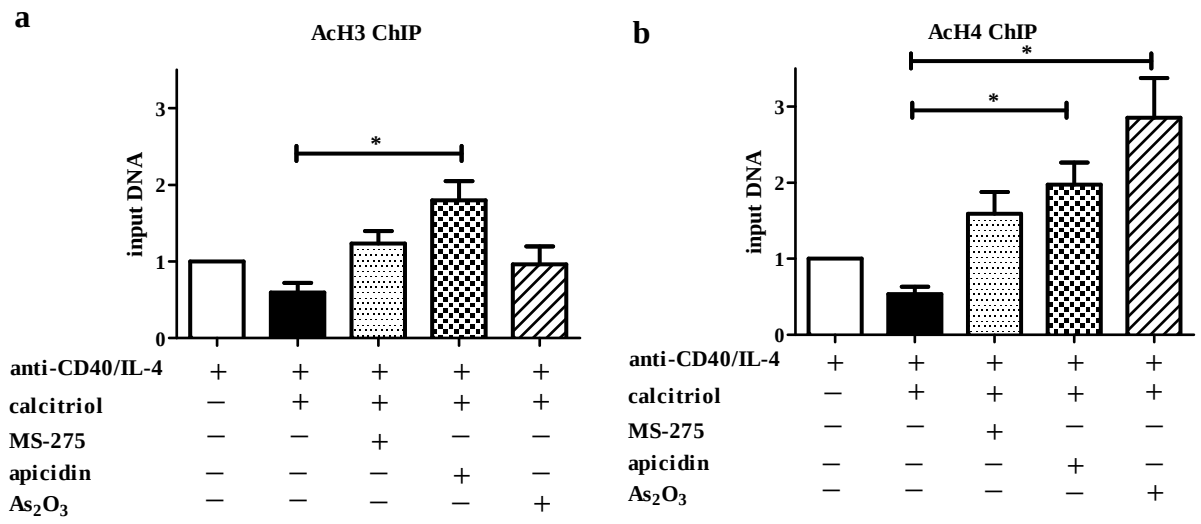

Figure 21. Inhibition of HDACs and SMRT inhibits the calcitriol-mediated deacetylation of the Iε region. CD23+ B cells were stimulated with +/- calcitriol +/-apicidin +/- MS-275 +/- As_2O_3 and AcH3 or AcH4 ChIP were performed, followed by qRT-PCR analyses of the Iε. Data are shown as the relative amount of Ab-bound to unprecipitated DNA as mean +/- SEM. N=4.

The biological relevance of changes in the acetlytion pattern for the εGLT expression was investigated next. Therefore, B cells were cultured with anti-CD40/IL-4 +/- calcitriol +/ As_2O_3 +/- MS-275, +/- apicidin for 48 h and εGLT was analysed by qRT-PCR. Indeed in the presence of these particular inhibitors the repression of the εGL transcription was disrupted (Figure 22). This suggest that calcitriol-mediated hypoacetylation is important for ε germline gene transrepression.

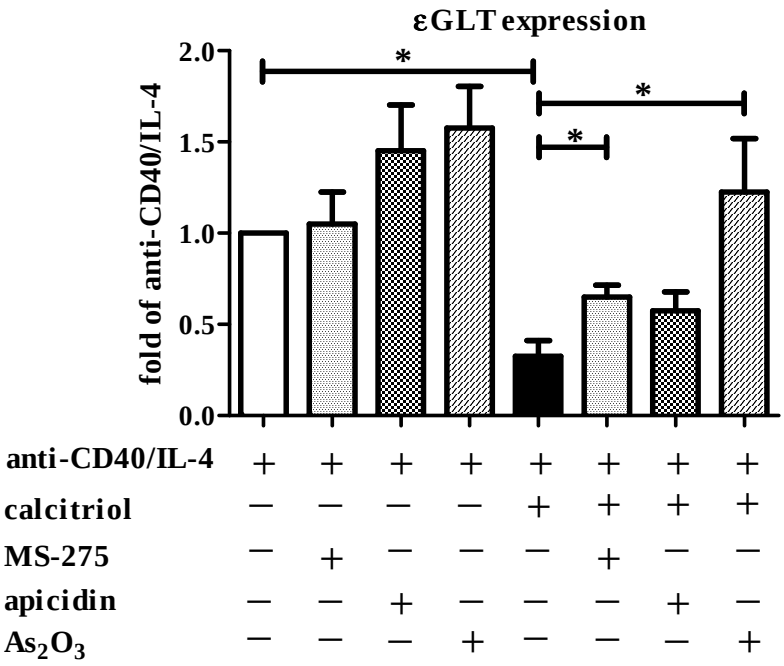

Figure 22. HDACs and SMRT inhibition reverses the calcitriol-mediated transrepression of εGLT. B cells were stimulated with CD40/IL-4+/- calcitiol +/- MS-275 +/-apicidin +/- As_2O_3 for 48 h and expression of the εGLT was analysed. Data are shown as relative to anti-CD40/IL-4 stimulated sample as mean +/- SEM. N=5; * $p<0.05$.

4.3.3. Repression of the biological activity of the ε germline gene promoter by calcitriol

For verifying the biological activity of the VDREs located in the Iε region a reporter gene assay was performed. It was hypothesised that the calcitriol-mediated recruitment of a transrepressive complex would lower the baseline luciferase experession of the SV40-pGL3-luciferase vector with inserted negative VDREs. As positive control the proximal promoter of the human cyp27B1 gene was chosen, as its calcitriol-mediated transrepression has been shown previously [32]. The region of cyp27b1 promoter with confirmed negative VDREs was inserted into the SV40-pGL3-luciferase vector. The construct cyp27-SV40-pGL3-luciferase together with VDR-expression vector and renilla-expression vector were cloned into HEK-293 cells. In the presence of calcitriol the basal activity of the Cyp27-SV40-pGL3-luciferase vector was significantly reduced by 43 % (Figure 23). Upon calcitriol stimulation of HEK-293 cells were transfected with the Iε-SV40-pGLT-luciferase construct (containing as insert of the Iε promoter with VDREs), VDR-

expression vector and renilla-expression vector analogous tendency was also observed (Figure 23). However, this effect did not reach the statistical significance, probably due to inter-assay variation. For that reason the impact of calcitriol on the construct with inserted region containing VDREs found in the Iε promoter (VDREs-SV40-pGLT-luciferase) was investigated. In this case the calcitriol-mediated transrepression of luciferase expression (by 38 %) reached statistically significance (Figure 23).

