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1,25D3 Prevents CD8+Tc2 Skewing and Asthma Development Through VDR Binding Changes to the *Cyp11a1* Promoter

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Abstract

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MS performed statistical analyses and revised the manuscript
JJL analyzed the data and revised the manuscript.
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Effector CD8$^+$ T cells convert from IFN-γ$^+$ (Tc1) to IL-13$^+$ (Tc2) cells in the presence of IL-4. Underlying regulatory mechanisms are not fully defined. Here, we show that addition of 1,25D3, the active form of Vitamin D3, during CD8$^+$ T cell differentiation prevents IL-4-induced conversion to IL-13-producers. Transfer of 1,25D3-treated CD8$^+$ T cells into sensitized and challenged CD8$^+$-deficient recipients fails to restore development of lung allergic responses. 1,25D3 alters Vitamin D receptor (VDR) recruitment to the *Cyp11a1* promoter *in vitro* and *in vivo* in the presence of IL-4. As a result, protein levels and enzymatic activity of CYP11A1, a steroidogenic enzyme regulating CD8$^+$ T-cell conversion, are decreased. An epistatic effect between *CYP11A1* and *VDR* polymorphisms may contribute to the predisposition of childhood asthma. These data identify a role for 1,25D3 in the molecular programming of CD8$^+$ T-cell conversion to an IL-13-secreting phenotype through regulation of steroidogenesis, potentially governing asthma susceptibility.

For a large percentage of asthmatics, inhaled corticosteroids (ICS) are the most effective first-line treatment to control airway inflammation and symptoms in persistent asthma, but an estimated 40% of asthmatics who fail to respond to corticosteroid show no improvement in airway function[1]. Hence, steroid-refractory asthma remains a clinical challenge. We and others have demonstrated an important role for type 2 (Tc2) CD8$^+$ T cells in the development of experimental asthma[2–9] as a result of their activation by IL-4-producing CD4$^+$ T cells[10]. In humans, increased numbers of CD8$^+$ T cells, which are more resistant than CD4$^+$ T cells to corticosteroids[11], have been detected in steroid-refractory asthmatics[12] and correlated with lower lung function and reticular basement membrane thickening[13].

Over the last decade, deficiency in Vitamin D, a member of the steroid family, has been associated with various inflammatory diseases[14–17] including steroid-refractory asthma[18, 19]. An association between lower levels of Vitamin D and increased asthma severity, reduced lung function, and poor asthma control has been suggested[19–25]. However, it is unclear if Vitamin D supplementation impacts the disease as seen in a recent trial in asthmatics[26] but a potential mechanism of action remains unknown.

Previously, we identified CYP11A1 as an essential component of a novel, pro-allergic mechanistic axis in the development of experimental asthma (CD8$^+$ T cells)[4, 27] and peanut-induced allergy (CD4$^+$ T cells)[28]. CYP11A1, a mitochondrial P450 cytochrome, is the first and rate-limiting enzyme in steroidogenesis converting cholesterol to pregnenolone[29]. In the presence of IL-4, CYP11A1 was identified as a critical regulator of CD8$^+$ T cell conversion. Together with antigen receptor signaling of differentiated CD8$^+$ T cells, CYP11A1 activation was essential for increased IL-13 and decreased IFN-γ production[4, 27]. These data linked for the first time steroidogenesis in CD8$^+$ T cells, a non-classical steroidogenic tissue, to a pro-allergic differentiation pathway.

In this study, we demonstrate the role of 1,25D3 as a key modulator of the functional conversion of CD8$^+$ T cells from IFN-γ- to IL-13-producing cells via a mechanistic link to CYP11A1 activity. This effect appears driven by 1,25D3-mediated changes in the recruitment of the VDR transcription factor to the promoter region of *Cyp11a1* paralleled by changes in the enzymatic activation of CYP11A1 and the prevention of lung allergic

responses. An epistasic effect between genetic variants in *CYP11A1* and *VDR* is implicated in humans due to protective effects on the development of asthma.

RESULTS

1,25D3 prevents conversion to IL-13-producing CD8$^+$ T cells

We previously demonstrated that in the presence of IL-4, CD8$^+$ T cells convert from IFN-γ CD8$^+$ effector T cells to pathogenic IL-13-producers, triggering the full spectrum of lung allergic responses[4, 27]. To investigate the effects of Vitamin D on this functional conversion of CD8$^+$ T cells, the active form of Vitamin D, 1,25$(OH)_2D_3$ (further referred to as 1,25D3, 100 nM, 1 μM), is added during cell differentiation. 1,25D3 has no significant effect on cell viability (Supplementary Fig. 1). When CD8$^+$ T cells are cultured with SIINFEKL and IL-2+IL-4 in the presence of 1,25D3 a dose-dependent decrease in the percentage of IL-13$^+$ cells and an increase in IFN-γ$^+$ cells is observed (Fig. 1). After adding 100 nM 1,25D3, IL-13-single-positive cells decrease from 23.8±9.3% (mean±SEM) to 11.3±4.8% whereas IFN-γ-single-positive cells increase from 16.8±5.6% to 24.5±4.8% (Fig. 1, Supplementary Table 1). This effect is even more pronounced after culture with 1 μM 1,25D3 (Fig. 1, Supplementary Table 1).

When 1,25D3 is added during the antigen (SIINFEKL) re-stimulation phase in the last 4 hours of culture, the cytokine profiles of differentiated CD8$^+$ T cells generated in the presence of IL-2+IL-4 and 100 nM or 1 μM of the drug are unaffected (Supplementary Fig. 2a,b). These results suggest a significant role for 1,25D3 only during the conversion of CD8$^+$ T cells in an IL-4-rich environment but not on differentiated cells.

1,25D3 alters functional activity of CYP11A1 in CD8$^+$ T cells

The major transcription factors, *Tbx21* and *Gata3*, regulate expression of IFN-γ and IL-13 in T cells[30]. During the differentiation of CD8$^+$ T cells with IL-2+IL-4, we observe decreased *Tbx21* mRNA levels while *Gata3* gene expression is increased compared to cells differentiated in IL-2 alone (Fig. 2a,b).

We previously demonstrated that the enzymatic activation of CYP11A1 plays a key role in the phenotypic conversion of CD8$^+$ T cells from IFN-γ- to IL-13-producing cells[4, 27]. A similar mechanism during the stages of CD8$^+$ T cell differentiation in the presence of 1,25D3 is postulated. CYP11A1 transcript (Fig. 2c) and protein (Fig. 2e, Supplementary Fig. 3) levels are decreased in the presence of 1,25D3 (100 nM or 1 μM) during the differentiation of CD8$^+$ T cells in IL-2+IL-4 to a greater extent than the lineage-specific transcription factors *Gata3* and *Tbx21*. In parallel, decreased levels of pregnenolone are detected in supernatants from cells cultured in the presence of 1,25D3 (Fig. 2f). These data strengthen the notion that in the presence of 1,25D3, CYP11A1 may be a functional regulator of the conversion of CD8$^+$ T cells from IFN-γ- to IL-13-producing cells. Of note, in CD8$^+$ T cells differentiated in IL-2+IL-4, *Vdr* gene expression is significantly enhanced while additionally adding 1,25D3 during the differentiation process reveals a trend towards lower *Vdr* levels (Fig. 2d).

1,25D3 alters VDR binding to the *Cyp11a1* promoter

1,25D3 primarily mediates signals in the cell through the transcription factor Vitamin D receptor (VDR), which regulates the transcriptional activity of many target genes[31, 32]. *In silico* transcription factor binding analyses of the *Cyp11a1* promoter region (5 kb) predict seven potential VDR binding sites (Supplementary Fig. 4). To define the molecular mechanism underlying the 1,25D3-mediated prevention of the conversion of $CD8^+$ T cells driven by CYP11A1, we evaluate recruitment of VDR to the *Cyp11a1* promoter region via chromatin immunoprecipitation (ChIP). In $CD8^+$ T cells cultured in IL-2 or IL-2+IL-4 in the absence or presence of 1,25D3 (100 nM or 1 μM), the relative abundance of *Cyp11a1* promoter DNA immunoprecipitated by a VDR-specific antibody is determined by qPCR using five *Cyp11a1*-specific primer pairs (Supplementary Fig. 4, Supplementary Table 5). Due to the close vicinity of some of the predicted VDR binding sites, primers cover multiple potential VDR binding sites (e.g., primer pair 4 and 5, Supplementary Fig. 4). A representative experiment of the percent input immunoprecipitated by the VDR and the negative control IgG antibody is presented in the Supplementary Fig. 5a–e. Considering the complexity of the nuclear environment in primary $CD8^+$ T cells, normalization between three independent experiments is performed via the percent input methodology and the relative percent input ratios are displayed using $CD8^+$ T cells stimulated with IL-2 as baseline (see more details in Materials and Methods). The data show that in the presence of IL-4 (black bars, Fig. 3b), the recruitment of VDR to the *Cyp11a1* promoter region is significantly reduced for all VDR binding sites in comparison to $CD8^+$ T cells stimulated with IL-2 alone (white bars, Fig. 3b). Intriguingly, $CD8^+$ T cells cultured in IL-2+IL-4 together with 1,25D3 (1 μM) increase the association of VDR to *Cyp11a1,* almost to levels seen in IL-2-differentiated $CD8^+$ T cells at baseline. In contrast, 1,25D3 has almost no effect on the VDR recruitment in $CD8^+$ T cells after IL-2 stimulation alone. These results suggest that VDR binding to the *Cyp11a1* promoter acts as a transcriptional repressor since the reduction in VDR recruitment to its promoter leads to increased CYP11A1 gene and protein expression (Fig. 2c,e) and elevated pregnenolone levels (Fig. 2f).

