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Vitamin D₃-Induced Promotor Dissociation of PU.1 and YY1 Results in FcεRI Reduction on Dendritic Cells in Atopic Dermatitis

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Atopic dermatitis (AD) is a severe inflammatory skin disease. Langerhans cells and inflammatory dendritic epidermal cells (IDEC) are located in the epidermis of AD patients and contribute to the inflammatory processes. Both express robustly the high-affinity receptor for IgE, FcεRI, and thereby sense allergens. A beneficial role of vitamin D₃ in AD is discussed to be important especially in patients with allergic sensitization. We hypothesized that vitamin D₃ impacts FcεRI expression and addressed this in human ex vivo skin, in vitro Langerhans cells, and IDEC models generated from primary human precursor cells. We show in this article that biologically active vitamin D₃ [1,25(OH)₂-D₃] significantly downregulated FcεRI at the protein and mRNA levels of the receptor's α-chain, analyzed by flow cytometry and quantitative RT-PCR. We also describe the expression of a functional vitamin D receptor in IDEC. 1,25(OH)₂-D₃-mediated FcεRI reduction was direct and resulted in impaired activation of IDEC upon FcεRI engagement as monitored by CD83 expression. FcεRI regulation by 1,25(OH)₂-D₃ was independent of maturation and expression levels of microRNA-155 and PU.1 (as upstream regulatory axis of FcεRI) and transcription factors Elf-1 and YY1. However, 1,25(OH)₂-D₃ induced dissociation of PU.1 and YY1 from the *FCER1A* promotor, evaluated by chromatin immunoprecipitation. We show that vitamin D₃ directly reduces FcεRI expression on dendritic cells by inhibiting transcription factor binding to its promotor and subsequently impairs IgE-mediated signaling. Thus, vitamin D₃ as an individualized therapeutic supplement for those AD patients with allergic sensitization interferes with IgE-mediated inflammatory processes in AD patients. *The Journal of Immunology*, 2021, 206: 531–539.

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a complex pathophysiology. Dendritic cells (DC) are highly specialized APCs that initiate and modulate the outcome of T cell responses (1). DC bridge innate and adaptive immune systems and thereby control the balance of inflammatory

and anti-inflammatory reactions (2–4). In the epidermis, Langerhans cells (LC) are the most prominent DC subtype and are assumed to be important inducers of tolerance (5, 6). Inflammatory dendritic epidermal cells (IDEC) are present in AD skin and exhibit a proinflammatory activity (7, 8). A hallmark of AD is the high expression of the high-affinity receptor for IgE, FcεRI, on both epidermal DC types (7, 9, 10). Via specific IgE, FcεRI⁺ DC can sense allergens and subsequently initiate, activate, and modulate immune responses by the induction of proinflammatory and regulatory mechanisms (8, 11–14). FcεRI on DC is composed of one IgE-binding α-chain and two γ-chains, the latter being responsible for surface expression and signaling (15). We have previously shown that in LC, TLR2 ligation induces a strong reduction of FcεRI at the transcriptional level of its α-chain, which is mediated by microRNA-155 upregulation and subsequent downregulation of transcription factor (TF) PU.1 (16, 17).

Vitamin D₃ is synthesized in the skin by the UV-induced conversion of 7-dehydrocholesterol or can be taken up by diet. Hydroxylation in the liver, kidneys, and by immune cells results in the biologically active forms 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂-D₃], the latter being more active and having the highest affinity to the vitamin D₃ receptor (VDR), which in turn acts as TF and gene regulator in the nucleus (18). The role of vitamin D₃ in AD is discussed controversially (reviewed in Ref. 19, 20). In contrast to psoriasis, topical treatment in AD is not recommended because of its irritative potential, but several studies report an improvement of the disease after oral vitamin D₃ supplementation as witnessed by reduced scoring for atopic dermatitis (SCORAD), reduced total IgE level, and reduced colonization of the skin with *Staphylococcus aureus* (21–24). Although some

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Abbreviations used in this article: 7-AAD, 7-amino-actinomycin D; AD, atopic dermatitis; CD34⁺ hematopoietic stem cell-derived LC; ChIP, chromatin immunoprecipitation; DC, dendritic cell; EC, epidermal cell; HC, healthy control; IDEC, inflammatory dendritic epidermal cell; ILT, Ig-like transcript; LC, Langerhans cell; moIDEC, in vitro monocyte-derived IDEC; 1,25(OH)₂-D₃, 1,25-dihydroxyvitamin D₃; Pam3Cys, *N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyll-[S]-seryl-[S]-[lysyl]3-[S]-lysine; qPCR, quantitative RT-PCR; SCORAD, scoring for atopic dermatitis; TF, transcription factor; VDR, vitamin D₃ receptor.