As negative control HEK-293 cells were transfected with an empty vector instead of VDR-expression vector, renila-expression vector and either Cyp27-SV40-pGL3-luciferase or Iε-SV40-pGLT-luciferase or VDREs-SV40-pGLT-luciferase constructs. In the presence of calcitriol the basal activity of the three mentioned constructs remained unchanged (data not shown).

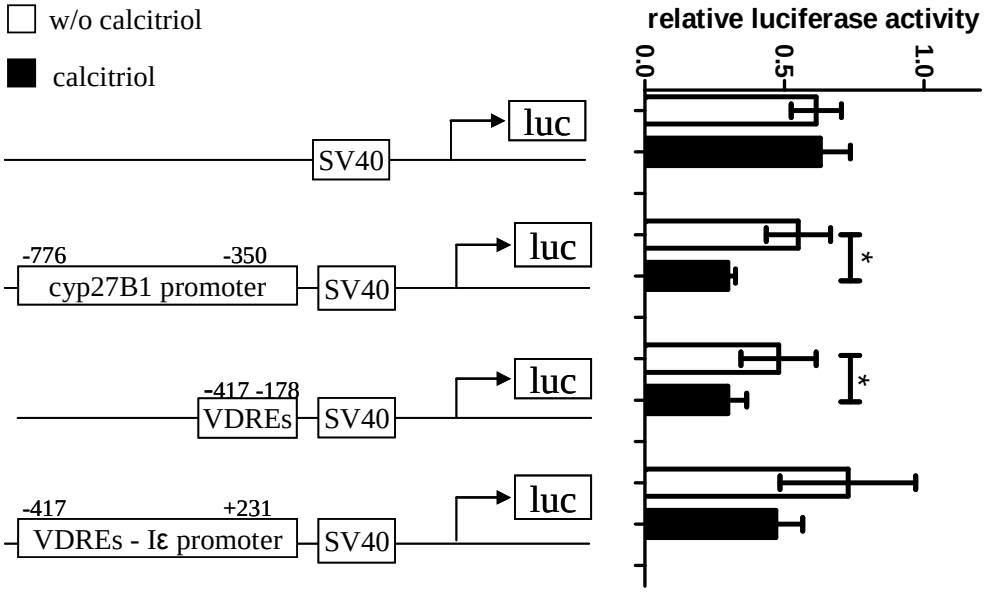

Figure 23. Functional analysis of VDREs in the Iε region. HEK-239 cells were transfected with SV40-pGL3-luciferase or cyp27b1-SV40-pGL3-luciferase or VDREs-Iε-SV40-pGLT-luciferase or VDREs-SV40-pGLT-luciferase. Cells were co-transfected with VDR- and renilla-expression vector. Upon calcitriol stimulation the luciferase activity was measured and normalised to the activity of the renilla-expression vector. Data are shown as mean +/- SEM from 5 independent experiments; * $p<0.05$.

To summarise, these results support the observed calcitriol-mediated inhibition of the εGLT expression.

5. DISCUSSION

Emerging studies are revealing the role of calcitriol in immunomodulation and its possible impact on the treatment of allergic diseases [23]. Moreover, vitamin D deficiency has been linked to the 'epidemic' of allergy present during the last decades [36-38, 41]. Therefore, the mechanisms of calcitriol-mediated actions in immune cells need to be described in detail.

5.1. Calcitriol-mediated inhibition of εGLT expression and IgE production

IgE is the key component of type-I-hypersensitivity underlying the pathogenesis of allergic manifestations, and therefore a promising target for therapeutic approaches [1]. CSR to IgE and IgE production are inefficient and tightly regulated processes, as is well illustrated by the minute amounts of IgE found in the circulation in comparison with other Ig subclasses [7]. In general, effective CSR to IgE requires simultaneous triggering of the CD40 and the IL-4R [5]. The concurrent activation of NF-κB and STAT6 initiates a cascade that culminates in the transcription of ε germline gene, and moreover initiation of a distinct genetic programme necessary for the differentiation of plasma cells [5, 6].

This work shows that calcitriol-mediated inhibition of IgE production and differentiation of IgE producing plasma-like cells is a direct consequence of a prominent inhibition of εGT. Furthermore, it was shown here that cells undergoing switching towards IgE are co-expressing CD23 on the cell surface, as well as up-regulating VDR expression and therefore indeed represent a target for calcitriol. This is in line with the gene expression analyses of germinal centre B cells [56]. The performed microarray revealed an up-regulation of only a limited number of the genes, like transcription factor B lymphocyte-induced maturation protein 1, X-box binding protein 1, interferon regulatory factor 4, and for up to now unexplained reasons VDR in centrocytes (B cells mainly undergoing CSR) compared to centroblasts (cells mainly undergoing somatic hypermutation) [56]. Furthermore, the here performed analyses of ex vivo derived memory B cells showed baseline levels of VDR interaction with Iε region (data not shown). Upon anti-CD40/IL-4 stimulation VDR disassociates from the promoter, whereas upon calcitriol addition this effect is reversed (data not shown). As the VDR upregulation in switching B cells was confirmed and the impact of calcitriol on CSR to IgE was clearly demonstrated, it is

intriguing to speculate that calcitriol may be a physiological regulator of CSR directly in the germinal centre.

As vitamin D deficiency is nowadays very prominent in the Western countries, its precise definition and interpretation of serum vitamin D is crucial for further discussion of its consequences for allergy. Although calcitriol is the biologically active form of vitamin D, its half-life is less than 5-8 h [21, 22]. In fact, calcitriol may remain normal or even increase in vitamin D-deficient states [21]. Thus, serum 25-hydroxy VD3 with a half-life of about 2 weeks is a routinely accepted biomarker for assessing vitamin D levels [21]. Determinants of circulating 25-hydroxy VD3 concentrations do include sun exposure and diet, but high heritability suggests that genetic factors could also have their part [55]. Due to the differing evidence regarding the relationship of the calcitriol levels with VDR polymorphism [56-58], the focus has been moved to the genetic variants in the vitamin D signalling pathway [55, 59, 60]. Recently common genetic variants associated with 25-hydroxy VD3 concentrations have been identified. The presence of certain alleles at the three confirmed loci of vitamin D pathway genes encoding: vitamin D binding protein (*GC*), 7-dehydrocholesterol (*DHCR7*), 25-hydroxylase (*cyp2R1*) and 24-hydroxylase (*cyp24a1)* more than doubled the risk of VD deficiency [55]. Interestingly, Hibler et al. demonstrated the linkage between single nucleotide polymorphisms (SNP) in RXRα and calcitriol concentrations [59]. These variants might provide useful genetic approaches to investigate the role of vitamin D deficiency in several chronic diseases, such as allergic diseases. An up to now not defeated obstacle for clarifying the importance vitamin D lays in the current definition of vitamin D deficiency, which is based on vitamin D effect in the context of musculoskeletal health [22]. The values are at this time interpreted as follows: <20 ng/ml (<50 nmol/l) is deficient; 21–29 ng/ml (51–74 nmol/l) is insufficient; and >30 (>75 nmol/l) is sufficient [39]. This classification is currently a subject of revision as further information is collected regarding effects of vitamin D on immune function. However, the association between vitamin D deficiency and increase in incidence of allergic disease implies that calcitriol could be an epigenetic environmental factor underlying or at least contributing to the development or manifestation of allergy.