1,25D3-treated $CD8^+$ T cells prevent lung allergic responses

Since 1,25D3 prevents the IL-4-induced conversion of $CD8^+$ T cells from IFN-γ to IL-13 production *in vitro*, we examine if 1,25D3 treatment of $CD8^+$ T cells attenuates restoration of lung allergic responses following adoptive transfer *in vivo* into CD8-deficient mice. Adoptive transfer of IL-2-differentiated $CD8^+$ T cells into sensitized and challenged CD8-deficient recipients followed by secondary allergen challenge fully restores lung allergic responses (Fig. 4a,b). In contrast, transfer of IL-2-differentiated $CD8^+$ T cells cultured in the presence of 1,25D3 (100 nM or 1 μM) fails to restore airway hyperresponsiveness (AHR) (Fig. 4a) or airway inflammation (Fig. 4b). Levels of IL-4, IL-5, and IL-13 are significantly lower in the bronchalveolar lavage (BAL) fluid of these recipient mice compared to recipients of untreated cells (Fig. 5a–c). Lung sections confirm that these recipients show less inflammation and significantly decreased numbers of PAS^+ mucus-containing goblet cells (Fig. 6a–e).

To confirm that the observed effects are dependent on the binding of VDR to the *Cyp11a1* promoter region, adoptively-transferred $CD8^+$ T cells are isolated from the lungs of OVA-

sensitized mice after allergen challenge followed by ChIP experiments. As seen above in the *in vitro* experiments, isolated 1,25D3 treated $CD8^+$ T cells from the lungs of sensitized and challenged recipients show increased recruitment of VDR to the *Cyp11a1* promoter region in comparison to recipients of untreated $CD8^+$ T cells differentiated in IL-2 alone for four out of the five VDR binding sites under study (Fig. 7). These *in vivo* results together with the *in vitro* findings support the hypothesis that CYP11A1 plays a key role in both preventing the conversion of $CD8^+$ T cells from IFN-γ- to IL-13-producers and the failure to induce AHR is the result of 1,25D3-triggered VDR binding to the *Cyp11a1* promoter region.

Genetic asthma predisposition of *CYP11A1* in children

Given the importance of CYP11A1 in the regulation of experimental asthma in mice, we determine if a predisposition to asthma in humans is influenced by *CYP11A1* single nucleotide polymorphisms (SNP). The 25 polymorphisms in *CYP11A1* cluster in 4 tagging bins and 4 single SNPs are identified (Supplementary Fig. 6 and Supplementary Table 2). A significant protective effect in asthma is observed for three (rs4886595, rs4432229, rs11632698) out of eight tested SNPs in the case-control population with an odds ratio (OR) ranging from 0.77 to 0.85 (Table 1). Association results for SNP rs4886595 remains significant after correction for multiple testing (Table 1). Seven out of the eight polymorphisms covered by the asthma-associated tagging SNPs are located in potential regulatory regions of *CYP11A1* (promoter: N=6, introns: N=1). Allele-specific *in silico* analyses predict changes in transcription factor binding for all of these SNPs (Supplementary Table 2). Computed score-based analyses of multiple high-throughput datasets (RegulomeDB Database, Supplementary Table 3) suggest a putative functional differentiation protocol regulatory role of some of the asthma-associated *CYP11A1* SNPs. Interestingly, for polymorphism rs8039957 (tagging bin 1, covered by rs4432229) located in the *CYP11A1* promoter region, a novel VDR binding site is predicted in the presence of the polymorphic allele. Three *CYP11A1* SNPs within this block and rs4886595 reveal allele-specific alterations on *CYP11A1* gene expression (Supplementary Table 3).

Because of these findings and the observed transcriptional regulation of *Cyp11a1* by VDR in mice, a combined analysis of SNPs in *CYP11A1* and *VDR* is performed to further determine if an epistatic phenomenon, here defined as the effect of one locus being dependent on the genotype of a second locus, may be involved in the development of childhood asthma. The most significant asthma-associated SNPs in our population for *VDR* rs2107301[33] and *CYP11A1* rs4886595 are selected for the analyses. Additionally, *CYP11A1* SNP rs4432229 is included, since rs8039957 which is within the same tagging bin, is predicted to affect allele-specific binding of VDR to the *CYP11A1* promoter region. Epistasis is observed when the effects of the respective protective alleles in *CYP11A1* or *VDR* are studied after stratification for individuals carrying the corresponding SNP located in the other gene (Table 2). Both *CYP11A1* SNPs, rs4886595 and rs4432229, are significantly associated with asthma only in individuals carrying homozygous asthma-risk alleles of rs2107301 (*VDR*). Thus, *CYP11A1* polymorphisms may alter the pro-allergic function of CYP11A1 specifically in combination with modifier SNPs in *VDR*.

DISCUSSION

Although cluster designations have identified some of the clinical features in asthmatics, actual definition of underlying pathobiology has lagged behind, thus limiting targeted therapy. To date, only one endotype has been well-characterized, a T_H2-high-signature associated with $CD4^+$ T cells, IL-13, and steroid-responsiveness. There has been accumulating evidence for the role of $CD8^+$ T cells in asthma, especially in steroid-refractory asthma[2–9, 11–13, 34]. We have shown *in vitro* and *in vivo* that in the presence of IL-4, mouse $CD8^+$ T cells can convert from IFN-γ-producers to a significant source of IL-13[4, 27]. This conversion was associated with changes in lineage-specific transcription factor signatures and histone modifications at key loci. In the terminal stage of differentiation to IL-13 production, the enzymatic activation of CYP11A1 was shown to play an essential role[4, 27]. Since Vitamin D deficiency has been implicated in refractory asthma, although without a defined molecular mechanism, we examined if 1,25D3 modulated the conversion of $IL\text{-}13^+CD8^+$ T cells, a pathway shown to play a role in asthma[4, 27]. We now demonstrate that differentiation of $CD8^+$ T cells in the presence of 1,25D3 prevented the IL-4-mediated skewing of $CD8^+$ T cells to IL-13-producing Tc2 cells. As a consequence, adoptively-transferred $CD8^+$ T cells, differentiated in the presence of 1,25D3, failed to induce lung allergic responses in sensitized and challenged CD8-deficient recipients. This failure appeared linked to 1,25D3 induced changes in the binding of the VDR transcription factor to the *Cyp11a1* promoter region. In humans, the data supported an epistatic effect between *CYP11A1* and *VDR* polymorphisms, diminishing the risk to develop childhood asthma.

Vitamin D has previously been shown to play a critical role in modulating effects of $CD4^+$ T cell subsets[35–37] and even enhanced the therapeutic response(s) to corticosteroids[38]. However, little mechanistic data are available on the impact of 1,25D3 on $CD8^+$ T cells. When 1,25D3 was added during $CD8^+$ T cell differentiation, the IFN-γ-producing capacity of Tc1 cells differentiated in IL-2 alone was not affected. However, the cytokine profile in $CD8^+$ T cells differentiated in IL-2+IL-4 was significantly modified, characterized by increased IFN-γ and decreased IL-13 production. Our findings suggested that the activity of 1,25D3 was dependent on the exposure to IL-4 during $CD8^+$ T cell skewing, which can mediate the epigenetic poising of $CD8^+$ T cells at loci sensitive to VDR activity. Thus, it is possible that fully differentiated $CD8^+$ Tc2 cells are insensitive to 1,25D3 due to previous changes in chromatin structure at critical loci, now insensitive to VDR.

In light of the immunomodulatory activities associated with Vitamin D[14, 39, 40], there has been increased interest in determining its role in asthma. However, results of clinical trials using Vitamin D as a therapeutic supplement[19–22, 24, 26, 41–43] or during pregnancy to attenuate development of atopic disease in progeny[44–49] have been conflicting. Our findings suggest that Vitamin D supplementation may exhibit preventive activities rather than increase the therapeutic potential during an asthma exacerbation as the molecular mechanisms implicated in responsive cells at that stage may have already been epigenetically poised; however, by modifying transcription of key pro-allergic transcription factor expression an early stage may attenuate disease progression. Further, Vitamin D as an

interventional strategy may be restricted to a well-defined subpopulation of steroid-refractory asthmatics with increased numbers of $CD8^+IL\text{-}13^+$ T cells (Tc2 cells).

During differentiation of $CD8^+$ T cells in the presence of IL-4 and 1,25D3, lineage-specific markers (e.g., *Gata3* and *Tbx21*) were only marginally altered whereas CYP11A1 gene and protein expression were significantly affected in a dose-dependent manner. Changes at a transcriptomic level appeared weaker, likely the result of analysis on day 4 of the $CD8^+$ T cell differentiation protocol. Importantly, the enzymatic activity of CYP11A1, as measured by generation of pregnenolone was markedly reduced. Together with TCR engagement, the relevance of this essential factor in the conversion of $CD8^+$ T cells to IL-13-producers was augmented. 1,25D3 was only effective when included during $CD8^+$ differentiation and was without effect when added during the terminal differentiation stage.