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reports show an inverse correlation between serum vitamin D₃ and disease severity and/or total serum IgE, this could not be confirmed by others (25–27). Recent reports started to shed light on this enigmatic role of vitamin D₃, showing inverse correlations only for a subgroup of AD patients characterized by IgE-mediated allergic sensitization, evaluated by specific IgE tests as well as skin prick tests (28, 29). We hypothesized that vitamin D₃ may affect AD via an IgE-related mechanism involving FcεRI expression and function on DC. We subjected 1,25(OH)₂-D₃ to ex vivo skin from AD patients and healthy, nonatopic donors (healthy control [HC]) and in vitro-generated LC and IDEC. We found reduced expression levels of FcεRI at the mRNA and protein levels without induction of maturation of the DC, a functional loss of IgE-mediated activation, and the dissociation of TF PU.1 and YY1 from the FcεRI promoter site after 1,25(OH)₂-D₃ treatment.

Materials and Methods

Patients

Human cord blood was obtained from the Johanniter-Hospital and the St. Marien-Hospital in Bonn, Germany. Blood samples were obtained from HC (four female, two male; age range: 29–62 y) and patients with AD (seven female, eight male; age range: 21–74 y). Normal skin samples were obtained from healthy individuals (eight female, two male; age range: 25–65 y) undergoing plastic surgery at the University of Bonn. Shave biopsies were obtained from patients with moderate to severe AD (SCORAD >30; age range: 42–64 y) diagnosed according to the criteria of Hanifin and Rajka (30). This study was approved by the local ethics committee of the University of Bonn and performed in accordance with the declaration of the Helsinki principle. Informed consent was obtained from all of the participants in this study.

Reagents

FBS and antibiotics/antimycotics were from Life Technologies (Karlsruhe, Germany), GM-CSF was from Bayer Healthcare (Leverkusen, Germany), iTaq Universal SYBR Green Supermix was from BioRad (Munich, Germany), TLR2 ligand *N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyll-[S]-seryl-[S]-[lysyl]-3-[S]-lysine (Pam3Cys) was from EMC Microcollections (Tübingen, Germany), and human myeloma IgE was from Merck (Darmstadt, Germany). For flow cytometry, mAb specific for CD1a (clone T6, RD1-coupled) and CD83 (clone HB15a) were from Beckman-Coulter (Krefeld, Germany), CD14-allophycocyanin mAb (clone Tük4) were from Miltenyi Biotec (Bergisch-Gladbach, Germany), FcεRI-specific mAb (clone AER-37) were from eBioscience (San Diego, CA), CD80 (clone L307.4) and CD86 (clone IT2.2) mAb were from BD Pharmingen (San Diego, CA), and mAb against HLA-DR were from L243 hybridoma supernatant. IgG2b mAb were used as isotype-matched negative controls. 2-ME, 7-amino-actinomycin D (7-AAD), and 1,25(OH)₂-D₃ were from Sigma-Aldrich (St. Louis, MO). FITC-conjugated goat anti-mouse IgG and normal mouse serum were from Dianova (Hamburg, Germany). Anti-human IgE was from Dako (Carpinteria, CA). For chromatin immunoprecipitation (ChIP), Abs specific for PU.1 (clone 9G7), YY1 (clone D5D9Z), VDR (clone D2K6W), and isotype controls mouse IgG1 (clone G3A1) and rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA); Elf-1 (clone sc-133096) was obtained from Santa Cruz Biotechnology (Dallas, TX). Amplicons and primer for *FCER1A*, *FCER1G*, *ACTB*, *PU.1*, *ELF1*, *YY1*, *HMGB1*, *HMGB2*, *SPI1*, *VDR*, *CYP24A1*, and *FCER1A*-promoter region were obtained from Invitrogen (Carlsbad, CA). cDNA clone for actin MGC 8647 (ID2961617) was from Thermo Fisher Scientific (Waltham, MA).