This work not only offers an explanation for the VDR up-regulation in cells undergoing CSR, but is also proposing that maintenance of certain levels of sufficient 25-hydroxy VD3 could indeed have a protective role at the early stages of IgE production and therefore pathogenesis of allergic diseases.

5.2. VDR binding to the ε germline gene promoter

The down-regulation of εGT in B cells by calcitriol has been shown previously [40]. However, it remained under discussion whether ε germline gene represents a primary or a secondary target of calcitriol [25]. In general, gene repression by steroid NRs is far less understood than gene activation. However, since VDR is the only nuclear protein that binds to calcitriol with high affinity, this NR has to be the central switch in the case of the primary gene repression [33].

VDR has been shown to bind to the DNA as a homodimer, but also heterodimer with the TR, RAR and by far the strongest and the most important binding partners RXRs (α, β or γ) [25]. The data obtained here using ChIP assay confirmed these previous findings and revealed that VDR binds as a heterodimer with RXRα to the Iε promoter. There were no previous data whether ε germline gene is the primary target of VDR and whether the machinery of the calcitriol-mediated εGT inhibition involves transrepression. Therefore, the focus was first and foremost directed on identifying these mechanisms, and the genetic variants were not considered in these experiments. More importantly, as these experiments involve addition of calcitriol, the biologically active metabolite, the changes regarding the metabolism of its precursors do not play a role in this system. To better compare the kinetics of the binding of different co-factors for each repetition of the experiment pooled stimulated B cells were used. VDR polymorphism among individuals has an influence on its signalling. Nevertheless, it is known that SNP can influence the rate of gene transcription, stability of the mRNA, quantity and the activity of the formed protein [55, 61]. These factors can quantitatively and qualitatively change the VDR impact on any target gene, and thereby ε germline gene as well. However, studies of VDR polymorphism and asthma associations made clear that VDR influences asthma and allergy susceptibility in a complex manner [57-60, 64]. Associations between multiple SNP in VDR, cyp2r1 and cyp24a1 have been linked to asthma and total IgE levels so far [57, 60]. No functional data has been shown that these polymorphisms change the amount or function of the mentioned proteins. Hibler et al. demonstrated the linkage between SNP in RXRα and calcitriol concentrations [59]. As data shows here VDR:RXRα binding to the Iε, it would be of interest to examine the role of RXRα SNP in IgE production or asthma. Further research is necessary to relate the determined genetic variations of the VDR gene to the VDR function, since this could be important for identifying the patients with potential benefits from vitamin D based therapy.

5.3. Composition of the VDR-transrepressive complex

NRs are able to positively or negatively regulate gene expression [25]. While the mechanisms of calcitriol-driven transactivation have been well described previously, the negative control of gene expression by VDR has not been completely unravelled yet. The negative regulation, which is here of particular interest, can be ligand independent and ligand dependent, whereas the latter can be driven in agonist or antagonist manner [26, 31, 35].

In the classical model, unoccupied non-steroidal NRs bind SMRT and NCoR, which are released upon ligand binding [29]. However, the ability of the unoccupied steroid NRs, such as VDR, to bind these co-repressors and silence transcription has been controversially discussed [25, 29]. Although VDR is able to interact, to some extent, with these co-repressors, the un-ligated VDR has, at most, a weak silencing activity [29]. In fact, the ability of this receptor to interact with NCoR and SMRT in the absence of ligand appears to be suppressed by heterodimerization with RXR. In contrast to other receptors such as thyroid receptor or RAR [29], no detectable binding of the co-repressors with the unoccupied heterodimer is observed. Recent reports have demonstrated strong binding of these co-regulators to antagonist- but as well agonist-bound steroid receptors [29, 32, 33].

Upon the recruitment of the co-repressors to the regulatory gene region the transrepressive function is modulated through deacetylation of lysines on histone tails by HDACs [29, 32, 33]. The two major co-repressors, SMRT and NCoR, are known to co-operate with VDR [28]. Other co-repressors, like alien and heirless co-repressors have also been described to interact with VDR. However, their repressive actions seem to be HDAC independent. Therefore, here the focus of attention was primarily on investigation SMRT and NCoR interactions. They both have the same core associated factors, including HDAC3, GPS2 and the transducin β-like factors, TBL1 and TBLR1 [12-16]. A recently described domain named the deacetylase activating domain in both SMRT and NCoR has been shown to promote both the enzymatic activity and the binding of HDAC3 [10]. It is important to emphasize that co-repressor complexes direct the substrate specificity of HDAC3. HDACs 1, 2, 4, 5 and 7 can also interact with SMRT or NCoR complexes [10]. Their exact roles in SMRT- and NCoR-dependent gene repression is still under discussion. In order to form an active SMRT/HDAC3 complex, association with the TCP1 ring complex chaperone is required [24]. This process is ATP-dependent and TCP1 ring complex dissociates from SMRT-HDAC3 after the complex formation.

Up to now the agonist-mediated VDR-transrepression has been described in detail for the cyp27b1 gene, in which case both SMRT and NCoR have been recruited upon calcitriol treatment [32]. Similarly, the down-regulation of the human *myc* gene by calcitriol is associated with TBL1X, SMRT, HDAC6, and HDAC11 [33]. Sanchez-Martinez et al. show that the VDR:RXR heterodimer recruits NCoR and SMRT to repress cyp24 gene strictly in a VDR agonist-dependent manner [29]. Likewise the results show here that in B cells the ε germline gene transrepression takes place in a calcitriol-dependent manner, by VDR:RXRα interaction with SMRT, but not NCoR. This complex harbours HDAC1 and HDAC3, which are known to deacetylate histone 3 and 4 [34]. With the means of HDACs and SMRT inhibitors the function of the recruited complex was verified and a novel mechanism of target gene dependent VDR-mediated deacetylation in B cells was identified (Figure 24).