Adoptive transfer of 1,25D3 treated $CD8^+$ T cells into sensitized and challenged CD8-deficient mice failed to induce lung allergic responses. Similar inhibitory effects on conversion were observed following inhibition of the enzymatic activity of CYP11A1 (with aminoglutethimide (AMG)) or silencing of the gene[4] as both were associated with the failure to restore lung allergic responses in sensitized and challenged CD8-deficient mice. These results indicated that 1,25D3, similar to AMG, acts upstream of CYP11A1. The effects on mouse $CD8^+$ T cells required 100 nM to 1μM concentrations of 1,25D3, concentration (500 nM calcitriol) previously demonstrated *in vitro* to be effective in $CD4^+$ T cells from steroid-resistant asthmatics[50]. These results suggest that *in vitro*, higher concentrations of 1,25D3 are needed in order to mimic potential *in vivo* effects.

Little is known about molecular mechanisms mediated by 1,25D3 in $CD8^+$ T cells, specifically in the conversion to IL-13 production and contributions to asthma pathogenesis. The active form of Vitamin D primarily transmits a signal through the transcription factor VDR, a member of the nuclear hormone receptor family[51]. By performing ChIP experiments with a VDR-specific antibody, we showed that VDR potentially acts as a transcriptional repressor of CYP11A1; in IL-4-activated $CD8^+$ T cells, the recruitment of VDR to *Cyp11a1* was decreased leading to higher CYP11A1 expression, whereas in $CD8^+$ cells cultured in IL-2 alone, more VDR binding paralleled by lower CYP11A1 expression was detected. A similar effect on IL-12B expression has previously been reported in lipopolysaccharide (LPS)-treated human monocytes (THP-1)[52]. Vitamin D induced alterations in the recruitment of VDR/RXR, the co-repressor NCOR2/SMRT, and histone deacetylase 3 paralleled by decreased histone 4 acetylation (H4ac), and increased histone 3 trimethylation (H3K27me3), potentially causing the downregulation of IL-12B. In the $CD8^+$ T cell differentiation model investigated here, we propose that 1,25D3-induced recruitment of VDR to the *Cyp11a1* promoter region results in lower CYP11A1 enzymatic activity, which in turn, is associated with attenuation of the IL-4-driven conversion of $CD8^+$ T cells to IL-13 production. Examination of adoptively-transferred $CD8^+$ T cells recovered from the lungs of sensitized and challenged recipient mice recapitulated our *in vitro* findings, as shown by the increased recruitment of VDR to the *Cyp11a1* promoter region in recovered cells from recipients of 1,25D3-treated cells. These results support the hypothesis that CYP11A1 is an important regulator of the conversion of $CD8^+$ T cells and accounts for the failure of transferred 1,25D3-treated cells to restore lung allergic responses.

To determine if these findings in mice are relevant to asthma, we investigated if *CYP11A1* is involved in the susceptibility of asthma in childhood. We observed three *CYP11A1* tagging SNPs that decreased the risk for asthma in a case-control population. Even though, the effect did not reach genome-wide statistical significance, our previous findings in experimental asthma supported the relevance of CYP11A1. Most of the *CYP11A1* polymorphisms covered by the asthma-associated tagging SNPs were located in regulatory regions of *CYP11A1*, indicating a potential allele-specific function. Depending on the genotype of rs8039957, located in the *CYP11A1* promoter region, a change in VDR binding was predicted. Hence, a similar regulatory mechanism of *CYP11A1* through VDR as observed in mice may be relevant to human disease. An interaction between asthma-associated SNPs in *CYP11A1* and *VDR* supported the relevance of *CYP11A1* and *VDR* predisposing to childhood asthma. However, genetic variants in VDR have been studied extensively in the context of asthma with conflicting results[31, 33, 53–54]. In order to validate our findings linking asthma susceptibility to *CYP11A1* and *VDR* SNPs, the association needs to be confirmed in an independent cohort and functional studies are needed to delineate the transcriptional regulation of *CYP11A1* through VDR in humans.

In summary, the data established for the first time a mechanistic role for Vitamin D in regulating the IL-4-mediated conversion of $CD8^+$ T cells to IL-13-producing pathogenic effector cells. This positions a novel role for 1,25D3 as a critical regulator of the development of lung allergic responses through a unique VDR-CYP11A1-IL-13 pathway in steroid-insensitive $CD8^+$ T cells. As a result, Vitamin D may be beneficial in asthmatics, but restricted to a subpopulation of asthmatics characterized by the presence of these $CD8^+$ T cells. Translating findings from the mouse model of experimental asthma, we identified similar mechanistic links which contribute to the susceptibility to childhood asthma. Further understanding of the molecular mechanisms by which 1,25D3, CYP11A1, and VDR interact to regulate the plasticity and/or stable conversion of $CD8^+$ T cells may provide new targets and novel therapeutic strategies in asthma.

METHODS

Animals

Pathogen-free, 8- to 10-week-old female OT-1 mice expressing a transgenic T cell receptor (TCR) specific for SIINFEKL peptide (ovalbumin ($OVA_{257-264}$)[4, 27] and homozygous CD8-deficient mice[6, 56] were bred in the animal facility at National Jewish Health (Denver, CO). Studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

$CD8^+$ T cell culture

$CD8^+$ effector memory T cells were generated *in vitro* as previously described[4, 6, 27, 56]. In brief, mononuclear cells (MNCs) were isolated from the spleens of OT-1 mice followed by stimulation with 1 μg/ml SIINFEKL peptide for 1.5 hours. Two days later, living cells were isolated using histopaque (Sigma-Aldrich, St. Louis, MO) and cultured in complete RPMI 1640 medium that contained recombinant mouse IL-2 (20 ng/ml, R&D, Minneapolis, MN) or IL-2+IL-4 (20 ng/ml, Peprotech, Rocky Hill, NJ). For some experiments, the active form

of Vitamin D3, 1α,25$(OH)_2D_3$ (referred to as 1,25D3, Sigma-Aldrich, St. Louis, MO) at a concentration of 100 nM or 1 μM was additionally added. Medium with cytokines was changed every day on 4 consecutive days. Cells were then re-stimulated with 1 μg/ml SIINFEKL in medium containing 2 μM monensin (Calbiochem, La Jolla, CA) for 4 hours. In some experiments, 100 nM or 1 μM 1,25D3 was additionally added to the medium during the antigen re-stimulation stage.

RNA preparation and qPCR

Total RNA was extracted from 5×10^6 differentiated $CD8^+$ T cells using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) isolation kit following the manufacture's protocol. Total RNA (1 μg) was converted into cDNA using QuantiTect reverse transcription kit (Qiagen, Valencia, CA). Specific primers and probes for qPCR of *Gata3*, *Tbx21*, *Cyp11a1*, *Vdr* and the housekeeping gene *18SrRNA* were designed with Vector NTI advance10 (Life Technologies, Carlsbad, CA) and for *Tbx21* a pre-designed assay (Mm00450960_m1, LifeTechnologies, Carlsbad, CA) was used (Supplementary Table 4). The determined cycle threshold (Ct) reflects the number of PCR cycles required for the fluorescence signal to exceed the detection threshold, which was set to the log-linear range of the amplification curve. The differences in Ct values of the gene of interest and the house keeping gene *18SrRNA* were used to calculate delta Ct (ΔCt). Relative fold changes (RFC) were then calculated using the $2^{-\Delta\Delta Ct}$ algorithm.

ELISA for pregnenolone measurements

Supernatants of differentiated $CD8^+$ T cells were collected after culturing 5×10^6/ml cells in 6-well plates for 24 hours with IL-2 or IL-2+IL-4 in the absence or presence of 1,25D3 (100 nM or 1 μM). Pregnenolone levels were measured using the pregnenolone ELISA kit (ALPCO Diagnostics, Salem, NH) following the manufactures protocol [6].

Immunoblot analyses

$CD8^+$ T cells (5×10^6) were lysed with RIPA buffer containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) on ice for 30 minutes[4, 27]. Samples were run by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked using buffer containing 2% BSA and 0.5% sodium azide in TBST for 1 hour and incubated with rabbit polyclonal CYP11A1 antibody (Lifespan Biosciences, Seattle, WA) overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Hertfordshire, UK) was used to detect CYP11A1 protein. Mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used as an internal control. Immunoreactive bands were quantified by densitometric quantification of autoradiographs using Image J (NIMH, Bethesda, MD), and expressed as relative CYP11A1 normalized to β-actin (Sigma-Aldrich, St. Louis, MO).

Chromatin immunoprecipitation

ChIP assays of cultured $CD8^+$ T cells (5×10^6) with IL-2 or IL-2+IL-4 in the presence or absence of 100 nM or 1 μM 1,25D3 were performed according to manufacturer protocols using the ChIP IT express kit (Active Motif, Carlsbad, CA). Briefly, chromatin was cross-

linked for 7 minutes at room temperature by adding 1% methanol-free formaldehyde (Thermo Fisher, Waltham, MA). Crosslinking was stopped by the addition of 1× glycine-stop fix solution at room temperature. Following incubation of cells for 30 minutes at 4°C, cell nuclei were pelleted and resuspended in 130 μl shearing buffer containing protease inhibitors. Shearing was conducted using an optimized 23-cycle treatment with a focused energy isothermal sonicator (Covaris S2 sonicator, Woburn, MA) leading to an average size of 300–500 base pairs, with highest density at 500 bp. Supernatant (10 μl) was saved for use as total input DNA.