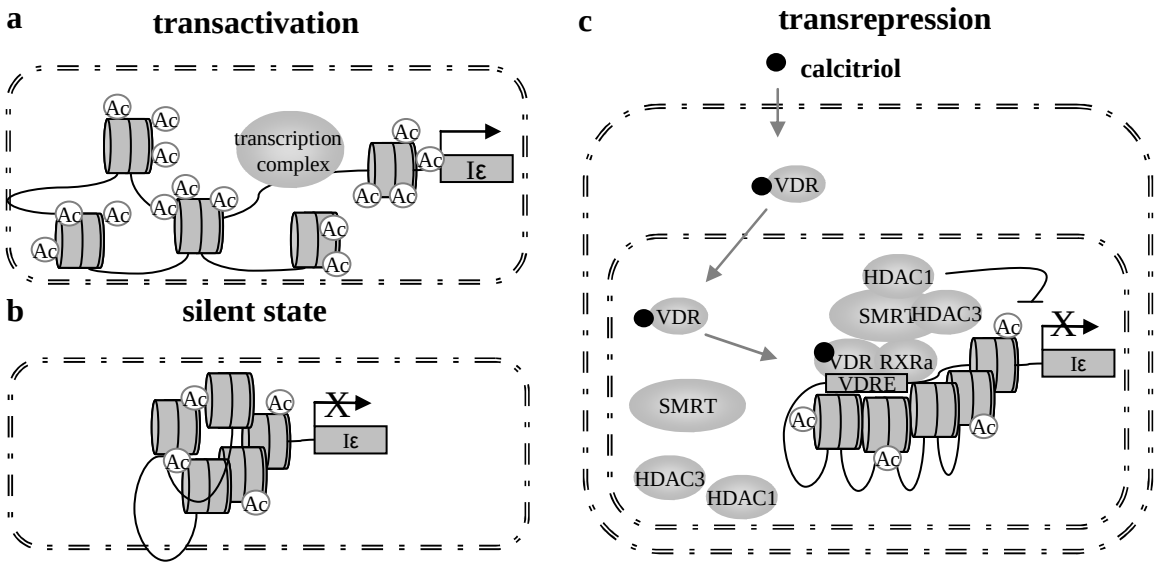

Figure 24. Model demonstrating the role of acetylation in εGT. a) In the resting B cells the histones surrounding the Iε promoter are deacetylated, chromatin is condensed, and the ε germline gene does not takes place. b) Upon activation the acetylation of the histones surrounding the Iε promoter takes places, leading to decondensation of the chromatin and εGT. c) During the calcitriol-induced transrepression of the ε germline gene VDR binds to the Iε, recruits SMRT harbouring HDAC1 and HDAC3, which subsequently deacetylate histones around the promoter leading to condensation of the DNA an inhibition of the transcription.

It is important to mention that SMRT represents a target for cell signalling pathways, which influence their expression levels, subcellular localization, association with other proteins, modification such as phosphorylation or their degradation. Dunlop et al. found that SMRT gene

is a direct transcriptional target of calcitriol due to the presence of DR3-type VDREs in its promoter [62]. However, a recent report ruled out the possibility that calcitriol inhibits NF-κB via SMRT induction [63].

5.4. Acetylation pattern of the ε germline promoter and its functional relevance

Recent studies are emphasizing the importance of HAc in allergy in general as well as in the induction of numerous proinflammatory genes in particular [64-66]. Recent data suggests that HDACs are relevant for maintenance of the pre-established Th1/Th2 ratio [67]. Moreover their inhibition favours the shift towards a Th2-like immune response. Covalent modifications of chromatin (in particular HAc) have been implicated in the Ig regulation in B cells as well [3]. Based on the “accessibility model” the modulation of the acetylation pattern is responsible for the initiation of germline gene transcription and CSR [3, 68]. These observations were proven in mutant mice, in which a replacement of the germline promoter, containing cytokine-response elements selectively blocks GT and CSR to those specific loci [14, 68]. Several reports indicated that I and S regions of all isotypes, except μ, are hypoacetylated (marker of condensed chromatin) in resting B cells and become hyperacetylated (marker of loose chromatin state) upon cytokine stimulation [15-17]. Moreover, the hyperacetylation of the S regions is promoting specific AID targeting and thereby increasing the CSR frequency [17, 52]. On the contrary, another group suggests that CH genes are in an ‘‘accessible’’ chromatin conformation prior to CSR [18]. However, to our knowledge it has not been investigated whether changes in HAc by calcitriol at the Iε have an impact on switching to IgE. The data show here not only the binding of HDAC, but also the histone 3 and 4 deacetylation in Iε region, which spreads into the Sε region. This implies that on the one hand the εGT is inhibited by chromatin compacting of the promoter and on the other hand that calcitriol-mediated deacetylation in the Sε region is furthermore reducing the CSR frequency, since the hyperacetylated S regions are preferentially targeted by AID [52].

With the help of different inhibitors it was possible to prove that both HDAC1 and HDAC3 have an impact not only on the acetylation pattern of Iε in principal, but also at the level of εGLT expression itself. Thereby, the results demonstrate a mechanism of calcitriol-mediated inhibition of εGLT expression and highlight the importance of the acetylation state for IgE production. Increased HAT activity has been suggested to be relevant in allergic patients, for example for

Th1, Th2, Th17 differentiation [35, 66, 69]. This is elucidating new perspectives for the prevention or treatment of allergic diseases at a genomic level. Likewise HDAC inhibitors are currently under investigation in controlled clinical trails for the treatment of lymphoma or neuromuscular diseases [74, 75], while the usage of HAT inhibitors are still under consideration [65].

5.5. Impact of calcitriol on ε germline gene promoter activity

The findings obtained using a reporter gene assay confirmed the VDR-mediated action on Iε promoter. Due to the difficulties of transfection of human B cells and the fact that cyp27b1 transrepression involving similar transcription factors was confirmed in HEK cells, this approach was chosen here as well. The activity of the construct with a shorter Iε fragment including the four putative VDREs was significantly repressed in the same range as a chosen cyp27B1 region with previously confirmed negative VDREs upon calcitriol stimulation. Therefore, the data demonstrate that calcitriol does indeed represent a strong repressor in this case as well. These observations support the findings obtained by ChIP technology.

VDR binds usually directly to specific VDREs or through the VDR interacting repressor (VDIR) to the regulatory regions of its target genes [34, 35]. Therefore in case of primary targeted genes there must be at least one VDRE within the regulatory region of the gene [25]. The optimal VDRE halfsite is defined as a direct repeat of two six-base half motifs separated by a spacer of three nucleotides (DR3). It has been previously shown to mediate mostly positive, but also negative transcriptional gene regulation [25, 29, 33]. When DR3 sequences mediating negative regulation are examined, it is apparent that these display a considerable deviation from the optimal VDRE sequence [70]. Not surprisingly, the base differences occurring in the negative VDREs often do not correspond to those found in the positive ones. How the uniquely negatively acting bases influence VDR-heterodimers to mediate transrepression is not known. However, it was shown that negative VDRE in chicken parathormone gene can be converted into positive element by simple exchange of 2 bases [70]. This suggests that VDRE sequence alone can determine whether the bound VDR will activate or repress transcription. Interestingly, the one negative VDRE in the rat parathyroid hormone related peptide gene is identical to the positive element [70]. However, this gene has another typical negative VDRE, emphasizing that several VDREs can function together to exert the final effect. Moreover, binding of VDR have been

confirmed for many untypical sequences as well, or example in case of cyp24 [29]. Increasing evidence is accumulating that variable sequences are inducing unique conformations in the VDR heterodimer complex thereby promoting association with distinct subsets of co-regulators or permitting differential actions in terms of diverse tissues [70].

The promoter assay demonstrated that the construct containing VDREs found in the Iε region mediated transrepressive activity. However, the function of each single VDRE as well as the potential role of VDRI remains to be determined in further research.

5.6. Clinical implications

Vitamin D levels can have an impact in the development and maintenance of allergic disease through multiple mechanisms [23]. Induction of Treg cell-differentiation, impairment of Th17 cell-differentiation [22, 23, 39], and as shown here IgE inhibition are some of the beneficial effects of vitamin D in allergy. Other mechanism might involve improved response to respiratory tract infections, because vitamin D has been shown to induce the production of antimicrobial and antiviral proteins, such as cathelicidin and defensin [71].

There are different vitamin D-based treatment strategies [72]. Adequate sunlight exposure is the most inexpensive way to obtain vitamin D. Whole body exposure to UVB radiation or sunlight to provide a mild skin reddening provides the equivalent of 10,000 international units (IU) vitamin D3 [73]. A half the dose required to produce a slight reddening of the skin or UVB radiation to the arms and legs, which can be achieved in 5–10 minutes on a bright summer day in a fair skinned individual in Boston, has been calculated to be the equivalent of 3000 IU VD3 [36]. However, concerns regarding the association between sunlight and skin cancer and/or aging have limited this approach. The current recommendations in Germany (proscribed by the Die Deutsche Gesellschaft für Ernährung) are daily intake of 400 international units (IU) (10 µg) for infants during the first year of life and senior older then 65 years [74]. For the rest of age groups the recommendations are 200 IU (5 µg) Vitamin D3. It was discovered in the USA that the daily vitamin D supplementation (200 IU for children and young adults, 400 IU for adults 51–70 years old, and 600 IU for adults older than 71 years of age) is too low and do not maintain 25-hydroxy VD3 at the desired level for many individuals [36]. Several studies demonstrated that for every 100 IU vitamin D3 supplementation administered on a daily basis for 4 months, 25-hydroxy

VD3 levels rise by 0.5-1 ng/ml [73, 75]. Thus, to increase a patient's 25-hydroxy VD3 level from 20 ng/ml to 30 ng/ml, the supplementation would need to be 1000–2000 IU/day [72]. The lower limit of vitamin D supplementation required to prevent fractures and falls seems to be 700–800 IU. Unfortified food contains little vitamin D with the exception of wild salmon and other fish products such as cod liver oil. Toxicity due to vitamin D supplementation has not been observed at doses less than 10,000 IU/day, although such doses are seldom required except in situations in which vitamin D is poorly absorbed (malabsorption syndromes) [76]. Toxicity manifests as hypercalcemia and hypercalciuria, leading consequently to renal failure and neurologicsymptoms including coma. Because of individual variation of vitamin D production in the skin and in dietary intake, it is difficult to make a 'one size fits all' recommendation as to levels of oral supplementation [72]. The goal is to achieve serum levels of 25-hydroxy vitamin D3 that are optimal for the numerous vitamin D functions in the body.

Up to now several clinical studies involving vitamin D supplementation investigated its role in the development and manifestations of asthma, atopic dermatitis and rhinitis.

The protective effects of higher maternal dietary vitamin D intakes in pregnancy on wheezing phenotypes in young children were demonstrated in three separate cohorts [38, 67, 77]. Additionally, one study discovered benefits for patients suffering from allergic rhinitis as well. These studies are limited by the fact that vitamin D intake was calculated from food frequency questionnaires (thus, no direct measure of vitamin D status in the mothers) and may be confounded by diet quality. However, the results were adjusted for total energy intake and for other nutrients associated with healthy diets. Another study of the population of North American children with mild-to-moderate persistent asthma showed an association with higher odds of severe exacerbation over a 4-year period [71]. On the other hand, few randomized controlled trial using vitamin D and/or E supplementation revealed benefits for patients suffering from atopic dermatitis [77].