Immunoprecipitations were performed according to manufacturer protocols with protein G magnetic beads overnight at 4°C with an estimated 2 μg sheared chromatin. Equal amounts of chromatin were used for the immunoprecipitation with the VDR-specific antibody (ab3508, Abcam, Cambridge, MA). After washing and eluting the beads with respective kit buffers, reverse-crosslinking was performed for 15 minutes at 95°C followed by a proteinase K treatment (10 μg/ml) for 1 hour at 37°C. The immunoprecipitated genomic DNA was purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) and analyzed via SYBR green (SYBR green Select Mastermix, Life Technologies, Carlsbad, CA) qPCR.

In silico transcription factor binding analyses were performed using MatInspector (Genomatrix, Munich, Germany) and Alibaba2.2 (http://www.gene-regulation.com) to identify putative *VDR* binding sites in the *Cyp11a1* promoter region. Respectively, *Cyp11a1* promoter-specific primers covering *VDR* binding sites were designed using the VectorNTI 11 software (Supplementary Table 5). qPCR data were analyzed via the percent input methodology: (2^ (CT of total input – CT of specific IP)) ×100 and relative fold change to percent input ratios using $CD8^+$ T cells stimulated with IL-2 as baseline.

Flow cytometry analyses

For intracellular staining, 1×10^6/ml cells were washed twice with PBS containing 1% BSA, stimulated with 1 μg/ml SIINFEKL in the presence of 2 μM monensin at 37°C for 4 hours. After fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and permeabilization with 0.1% saponin (Sigma-Aldrich, St. Louis, MO), cells were washed twice with PBS containing 1% BSA, then incubated with anti-mouse CD16/CD32 (0.5 mg/ml, 2 μl, 2.4G2, BD Bioscience, San Jose, CA) at 4°C for 5 minutes, then stained with FITC labeled anti-mouse IFN-γ (0.5 mg/ml, 5 μl, XMG 1.2, eBioscience, San Diego, CA) and PE-labeled anti-mouse IL-13 (0.5 mg/ml, 5 μl, eBio13A, eBioscience, San Diego, CA). Cell staining was monitored on a FACSCalibur (BD Bioscience, San Jose, CA) and analyzed using Flowjo software (Tree Star, Inc, Ashland, OR)[4, 6, 27, 56].

Secondary allergen challenge model and adoptive transfer

The experimental protocol for sensitization and challenge to OVA was performed as described previously[4, 6, 27, 56] with some modifications. CD8-deficient mice were sensitized with 20 μg of OVA (Calbiochem, La Jolla, CA) emulsified in 2.25 mg of alum (AlumImuject; Pierce, Rockford, IL) on days 0 and 14 by intraperitoneal injection. Sensitized mice were challenged with 0.2% OVA for 20 minutes on days 28, 29, and 30 using an ultrasonic nebulizer (model NE-U07; Omron Healthcare, Kyoto, JP). To address

the effect of 1,25D3 on $CD8^+$ T cell-mediated AHR, $CD8^+$ T cells (5×10^6) generated in medium containing IL-2 without or with 1,25D3 (100 nM or 1 μM) were injected into naive or OVA-sensitized CD8-deficient mice intravenously on day 44. Two hours after cell transfer, naive and sensitized mice were re-challenged (secondary) with 1% OVA for 20 minutes by nebulization. Airway function was measured and samples were collected 48 hours after the secondary challenge.

For some ChIP experiments, $CD8^+$ T cells cultured in the presence of IL-2 without or with 1 μM 1,25D3 and adoptively transferred into sensitized and challenged recipients were isolated from the lungs. T cells were isolated from the lung after lung digestion[27, 57] with magnetic-activated cell sorting beads by positive selection using $CD8^+$(Ly-2) microbeads (Miltenyi Biotec, San Diego, CA) providing 99% $CD8^+$ T cells[27, 57].

Assessment of airway function

Airway function was assessed as described previously[6, 56] by measuring changes in airway resistance (RL) in response to increasing doses of inhaled methacholine (MCh, Sigma-Aldrich, St. Louis, MO). Data were presented as percentage change from the baseline RL values after saline inhalation. Baseline RL values were not significantly different among the various groups.

Bronchoalveolar lavage fluid analyses

After measurement of AHR, lungs were lavaged via the tracheal tube with 1 ml of HBSS. The supernatants were collected and IL-4, IL-5, and IL-13 (eBiosicence, San Diego, CA) levels were measured by ELISA following the manufactures protocols[6, 56]. The limits of detection for IL-4, IL-5, and IL-13 are 4 pg/ml. Cytospin slides of leukocytes were stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures in a blinded fashion [6, 56].

Lung histology

Lungs were fixed in 10% formalin, and then embedded in paraffin. Paraffin sections (5 μm thick) were stained with periodic acid-Schiff (PAS). Mucus-containing goblet cells were quantified. Histology analysis was done in a blinded manner under light microscopy linked to an image system. The number of PAS-positive goblet cells was determined in cross-sectional areas of the airway wall. Six to 10 different sections were evaluated per animal. The obtained measurements were averaged for each animal and the mean values and SE were determined for each group[4, 27].

Statistical analyses for *in vitrolin vivo* mouse experiments

For outcome variables with multiple measures within mice (either within or between treatments), linear mixed models were employed, using the best available covariance structure for repeated measures; for outcome variables with one measure per mouse, general linear models were used, allowing separate variances by treatment (i.e., weighted least squares). Pairwise comparisons were performed using t-tests derived from these models. Planned tests (5 for each outcome) for AHR, differential cell count, BAL cytokine measurements, and lung histology (PAS) included comparisons using sensitized and

challenged CD8-deficient mice either before or after adoptive transfer of untreated $CD8^+$ T cells as baseline. For gene expression, CYP11A1 Western blot, pregnenolone, and ChIP experiments, $CD8^+$ T cells differentiated either with IL-2 alone or in the presence of IL-2+IL-4 were used as baseline for all pairwise comparisons (7 tests for each outcome). The Benjamini-Hochberg[58] procedure was used for the set of 5 or 7 comparisons within each outcome variable to help control for false-positive test results, using a false discovery rate (FDR) of 0.05.

Data for each outcome variable were based on at least 3 independent experiments, often with multiple mice per treatment in each experiment. For the purposes of the analyses, data from each experiment were pooled together. Analyses for BAL and cytokine levels utilized a minimum of 6 mice per treatment, with unique mice across treatments; experiments for ChIP, gene expression, Western blot, and pregnenolone variables used a minimum of 3 independent experiments per treatment, with repeated measures across treatments; for lung histology (PAS), 3–5 mice per treatment were used, with independent mice across treatments, but where mice had multiple measurements within treatments (up to 9). *In vivo* experiments including AHR were performed with 11–13 mice per treatment group. For gene expression, Western blot, pregnenolone, and ChIP analyses, mice had repeated measures across different treatments. For the outcome variables in these analyses, a banded main diagonal structure was used that allowed for separate variances by treatment and no covariances between treatments; random intercepts for mice were also included in these models to account for within-mice correlation, but were excluded from final models since they did not improve model fits. For lung histology (PAS), mice had repeated measures within but not across treatments. For this outcome variable, a compound symmetric structure was used, allowing a separate variance parameter for the control treatment (sensitized and challenged CD8-deficient mice) relative to the other groups to account for the much lower variability for that condition. Results were expressed as the mean± SEM.

Human study populations and *CYP11A1* and *VDR* genetic analyses

Based on the linkage disequilibrium ($r^2 \geq 0.8$, HapMap database, release #28, CEU population) within the *CYP11A1* locus (10 kb up- and 5 kb down-stream, Supplementary Figure 4), we identified 25 *CYP11A1* SNPs with a minor allele frequency (MAF) ≥ 0.03 and 2 SNPs leading to an amino acid change (rs6161 and rs1130841, MAF=0.005, Supplementary Fig. 6, black box). These *CYP11A1* polymorphisms clustered in 4 tagging bins and 4 single SNPs (Supplementary Figure 5). Genotyping of rs9806234 failed but for the association analyses an imputation based dataset originated from HapMap II was available[33]. None of the SNPs deviated from Hardy-Weinberg Equilibrium (Table 1). No significant associations with asthma were observed for rs6161 (MAF=0.004, n=11 heterozygous subjects, 5 cases, 6 controls) and rs1130841 was monomorphic in the cohort under study. Thus, these mutations were not studied further.

Most of the asthmatics (n=655) for the association study included children of German or Austrian origin from the Multicenter Asthma Genetic In Childhood (MAGIC) study (mean age of 11±2.9years SD)[33,59,60] and 73 asthmatics and 767 healthy controls were derived from of the International Study of Asthma and Allergies in Childhood (ISAAC) Phase II

(n=5,629, mean age of 9.6±0.6 years SD)[61]. As previously described[60], the subset of asthmatics was combined with asthmatics from the MAGIC study to test for associations in a case-control setting. No significant demographic differences between the populations under study were observed[60]. Genotyping of *CYP11A1* was performed by Illumina HumanHap300Chip (rs2279357, rs11632698, rs2073475, rs4432229, N=1,311)[33,59] or by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (rs1484215, rs16968478, rs4432229, rs9806234, n=1,454, Table 1)[33,62]. Detailed information on the case-control dataset and genotyping methodology including oligonucleotide sequences are available from the authors upon request.