Recently vitamin D has been considered as an adjuvant in the allergen-specific immunotherapy (SIT) [78], the only causal therapy for allergic diseases up to now. Currently, IL-10 producing Treg cells are considered to be responsible for clinically successful SIT and the underlying immunological responses, e.g. increased IgG4 levels [3, 4]. The main rationale to use vitamin D as adjuvant is the previously reported induction of IL-10 production by T regs as well as activated B cell [24, 79]. Moreover, calcitriol has been shown to be effective as adjuvant in an

allergic mouse model of immunotherapy, potentiating the suppression of airway hypersensitivity, eosinophilic airway inflammation and serum IgE levels [80]. Majak et al. used, for the first time, pharmacological agents, glucorticosteroids (GCS) alone or in combination with VD3, as adjuvants for SIT [80, 81]. In a randomized, double-blind, placebo-controlled trial with children suffering from IgE-mediated asthma, they examined prednisone alone or in combination with VD3 as adjuvants for SIT. Surprisingly, the group that received house-dust mite SIT with GCS as adjuvant, showed significantly less clinical improvement compared with the SIT control group. On the other hand, VD3 seemed to neutralize this negative effect, as the group that received SIT with GCS/VD3 adjuvant displayed similar improvement as the SIT control group. It can be concluded that despite strong in vitro and in vivo evidence in favour of a putative adjuvant effect for GCS and VD3 in SIT, this first translational study did not observe beneficial effects using the combination of these adjuvants. Given all the limitations, further clinical studies are needed to determine whether these immunosuppressive drugs can be useful as adjuvant for SIT. One such large scale clinical trial “VITamin D and omega-3 TriaL” (VITAL) is on-going [82]. The study is funded by the National Institutes of Health and being run by Harvard Medical School and the Brigham and Women’s Hospital in Boston, MA. It will involve 20,000 men (older then 60 years) and women (older than 65 years) who would be supplemented with 2000 IU vitamin D and/or 1 g eicosapentaenoic acid. This study will test biological mechanisms and evidence based data. This study will be able to verify the biological mechanisms and evidence based data previously described on the role of vitamin D and omega-3 fatty acids in prevention of carcinoma, cardiovascular diseases as well as other chronic diseases, such as asthma.

5.7. Future perspectives

During the last years the knowledge on epigenetic modifications which can induce adaptive changes in gene expression and therefore determine the phenotype is rapidly growing [35]. There is strong evidence that early environmental exposure plays a key role in activating or silencing genes by altering DNA and histone methylation, histone acetylation and chromatin structure. These modifications determine the degree of DNA compaction and accessibility, altering gene expression, phenotype and disease susceptibility [34]. Epigenetic mechanisms provide new insights into how environmental changes may mediate the increasing predisposition for complex immune diseases such as allergic disease [35].

According to the current opinion many environmental risk factors (including reduced microbial exposure, dietary changes and environmental pollutants) as well as family predisposition are implicated in allergy, which is reaching epidemic proportions in the Western countries [35]. Co-occurring with these findings is the vitamin D deficiency which is very prominent in the Western countries [36]. Importantly, numerous studies revealed that vitamin D, has considerable impact on the immune system [22, 23]. The determination of serum vitamin D is crucial for interpretation of its effects. However, a major difficulty for clarifying its importance lays in the current definition of vitamin D deficiency, which is based on calcitriol-mediated actions on bone health. Current studies considering the immunomodulatory effects of vitamin D are revising the old definitions of vitamin D sufficiency and the recommendations for the vitamin D intake. Moreover, it would be of particular interest to determine whether the various actions of VDR have an optimal 25-hydroxy VD3 serum level. Emerging evidence is potentiating the importance of in utero exposure to 25-hydroxy VD3 for the foetal development [35]. Whether this is important for allergy and its long term effects remains to be determined.

Interestingly, only about a quarter of the interindividual variability in 25-hydroxy VD3 concentration is attributable to season of measurement, geographical latitude, or reported vitamin D intake [55]. Recently polymorphism in the genes encoding molecules involved in the vitamin D metabolism and signalling were associated with 25-hydroxy VD3 levels as well as asthma and total IgE [55, 59, 60]. These findings improve the understanding of vitamin D homoeostasis and could assist identification of a subgroup of the white population who are at risk of vitamin D insufficiency. However, the questions remain: should everyone have their 25-hydroxy VD3 levels measured and is vitamin D supplementation given orally equally effective as the vitamin D produced endogenously in the skin? Also the lingering topics, such as the in utero exposure to vitamin D, supplementation at the early age, prevention and/or treatment throughout the life have need to be further investigated.

This dissertation demonstrates that histone deacetlyation plays a role in the IgE production by calcitriol. The HAc, the epigenetic mechanism, has already been implicated in allergy [67]. For instance, bronchial biopsies from asthmatic patients show increased HAT activity and reduced HDAC activity [67]. Similar changes have been found in alveolar macrophages from asthmatic patients [67, 69]. Thus, the above mentioned together with here shown results shows the importance of acetylation for εGT and supports the initiatives for HAT inhibitor-based treatment.

It is important to mention that VDR is a broadly expressed transcription factor regulating several hundreds of genes [26]. Further studies are needed to identify each and every calcitriol-induced change in gene acetylation in the aetiological context of allergic disease. The challenge of ongoing research will be to identify precise networks of VDR regulated genes and to define to what extent are the calcitriol levels driving the susceptibility to allergy. The possibility to modify the epigenetic factors also provides opportunities for environmental strategies that can re-programme gene expression for disease prevention, such as dietary supplementation with 25-hydroxy VD3, especially since it represents a natural compound. This is of importance for reversing the epidemic rates of immune-mediated disorders.

6. CONCLUSION

Novel approaches to better understanding the development of allergic diseases are focusing on epigenetic modifications [35]. Changes of environmental factors leading to phenotype determination withhold a great potential for prevention and therapy of these diseases.

The ‘allergy epidemic’ and vitamin D deficiency are co-occurring during the last years [37, 38]. As vitamin D exerts potent immunomodulatory properties, its action in allergy is of great interest. This work contributes to understanding the actions of vitamin D in B cells as well as in the humoral immune response.

A detailed investigation of CSR, IgE production at the level of IgE secretion and plasma cell-differentiation implied that calcitriol is inhibiting the IgE production by interfering with the early events preceding the initiation of εGT. It is discovered here that ε germline promoter represents a primary target of VDR in human B cells, which in turn leads to its transpression. The calcitriol-mediated inhibition of the εGT relies initially on the recruitment of VDR:RXRα to the DNA, which engages SMRT co-repressor and consequently histone modifying enzymes. HDAC1 and HDAC3 are targeting the histones round the Iε region leading to deacetylation of the histone 3 and 4. Thus, by chromatin compacting changes the DNA accessibility for the transcription machinery.

The results demonstrate not only a mechanism of calcitriol-mediated inhibition of εGLT expression, but also highlight the importance of the acetylation state for IgE production in principal. Moreover, this work offers an explanation for VDR up-regulation in cells undergoing CSR [83]. Therefore, the maintenance of certain levels of sufficient 25-hydroxy VD3 may indeed have an autoregulatory role at the early stages of IgE production.

Taken together, this work shed new light on the mechanisms of gene repression by nuclear receptors in general and the role of epigenetic imprinting by VDR on IgE regulation in particular.

7. ABSTRACT

Recently an increased incidence of allergic diseases has been associated to vitamin D deficiency. It was previously demonstrated that calcitriol, the active form of vitamin D, inhibits epsilon germline transcription, a prerequisite for IgE production. However, the underlying mechanisms remained unexplored.

The aim of this work was to investigate the epsilon germline promoter regarding vitamin D receptor (VDR)-DNA-complex formation, changes in the chromatin acetylation and its impact on the epsilon germline transcription in human B cells.

The VDR binding to the epsilon germline promoter in human B cells, the composition of the VDR-recruited complex and the acetylation pattern were investigated by chromatin immunoprecipitation. The calcitriol mediated action on Iε were analysed using a reporter gene assay.

The data demonstrate that calcitriol-activated VDR binds together with retinoid X receptor α to the Iε. The heterodimer interacts with silencing mediator for retinoid and thyroid hormone receptors (SMRT), which is recruiting histone deacetylase (HDAC) 1 and HDAC3. The inhibition of SMRT or HDACs reversed the site specific deacetylation of histone 3 and 4 and the Iε transrepression driven by calcitriol. These VDR-complex transrepressive actions on Iε were confirmed in a reporter assay.

We show here that calcitriol-mediated inhibition of Iε involves the recruitment of VDR-heterodimers, which engage co-repressors and histone modifying enzymes. Our findings shed new light on mechanisms of VDR-transrepression in principal and understanding of IgE regulation in particular.

Key words: Vitamin D receptor, calcitriol, ε germline transcript

ZUSAMMENFASSUNG

Kürzlich wurde der Zusammenhang zwischen der gestiegenen Inzidenz allergischer Erkrankungen und Vitamin D-Defizienz beschrieben. Es wurde gezeigt, dass Calcitriol, als bioaktive Form von Vitamin D, die Epsilon-Keimbahntranskription, die Voraussetzung für den Klassenwechsel zu IgE, hemmt. Allerdings sind die zugrunde liegenden Mechanismen nicht aufgeklärt.

Zielsetzung dieser Arbeit war die Untersuchung des epsilon-Keimbahnpromotors hinsichtlich Vitamin-D-Rezeptor(VDR)-DNA-Komplexbildung, Modifikation der Chromatin-Acetylierung, sowie deren Einfluss auf die Epsilon-Keimbahntranskription in humanen B-Zellen.

Die Bindung des VDR an den epsilon-Keimbahnpromotor (Iε) in humanen B-Zellen, die Zusammensetzung des durch den VDR rekrutierten Komplexes sowie das Acetylierungsmuster wurden mittels Chromatin-Immunpräzipitation untersucht. Die Calcitriol-vermittelten Effekte am Iε wurden durch Reportergen-Untersuchungen analysiert.

Die Daten zeigen hier, dass der Calcitriol-aktivierte VDR gemeinsam mit dem Retinsäure-X-Rezeptor an Iε bindet. Die Interaktion dieses Heterodimers mit SMRT (engl.: silencing mediator for retinoid and thyroid hormone receptors) führt weiter zur Rekrutierung von Histon-Deacetylase (HDAC) 1 und HDAC3. Die Hemmung von SMRT und HDACs führt zur Umkehrung der Calcitriol-vermittelten spezifischen Deacetylierung von Histon 3 und 4 sowie der Transrepression von Iε. Die transrepressiven Effekte des VDR-Komplexes auf Iε wurden in Reportergen-Analysen bestätigt.

Es ist hier verdeutlicht, dass die Calcitriol-vermittelte Hemmung des epsilon-Keimbahntranskripts auf der Rekrutierung von VDR-Heterodimeren mit Einbindung von Co-Repressoren und Histon-modifizierenden Enzymen beruht. Diese Arbeit zeigt neue Aspekte der VDR-vermittelten Transrepression und Regulation des IgE-Klassenwechsels.

Schüsselwörte: Vitamin D Rezeptor, Calcitriol, Epsilon-Keimbahntranskript

8. APPENDIX

8.1. Abbreviations

Ab: antibody

Ag: antigen

AcH: acetylated histone

AID: activation induced (cytidin) desaminase

BSA: bovine serum albumin

ChIP: chromatin immunoprecipitation assay

CSR: class switch recombination

DC: dendritic cell

GCS: glucocorticoids

GT: germline gene transcription

GLT: epsilon germline gene transcript

HAT: histone acetylase

HDAC: histone deacetylase

HEK: human embyogenic kidney fibroblasts

Ig: Immunoglobulin

IU: international units

NCoR: nuclear receptor corepressor

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NR: nuclear receptor

qRT-PCR: quantitative real-time PCR

PBMCs: peripheral mononuclear cells

RAR: retinoic acid receptor

RXR: retinoid X receptor

SDS: sodium dodecyl sulphate

SNP: single nucleotide polymorphism

STAT6: Signal transducer and activator of transcription 6

SMRT: silencing mediator for retinoid and thyroid hormone receptors

TSS: transcription start

VDR: vitamin D receptor

8.2. Antibodies

Antibody	Species	Clone	Manufacturer
Anti-human CD23 FITC	mouse	EBVCS2	eBioscience, San Diego, USA
Anti-human CD27 PE	mouse	O323	eBioscience, San Diego, USA
Anti-human CD38 PE-Cy5	mouse	HIT2	eBioscience, San Diego, USA
Anti-human CD40	mouse	82111	R&D, Minneapolis, USA
Anti-human IgA	goat	G20-359	BD PharMingen, San Diego, USA
Anti-human IgG	goat	G18-145	BD PharMingen, San Diego, USA
Anti-human IgE	mouse	HP6061	Southern Biotech, Birmingham, USA
Anti-human IgE	mouse	HP6029	Southern Biotech, Birmingham, USA
Anti-human IgE FITC	mouse		DRFZ
Anti-human IgA, IgG, IgM	goat		Jackson ImmunoResearch, West Grove, USA
Anti-human AcH3	rabbit		Millipore, Billerica, MA, USA
Anti-human AcH4	rabbit		Millipore, Billerica, MA, USA
Anti-human HDAC1	rabbit	H-51	Santa Cruz, Heidelberg, Germany
Anti-human HDAC2	rabbit	H-54	Santa Cruz, Heidelberg, Germany
Anti-human HDAC3	rabbit	H-99	Santa Cruz, Heidelberg, Germany
Anti-human NCOR	rabbit		Affinity bioreagents, Rockford, IL, USA
Anti-human RARα	rabbit	C-20	Santa Cruz, Heidelberg, Germany
Anti-human RARβ	rabbit	C-19	Santa Cruz, Heidelberg, Germany
Anti-human RXRα	rabbit	D-20	Santa Cruz, Heidelberg, Germany
Anti-human RXRβ	rabbit	C-20	Santa Cruz, Heidelberg, Germany
Anti-human SMRT	rabbit		Santa Cruz, Heidelberg, Germany
Anti-human VDR	rat	9-7ad	Affinity bioreagents, Rockford, IL, USA
Anti-human VDR	rabbit	C-20	Santa Cruz, Heidelberg, Germany