Genotyping data for each of the SNPs under study can be accessed using the following link http://www.share.asthmagene.org/permanentDownload/natComm15 (Phenotype: 1=asthma, 0=healthy control, Genotype: 0=homozygous wild-type allele, 1=heterozygous, 2=homozygous polymorphic allele). In order to comply with patient confidentiality, displayed ID numbers were randomly assigned for each SNP independently.

Deviation from Hardy-Weinberg equilibrium was analyzed by chi-square test. Associations of binary traits were evaluated by logistic regression (Plink, version 1.07: http://pngu.mgh.harvard.edu/purcell/plink). Odds ratios (OR), 95% confidence intervals (CI) and p-values are reported. Adjustment for multiple testing for the association analyses for *CYP11A1* SNPs was performed taking the LD into account. The effective number (M_{eff}) of independent SNPs was calculated (SNPSpD (http://gump.qimr.edu.au/general/daleN/SNPSpD)[63] to control for the experiment-wise significance level with an adjusted p-value of p=0.010[63,64]. Selection for SNPs within *VDR* for gene-by-gene-interaction analyses with *CYP11A1* polymorphisms was based on previously studied *VDR* SNPs[33].

All study subjects from both populations were of German descent. Written informed consent was obtained from all parents of children included in these studies. Study methods which were very similar in both populations were approved by the ethics committees of each of the study centers (ISAAC II: Dresden, and Munich; MAGICS: Bochum, Cologne, Feldkirch, Freiburg, Munich/Rosenheim, Vienna and Wesel).

In silico analyses of *CYP11A1* SNPs

Functional annotation was performed of the tagging and tagged *CYP11A1* SNPs associated with asthma. Transcription factor binding analyses depending on the genotype status were conducted by Matinspector (www.genomatix.de) and Alibaba (www.gene-regulation.com). The putative regulatory role was further evaluated by the regulomeDB[65]. The regulomeDB score and potential transcription role of histone modifications in naive $CD8^+$ T cells isolated from peripheral blood is presented. Allele-specific analyses on *CYP11A1* gene expression in EBV-transformed lymphoblastoid cell lines of unrelated samples of the HapMap population were performed (http://app3.titan.uio.no/biotools).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Martin RJ, et al. The Predicting Response to Inhaled Corticosteroid Efficacy (PRICE) trial. J Allergy Clin Immunol. 2007; 119:73–80. [PubMed: 17208587]
2. Hamelmann E, et al. Requirement for $CD8^+$ T cells in the development of airway hyperresponsiveness in a murine model of airway sensitization. J Exp Med. 1996; 183:1719–1729. [PubMed: 8666929]
3. Isogai S, et al. $CD8^+$ alphabeta T cells can mediate late airway responses and airway eosinophilia in rats. J Allergy Clin Immunol. 2004; 114:1345–1352. [PubMed: 15577833]
4. Jia Y, et al. Steroidogenic enzyme CYP11A1 regulates type 2 $CD8^+$ T cell skewing in allergic lung disease. Proc Natl Acad Sci USA. 2013; 110:8152–8157. [PubMed: 23630275]
5. Miyahara N, et al. Leukotriene B4 release from mast cells in IgE-mediated airway hyperresponsiveness and inflammation. Am J Respir Cell Mol Biol. 2009; 40:672–682. [PubMed: 19029019]
6. Miyahara N, et al. Effector $CD8^+$ T cells mediate inflammation and airway hyper-responsiveness. Nat Med. 2004; 10:865–869. [PubMed: 15258576]
7. Miyahara N, et al. Contribution of antigen-primed $CD8^+$ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL–13. J Immunol. 2004; 172:2549–2558. [PubMed: 14764728]
8. Miyahara N, et al. Requirement for leukotriene B4 receptor 1 in allergen-induced airway hyperresponsiveness. Am J Respir Crit Care Med. 2005; 172:161–167. [PubMed: 15849325]
9. Taube C, et al. The leukotriene B4 receptor (BLT1) is required for effector $CD8^+$ T cell-mediated, mast cell-dependent airway hyperresponsiveness. J Immunol. 2006; 176:3157–3164. [PubMed: 16493075]
10. Koya T, et al. $CD8^+$ T cell-mediated airway hyperresponsiveness and inflammation is dependent on $CD4^+IL\text{-}4^+$ T cells. J Immunol. 2007; 179:2787–2796. [PubMed: 17709492]
11. Li LB, Leung DY, Strand MJ, Goleva E. ATF2 impairs glucocorticoid receptor-mediated transactivation in human $CD8^+$ T cells. Blood. 2007; 110:1570–1577. [PubMed: 17525285]
12. Gelfand EW, Dakhama A. $CD8^+$ T lymphocytes and leukotriene B4: novel interactions in the persistence and progression of asthma. J Allergy Clin Immunol. 2006; 117:577–582. [PubMed: 16522456]
13. van Rensen EL, et al. Bronchial CD8 cell infiltrate and lung function decline in asthma. Am J Respir Crit Care Med. 2005; 172:837–841. [PubMed: 16085937]
14. Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. Am J Clin Nutr. 2004; 80:1717S–1720S. [PubMed: 15585793]
15. Correale J, Ysrraelit MC, Gaitan MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. J Immunol. 2010; 185:4948–4958. [PubMed: 20855882]
16. Munger KL, et al. Vitamin D intake and incidence of multiple sclerosis. Neurology. 2004; 62:60–65. [PubMed: 14718698]

17. Zhu Y, Mahon BD, Froicu M, Cantorna MT. Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease. Eur J Immunol. 2005; 35:217–224. [PubMed: 15593122]
18. Gupta A, et al. Defective IL-10 expression and *in vitro* steroid-induced IL-17A in paediatric severe therapy-resistant asthma. Thorax. 2014; 69:508–515. [PubMed: 24347461]
19. Gupta A, et al. Relationship between serum Vitamin D, disease severity, and airway remodeling in children with asthma. Am J Respir Crit Care Med. 2011; 184:1342–1349. [PubMed: 21908411]
20. Brehm JM, et al. Serum Vitamin D levels and severe asthma exacerbations in the Childhood Asthma Management Program study. J Allergy Clin Immunol. 2010; 126:52–58 e55. [PubMed: 20538327]
21. Goleva E, Searing DA, Jackson LP, Richers BN, Leung DY. Steroid requirements and immune associations with Vitamin D are stronger in children than adults with asthma. J Allergy Clin Immunol. 2012; 129:1243–1251. [PubMed: 22330698]
22. Korn S, Hubner M, Jung M, Blettner M, Buhl R. Severe and uncontrolled adult asthma is associated with Vitamin D insufficiency and deficiency. Respir Res. 2013; 14:25. [PubMed: 23432854]
23. Salas NM, Luo L, Harkins MS. Vitamin D deficiency and adult asthma exacerbations. J Asthma. 2014; 51:950–955. [PubMed: 24926743]
24. Searing DA, et al. Decreased serum Vitamin D levels in children with asthma are associated with increased corticosteroid use. J Allergy Clin Immunol. 2010; 125:995–1000. [PubMed: 20381849]
25. Sutherland ER, Goleva E, Jackson LP, Stevens AD, Leung DY. Vitamin D levels, lung function, and steroid response in adult asthma. Am J Respir Crit Care Med. 2010; 181:699–704. [PubMed: 20075384]
26. Castro M, et al. Effect of Vitamin D3 on asthma treatment failures in adults with symptomatic asthma and lower Vitamin D levels: the VIDA randomized clinical trial. J Am Med Association. 2014; 311:2083–2091.
27. Jia Y, et al. Stepwise epigenetic and phenotypic alterations poise $CD8^+$ T cells to mediate airway hyperresponsiveness and inflammation. J Immunol. 2013; 190:4056–4065. [PubMed: 23509358]
28. Wang M, et al. The steroidogenic enzyme CYP11A1 is essential for development of peanut-induced intestinal anaphylaxis. J Allergy Clin Immunol. 2013; 132:1174–1183 e1178. [PubMed: 23870673]
29. Lavoie HA, King SR. Transcriptional regulation of steroidogenic genes: *STARD1*, *CYP11A1* and *HSD3B*. Exp Biol Med (Maywood). 2009; 234:880–907. [PubMed: 19491374]
30. Ngoc PL, Gold DR, Tzianabos AO, Weiss ST, Celedon JC. Cytokines, allergy, and asthma. Curr Opin Allergy Clin Immunol. 2005; 5:161–166. [PubMed: 15764907]
31. Bosse Y, et al. Asthma and genes encoding components of the Vitamin D pathway. Respir Res. 2009; 10:98. [PubMed: 19852851]
32. Wang TT, et al. Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol. 2005; 19:2685–2695. [PubMed: 16002434]
33. Michel S, et al. Unifying candidate gene and GWAS approaches in asthma. PloS One. 2010; 5:e13894. [PubMed: 21103062]
34. Chung EH, et al. Leukotriene B4 receptor 1 is differentially expressed on peripheral T cells of steroid-sensitive and -resistant asthmatics. Ann Allergy Asthma Immunol. 2014; 112:211–216 e211. [PubMed: 24428972]
35. Chambers ES, et al. Serum 25-dihydroxyvitamin D levels correlate with $CD4^+Foxp3^+$ T-cell numbers in moderate/severe asthma. J Allergy Clin Immunol. 2012; 130:542–544. [PubMed: 22656048]
36. Matheu V, Back O, Mondoc E, Issazadeh-Navikas S. Dual effects of Vitamin D-induced alteration of T_H1/T_H2 cytokine expression: enhancing IgE production and decreasing airway eosinophilia in murine allergic airway disease. J Allergy Clin Immunol. 2003; 112:585–592. [PubMed: 13679819]
37. Nanzer AM, et al. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. J Allergy Clin Immunol. 2013; 132:297–304 e293. [PubMed: 23683514]

38. Xystrakis E, et al. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. J Clin Invest. 2006; 116:146–155. [PubMed: 16341266]
39. Cantorna MT, Mahon BD. D-hormone and the immune system. J Rheumatol Suppl. 2005; 76:11–20. [PubMed: 16142846]
40. Hayes CE, Nashold FE, Spach KM, Pedersen LB. The immunological functions of the Vitamin D endocrine system. Cell Mol Biol. 2003; 49:277–300. [PubMed: 12887108]
41. Carraro S, et al. Asthma severity in childhood and metabolomic profiling of breath condensate. Allergy. 2013; 68:110–117. [PubMed: 23157191]
42. Gergen PJ, et al. Lack of a relation between serum 25-hydroxyvitamin D concentrations and asthma in adolescents. Am J Clin Nutr. 2013; 97:1228–1234. [PubMed: 23595876]
43. Tolppanen AM, et al. Prospective association of 25-hydroxyvitamin D3 and D2 with childhood lung function, asthma, wheezing, and flexural dermatitis. Epidemiology. 2013; 24:310–319. [PubMed: 23377091]
44. Chawes BL, et al. Cord blood 25(OH)-vitamin D deficiency and childhood asthma, allergy and eczema: the COPSAC2000 birth cohort study. PLoS One. 2014; 9:e99856. [PubMed: 24925304]
45. Goldring ST, et al. Prenatal Vitamin D supplementation and child respiratory health: a randomised controlled trial. PLoS One. 2013; 8:e66627. [PubMed: 23826104]
46. Jones AP, Palmer D, Zhang G, Prescott SL. Cord blood 25-hydroxyvitamin D3 and allergic disease during infancy. Pediatrics. 2012; 130:e1128–1135. [PubMed: 23090338]
47. Nwaru BI, et al. Maternal diet during pregnancy and allergic sensitization in the offspring by 5 yrs of age: a prospective cohort study. Pediatr Allergy Immunol. 2010; 21:29–37. [PubMed: 20003068]
48. Rosenwasser LJ, Borish L. Genetics of atopy and asthma: the rationale behind promoter-based candidate gene studies (*IL-4* and *IL-10*). Am J Respir Crit Care Med. 1997; 156:S152–155. [PubMed: 9351597]
49. Weisse K, et al. Maternal and newborn Vitamin D status and its impact on food allergy development in the German LINA cohort study. Allergy. 2013; 68:220–228. [PubMed: 23253182]
50. Xystrakis E, et al. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. J Clin Invest. 2006; 116:146–55. [PubMed: 16341266]
51. Campbell FC, Xu H, El-Tanani M, Crowe P, Bingham V. The yin and yang of vitamin D receptor (VDR) signaling in neoplastic progression: operational networks and tissue-specific growth control. Biochem Pharmacol. 2010; 79:1–9. [PubMed: 19737544]
52. Gynther P, et al. Mechanism of 1alpha,25-dihydroxyvitamin D(3)-dependent repression of interleukin-12B. Biochim Biophys Acta. 2011; 1813:810–818. [PubMed: 21310195]
53. Maalmi H, et al. Association of vitamin D receptor gene polymorphisms with susceptibility to asthma in Tunisian children: A case control study. Hum Immunol. 2013; 74:234–240. [PubMed: 23200756]
54. Raby BA, et al. Association of vitamin D receptor gene polymorphisms with childhood and adult asthma. Am J Respir Crit Care Med. 2004; 170:1057–1065. [PubMed: 15282200]
55. Vollmert C, et al. Single nucleotide polymorphism screening and association analysis--exclusion of integrin beta 7 and vitamin D receptor (chromosome 12q) as candidate genes for asthma. Clin Exp Allergy. 2004; 34:1841–1850. [PubMed: 15663557]
56. Ohnishi H, et al. Corticosteroids enhance $CD8^+$ T cell-mediated airway hyperresponsiveness and allergic inflammation by upregulating leukotriene B4 receptor 1. J Allergy Clin Immunol. 2008; 121:864–871 e864. [PubMed: 18395551]
57. Kanehiro A, et al. Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge. Am J Respir Crit Care Med. 2001; 163:173–184. [PubMed: 11208644]
58. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. J Royal Stat Soc Ser B. 1995; 57(1):289–300.
59. Moffatt MF, et al. Genetic variants regulating *ORMDL3* expression contribute to the risk of childhood asthma. Nature. 2007; 448:470–473. [PubMed: 17611496]

60. Potaczek DP, et al. Different FCR1A polymorphisms influence IgE levels in asthmatics and non-asthmatics. Pediatr Allergy Immunol. 2013; 24:441–449. [PubMed: 23725541]

61. Weiland SK, et al. Prevalence of respiratory and atopic disorders among children in the East and West of Germany five years after unification. Eur Respir J. 1999; 14:862–870. [PubMed: 10573234]

62. Schedel M, et al. A signal transducer and activator of transcription 6 haplotype influences the regulation of serum IgE levels. J Allergy Clin Immunol. 2004; 114:1100–1105. [PubMed: 15536416]

63. Li J, et al. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity. 2005; 95:221–227. [PubMed: 16077740]

64. Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet. 2004; 74:765–769. [PubMed: 14997420]

65. Boyle AP, et al. Annotation of functional variation in personal genomes using RegulomeDB. Genome Research. 2012; 22:1790–1797. [PubMed: 22955989]

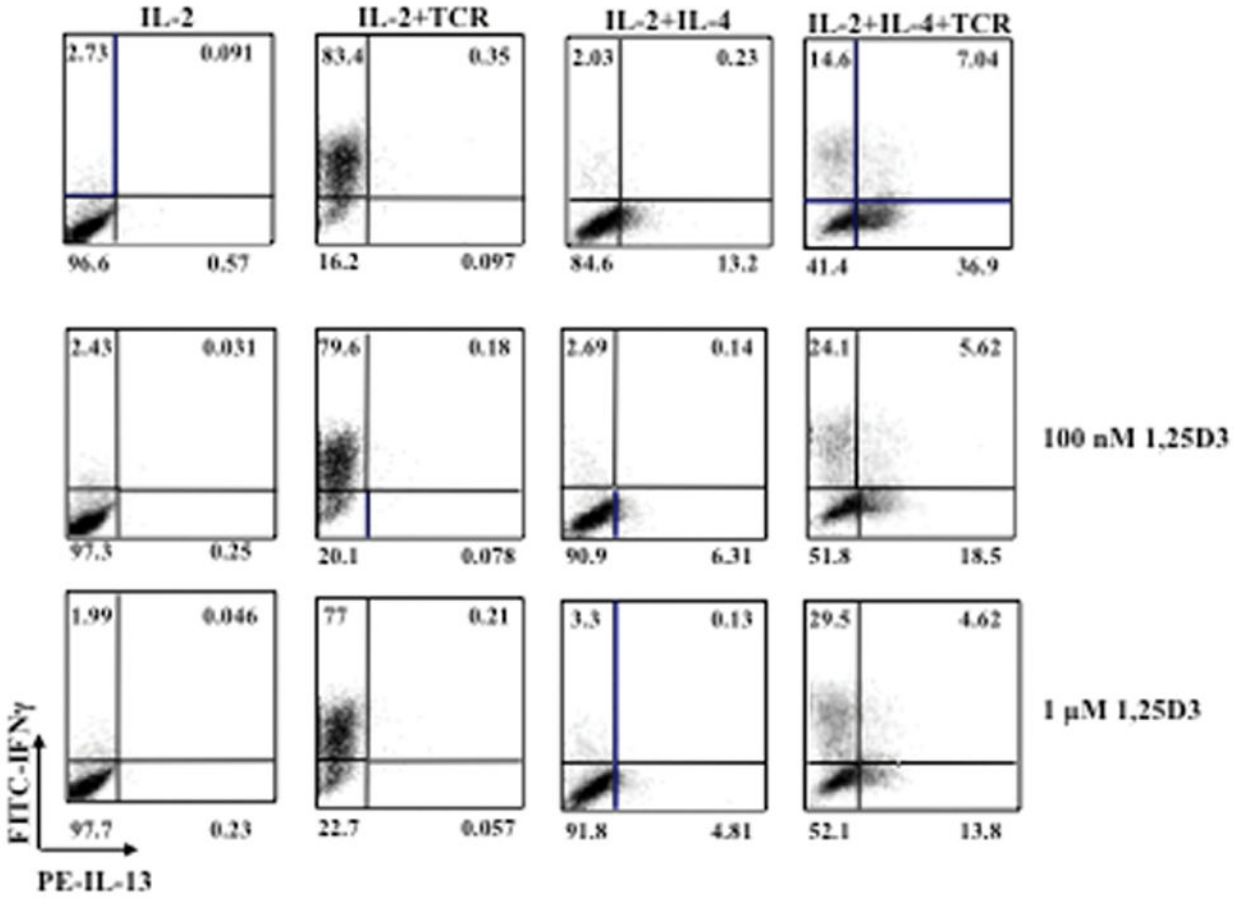

Figure 1. IFN-γ and IL-13 expression in CD8$^+$ T cells differentiated in IL-2 or IL-2+IL-4 in the presence or absence of 1,25D3 at 100 nM or 1 μM

Representative results of intracellular staining of IFN-γ and IL-13 expression in CD8$^+$ T cells with or without SIINFEKL (T cell receptor, TCR) restimulation.