8.3. Reagents

Reagents	Manufacturer
Anti-FITC Microbeads	Miltenyi Biotec, Bergisch-Gladbach, Germany
Anti-human CD19 Multisort Microbeads	Miltenyi Biotec, Bergisch-Gladbach, Germany
Arsenic(III)-oxide	Sigma-Aldrich, Steinheim, Germany
Apicidin	Calbiochem, Darmstadt, Germany
Litium-chloride	Sigma-Aldrich, Steinheim, Germany
MS-275	eBioscience, San Diego, USA
3-Amino-9-ethyl-carbazole	Sigma-Aldrich, Steinheim, Germany
Bacto Yeast Extract	Difco Laboratories, Detroit, USA
Beta mercapto ethanol	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumine	SERVA, Heidelberg, Germany
Brefeldin A	Sigma Aldrich, Steinheim, Germany
Calcium Ionophore	Calbiochem Merck, Darmstadt, Germany
Complete Protease Inhibitor	Roche, Mannheim, Germany
CytofixTM Buffer	BD PharMingen, San Diego, USA
ECL Plus Western Blot Detection Reagents	Amersham, Buckinghamshire, United Kingdom
Extra Avidine Peroxidase	Sigma-Aldrich, Steinheim, Germany
FastStart DNA Master SYBR® Green	Roche, Mannheim, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Ficoll, sterile, d = 1,077 g/mL	PAA, Pasching, Austria
Glucose	Sigma-Aldrich, Steinheim, Germany
L-Glutamine	Biochrom, Berlin, Germany
Glycine	SERVA, Heidelberg, Germany
Hydrochloric acid, HCl , 25%	Merck, Darmstadt, Germany
Hydrogen peroxide, H2O2, 30%	Merck, Darmstadt, Germany
Kaliumchloride, KCl	Merck, Darmstadt, Germany
Magnesiumchloride, $MgCl_2$	Merck, Darmstadt, Germany
Magnesiumsulfat, $MgSO_4$	Merck, Darmstadt, Germany
Milk powder (Blotting Grade)	Roth, Karlsruhe, Germany
Nucleospin RNA II Kit	Macherey-Nagel, Düren, Germany
Nucleospin Extract II Kit	Macherey-Nagel, Düren, Germany
NucleoleoSpin Plasmid Kit,	Macherey-Nagel, Düren, Germany
Nucleobond Ax Kit	Macherey-Nagel, Düren, Germany
Paraformaldehyde (PFA)	Sigma Aldrich, Steinheim, Germany
Pellet Paint™ Co	Precipitant Novagen, Madison, USA
Penicillin, 10.000 E	Biochrom, Berlin, Germany
Dulbecco´s Phosphate-Buffered Saline(PBS), without Ca2+/Mg2+, sterile	PAA, Pasching, Austria
Propidium iodide	Sigma Aldrich, Steinheim, Germany
Recombinant human IL-4	Immunotools, Friesoythe, Germany
RPMI 1640, without Ca2+/Mg2+, sterile	Biochrom, Berlin, Germany
Saponin	Sigma Aldrich, Steinheim, Germany

Sodiumchloride, NaCl	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, Steinheim, Germany
Sodium hydrogen carbonate, $NaHCO_3$	Merck, Darmstadt, GermanyParaformaldehyde
StreptavidinHorseradish Peroxidase	R&D, Minneapolis, USA
Streptavidin Alkaline Phosphatase	ZYMED, San Francisco, USA
Streptomycin	Biochrom, Berlin, Germany Germany
Sulphuric acid, H2SO4, 95-97%	Riedel de Haen, Seelze, Germany
T4 ligase kit	New England Biolabs, Frankfurt am Main, Germany
Tris (hydroxymethyl) aminomethane (Tris-Base)	Sigma Aldrich, Steinheim, Germany
Tris (hydroxymethyl) aminomethane Hydrochloride (Tris HCl)	Sigma Aldrich, Steinheim, Germany
Trypton	Merck, Darmstadt, Germany
Triton X-100	Merck, Darmstadt, Germany
Tween20	Bio-Rad, Munich, Germany

8.4. Equipment

Equipment	Model	Manufacturer
ELISA reader	Dynex MRX version 1.33	DYNATECH, Chantilly, USA
ELISPOT reader	CTL ImmunoSpot® S4 Analyzer	C.T.L Cellular Technology, Cleveland,USA
flow-cytometre	FACS Calibur	Becton Dickinson, Heidelberg, Germany
Light Cycler	LightCycler 1.5	Roche, Mannheim, Germany
SONOPULS	HD 2070	Bandelin, Berlin, Germany
Lumat	LB9501	Berthold

8.5. Software

Software	Manufacturer
ImmunoSpot v4.0.13 C.T.L	Cellular Technology, Cleveland, USA
Light Cycler Software Version 3	Roche, Mannheim, Germany
Primer 3	SourceForge, Mountain View, USA
Revelation G3.2	DYNEX, Berlin, Germany
GraphPad Prism	GraphPad Software, Inc, La Jolla, CA, USA
Genomatix	Genomatix Software GmbH, München, Germany
Gene-regulation, Patch	BIOBASE GmbH, Wolfenbuettel Germany

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11. CURRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

12. LIST OF PUBLICATIONS

1. **Milovanovic M,** Heine G, Zuberbier T, Worm M. Allergen extract-induced interleukin-10 in human memory B cells inhibits immunoglobulin E production. Clin Exp Allergy. 2009 May;39(5):671-8.

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13. ERKLÄRUNG

„Ich, Milena Milovanovic, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Characterization of the vitamin D receptor complex at the epsilon germline promoter selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift Milena Milovanovic