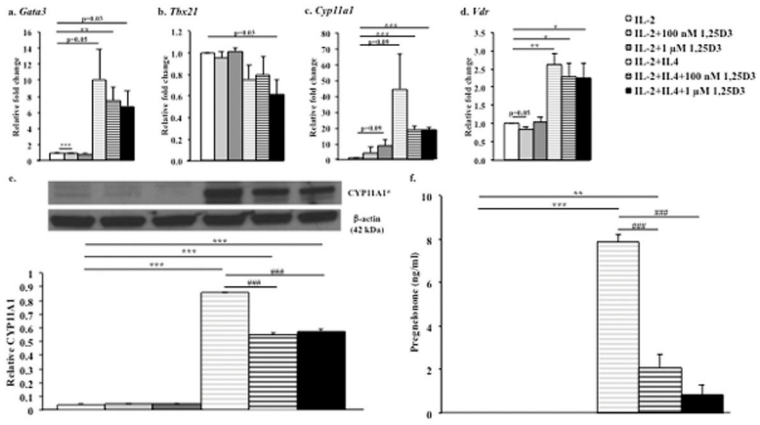

Figure 2. 1,25D3 treatment of CD8$^+$ T cells alters gene expression of transcription factors and the functional activity of CYP11A1

Gene expression of **(a)** *Gata3*, **(b)** *Tbx21*, **(c)** *Cyp11a1*, and **(d)** *Vdr* was measured by quantitative PCR (qPCR) in CD8$^+$ T cells in IL-2 or IL-2+IL-4 in the presence of absence of 100 nM or 1 μM 1,25D3. Results (relative fold change+SEM) are from three independent experiments. **(e)** CYP11A1 protein levels (mean+SEM) detected by immunoblot analyses and densitometry of autoradiographs in CD8$^+$ T cells differentiated in IL-2 or IL-2+IL-4 in the presence of absence of 100 nM or 1 μM 1,25D3. Results are from three independent experiments, *calculated molecular weight (MW) for CYP11A1 (13363-1-AP, Proteintech, Chicago, IL): 60 kDa, observed MW: 49 kDa, **(f)** Pregnenolone levels (mean+SEM) determined by ELISA in supernatants from CD8$^+$ T cells differentiated in IL-2 or IL-2+IL-4 in the presence or absence of 100 nM or 1 μM 1,25D3. Results are from six independent experiments. Linear mixed models were employed; pairwise comparisons were performed using *t-tests* derived from these models. *p<0.05, **p<0.01, ***p<0.001 compared to the IL-2 group, ###p<0.001 compared to the IL-2+IL-4 group, these p-values remained significant after correction for multiple comparisons (Benjamini-Hochberg[57] correction); p-values that did not reach the threshold p-value after adjustment for multiple comparisons are shown numerically.

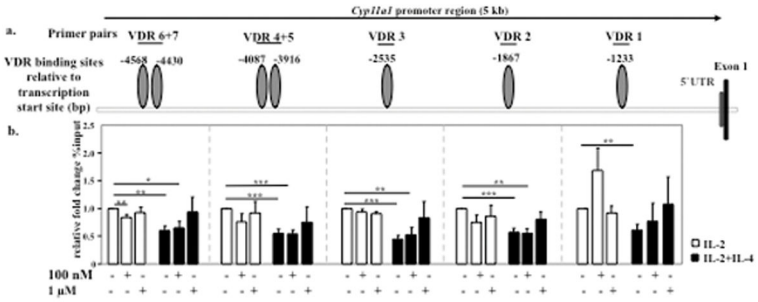

Figure 3. 1,25D3 mediated VDR recruitment to the *Cyp11a1* promoter region is altered in CD8$^+$ T cells differentiated in IL-2 or IL-2+IL-4 in the presence or absence of 100 nM or 1 μM 1,25D3

(a) Localization of VDR binding sites and qPCR primers in the *Cyp11a1* promoter region. **(b)** qPCR was performed using five *Cyp11a1* promoter-specific primers covering seven VDR binding sites. Data were analyzed via the percent input methodology: (2^ (CT of total input – CT of specific IP)) x100 and relative percent input ratios using CD8$^+$ T cells stimulated with IL-2 as baseline. Data (relative fold change+SEM) are from three independent experiments. Linear mixed models were employed; pairwise comparisons were performed using *t-tests* derived from these models. *p<0.05, **p<0.01, ***p<0.001 compared to the IL-2 group, these p-values remained significant after correction for multiple comparisons; p-values that did not reach the threshold p-value after adjustment for multiple comparisons (Benjamini-Hochberg[57] correction) are shown numerically. No statistical significance was detected compared to the IL-2+IL-4 group.

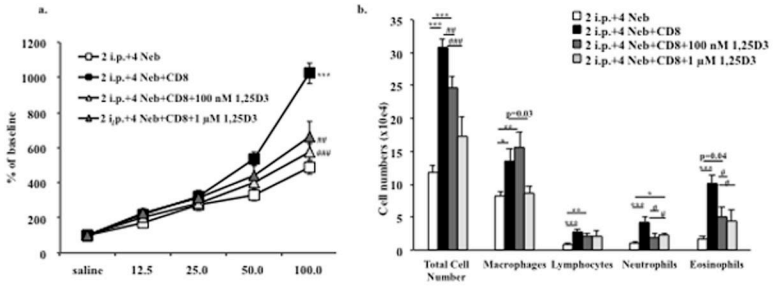

Figure 4. Adoptive transfer of 1,25D3-treated CD8$^+$ T cells into CD8-deficient recipients fails to induce AHR

Recipient mice were sensitized (2 intraperitoneal, i.p.) and challenged (4 nebulizations, Neb) using secondary allergen challenge model and received no cells, CD8$^+$ T cells differentiated in IL-2 alone (CD8) or in the presence of 100 nM (CD8+100 nM 1,25D3) or 1 μM 1,25D3 (CD8+1 μM 1,25D3). **(a)** Changes in airway resistance (RL) were measured in response to increasing concentrations of methacholine. **(b)** Cell composition in BAL fluid. Data (mean +SEM) are from two to three experiments with three to four mice per experiment. *p<0.05, **p<0.01, ***p<0.001; compared to sensitized and challenged CD8-deficient recipients which received no cells. General linear models were employed; pairwise comparisons were performed using *t-tests* derived from these models. #p<0.05, ##p<0.01, ###p<0.001 compared to sensitized and challenged CD8-deficient recipients which received CD8$^+$ T cells differentiated in IL-2 alone, these p-values remained significant after correction for multiple comparisons (Benjamini-Hochberg[57] correction); p-values that did not reach the threshold p-value after adjustment for multiple comparisons are shown numerically.

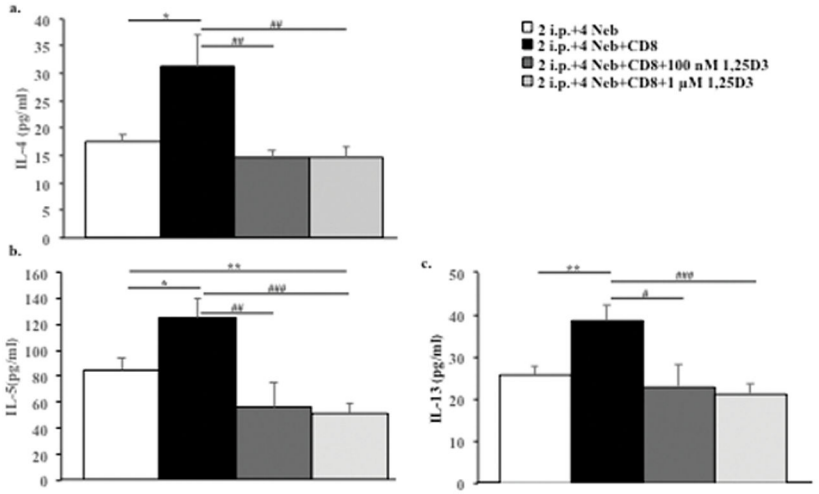

Figure 5. Adoptive transfer of 1,25D3-treated CD8$^+$ T cells into CD8-deficient recipients decreased cytokine levels in the BAL

Cytokine levels in BAL fluid (mean+SEM): **(a)** IL-4, **(b)** IL-5, and **(c)** IL-13. *p<0.05, **p<0.01; compared to sensitized and challenged CD8-deficient recipients which received no cells. Data (mean+SEM) are from two to three experiments with three to four mice per experiment. General linear models were employed; pairwise comparisons were performed using *t-tests* derived from these models. #p<0.05, ##p<0.01, ###p<0.001 compared to sensitized and challenged CD8-deficient recipients which received CD8$^+$ T cells differentiated in IL-2 alone, p-values remained significant after correction for multiple comparisons (Benjamini-Hochberg[57] correction).

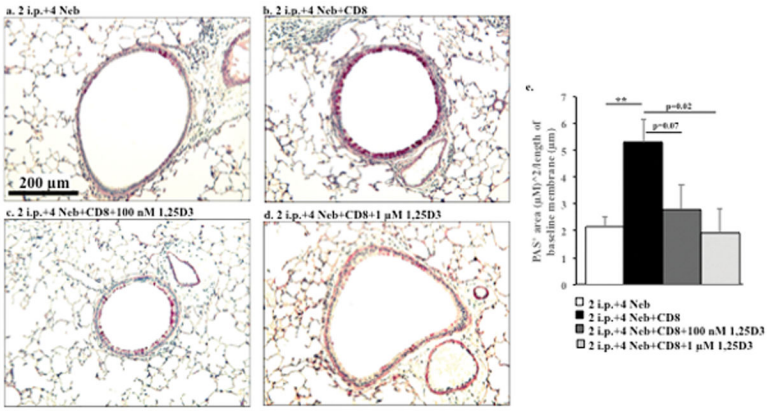

Figure 6. Adoptive transfer of 1,25D3-treated CD8$^+$ T cells into CD8-deficient recipients prevents goblet cell metaplasia

(a–d) Representative photomicrographs of lung histology (original magnification x3200, scale bar 200 µm). **(e)** Quantitative analysis of PAS-positive goblet cells was determined in cross-sectional areas of the airway wall. Data (mean+SEM) are from two experiments with three mice per experiment. Linear mixed models were employed; pairwise comparisons were performed using *t-tests* derived from these models. **p<0.01 these p-values remained significant after correction for multiple comparisons; p-values that did not reach the threshold p-value after adjustment for multiple comparisons (Benjamini-Hochberg[57] correction) are shown numerically.

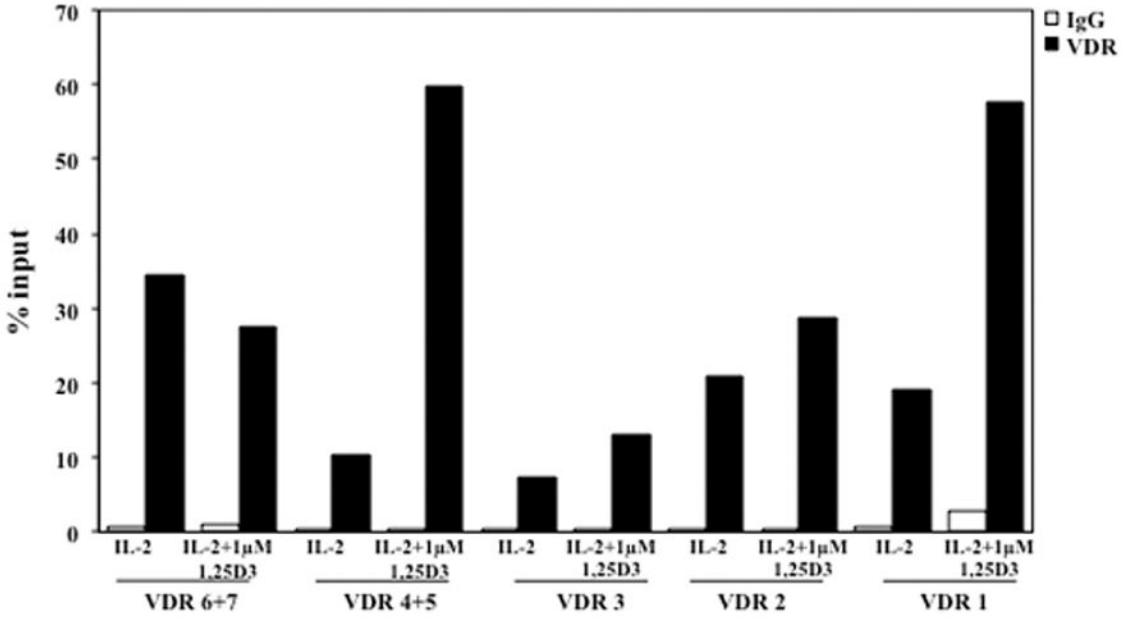

Figure 7. 1,25D3-mediated VDR recruitment to the *Cyp11a1* promoter region is altered in CD8$^+$ T cells after adoptive transfer into sensitized and challenged CD8-deficient mice

CD8$^+$ T cells were differentiated *in vitro* in IL-2 in the presence or absence of 1 μM 1,25D3. CD8$^+$ T cells from the lungs were recovered after adoptive transfer into sensitized and challenged CD8-deficient mice (n=3 mice/group). qPCR was performed using five *Cyp11a1* promoter specific primers covering seven VDR binding sites. Data were analyzed via the percent input methodology: (2^ (CT of total input – CT of specific IP)) ×100 and relative percent input ratios using CD8$^+$ T cells stimulated with IL-2 as baseline.

Table 1

Asthma associations for *CYP11A1* SNPs in the case-control study population.

SNP	Allele[1]	MAF[2]	Call rate (%)	LD[3] bin	p-value (HWE[4])	Asthma (n=763) OR[5] (95% CI) p-value
rs2279357 *	G/A	0.28	99.7	5	0.64	0.85 (0.71–1.00) p=0.0554
rs6161	G/A	0.004	98.6		1.00	
rs11632698 *	C/T	0.40	99.9	4	0.42	**0.85 (0.73–1.00) p=0.0496**
rs1484215	G/A	0.08	98.1	3	0.58	1.00 (0.76–1.32) p=0.9865
rs1130841	G/A	0	98.3			
rs2073475 *	G/A	0.14	98.9	2	0.26	0.98 (0.78–1.23) p=0.8513
rs16968478	T/C	0.17	97.6		1.00	0.94 (0.77–1.14) p=0.5293
rs9806234 #	T/C	0.26	100		0.76	0.99 (0.84–1.18) p=0.9478
rs4886595	T/G	0.19	97.3		0.91	**0.77 (0.64–0.93) p=0.0079**&
rs4432229 *	T/C	0.16	99.9	1	0.49	**0.80 (0.65–0.98) p=0.0351**

Significant associations in the case control-population (N=1,454 or N=1,311*) are marked in bold letters.

The statistical analyses were performed by logistic regression modeling additive effects. To control for the experiment-wise significance level, an adjusted p-value of p=0.010 was used.

[1] Allele: wild-type/polymorphic allele.

[2] MAF: minor allele frequency.

[3] LD: linkage disequilibrium.

[4] HWE: Hardy Weinberg Equilibrium, *p*-value of χ^2-test for deviation of HWE in controls.

[5] Odds ratios (OR) and 95% confidence intervals (95% CI): polymorphic (minor) allele used as basis for the calculation of the effect size.

Data obtained from imputed data set.

& Association between rs4886595 and asthma remains significant after correction for multiple testing with an adjusted p-value of 0.010.

Table 2

Epistatic effects on asthma susceptibility of polymorphisms in *CYP11A1 and VDR*.

Effect of	In individuals carrying	Reference allele[1]	Odds ratio (95% CI)	p-value
CYP11A1 rs4886595		G	0.77 (0.64–0.93)	**0.0079**
CYP11A1 rs4432229		C	0.80 (0.65–0.98)	**0.0351**
VDR rs2107301		A	0.76 (0.64–0.89)	**0.0010**
CYP11A1 rs4886595	*VDR* rs2107301 GG (n=660)	*G*	0.71 (0.54–0.94)	**0.0176**
CYP11A1 rs4886595	*VDR* rs2107301 GA+AA (n=608)	*G*	0.82 (0.62–1.10)	0.1894
VDR rs2107301	*CYP11A1* rs4886595 TT (n=835)	*A*	0.75 (0.60–0.93)	**0.0082**
VDR rs2107301	*CYP11A1* rs4886595 TG+GG (n=433)	*A*	0.73 (0.54–0.98)	**0.0372**
CYP11A1 rs4432229	*VDR* rs2107301 GG (n=678)	*C*	0.73 (0.54–0.98)	**0.0354**
CYP11A1 rs4432229	*VDR* rs2107301 GA+AA (n=624)	*C*	0.90 (0.67–1.21)	0.4785
VDR rs2107301	*CYP11A1* rs4432229 TT (n=928)	*A*	0.75 (0.61–0.91)	**0.0051**
VDR rs2107301	*CYP11A1* rs4432229 TC+CC (n=374)	*A*	0.76 (0.56–1.05)	0.0970

The entire case-control study population was stratified for the *CYP11A1* polymorphisms rs4886595, and rs4432229 *a*nd rs2107301 (*VDR*) to investigate the SNP effect on asthma susceptibility of one SNP in relation to the other polymorphism. The statistical analyses were performed by logistic regression modeling additive effects.

[1] Allele: polymorphic (minor) allele was used as basis for the calculation of the effect size.