Ex vivo skin

Split skin from HC was prepared from skin using a dermatome (thickness 0.4 mm). Shave biopsies of lesional AD skin were taken after local anesthesia and had an average thickness of 0.4 mm. Six-millimeter punch biopsies from HC split skin or AD shave biopsies were prepared and cultured for 24 h in the presence or absence of 10 or 100 nM 1,25(OH)₂-D₃. The epidermis was then dissected from the dermis, and an epidermal cell (EC) suspension was prepared by trypsinization as described (31). EC suspension was analyzed by flow cytometry.

Generation and stimulation of in vitro monocyte-derived IDEC and CD34⁺ hematopoietic stem cell-derived LC

For the generation of in vitro monocyte-derived IDEC (moIDEC), monocytes were isolated from blood of healthy donors or AD patients and

cultured for 6 d in RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 μM 2-ME, 1% antibiotics/antimycotics in the presence of GM-CSF (300 U/ml), IL-4 (500 U/ml), and 10 μg/ml human myeloma IgE. CD34⁺ hematopoietic stem cell-derived LC (CD34LC) were generated from CD34⁺ progenitor cells as described elsewhere (17). On day 6 (moIDEC) or day 8–10 (CD34LC), cell cultures were left untreated or stimulated with 2.5 or 10 nM 1,25(OH)₂-D₃ or 1 μg/ml Pam3Cys for 24 h or for indicated time periods. For some experiments, cells were pretreated with 10 nM 1,25(OH)₂-D₃ for 24 h, washed, and stimulated by anti-human IgE Abs for another 24 h. Cells were subjected to flow cytometry for surface expressions of proteins, ChIP assay for TF binding to promoter sequence, or magnetic cell sorting with anti-CD1a-coupled microbeads (Miltenyi Biotec) to enrich CD1a⁺ cells followed by mRNA and microRNA analyses.

Flow cytometry

EC suspensions from ex vivo skin were stained with Abs against the respective surface marker of interest or isotype control followed by goat-anti-mouse-FITC Abs. After blocking with mouse serum (1:20), allophycocyanin-conjugated Abs against CD1a and PE-conjugated Abs against Langerin were added to discriminate LC (CD1a⁺/Langerin⁺) from IDEC (CD1a⁺/Langerin⁻) and 7-AAD to exclude dead cells. CD34LC and moIDEC were stained with unconjugated Abs against the respective surface marker of interest or isotype control followed by goat-anti-mouse-FITC. After blocking with mouse serum (1:20), 7-AAD, allophycocyanin-conjugated Abs against CD14, and RD1-conjugated Abs against CD1a were added to gate on viable CD1a⁺ cells. Cells were analyzed using a FACS Canto, FACS Diva software (Becton Dickinson, San Jose, CA), and FlowJo (FlowJo, Ashland, OR). The relative fluorescence index was calculated as follows: (mean fluorescence intensity surface marker – mean fluorescence intensity isotype control)/mean fluorescence intensity isotype control.

mRNA and microRNA analyses

Total mRNA was purified from TRIzol lysates of CD1a-enriched cells. Differential gene expression was determined by SYBR Green-based quantitative RT-PCR (qPCR) as described (17). Relative expression levels of mRNA are given and calculated as the ratios of the copy numbers of the respective mRNA per 1000 actin copies. The oligonucleotides (5' to 3') used as primers are summarized in Supplemental Table I. For microRNA analyses, TaqMan MicroRNA Assays for hsa-miRNA-155-5p and control RNU48 were used according to the manufacturer's instructions (Thermo Fisher Scientific).

ChIP assay

CD34LC were lysed and subjected to ChIP with Abs of the respective TF according to the manufacturer's instructions using the SimpleChIP Plus Enzymatic Chromatin IP kit with agarose beads from Cell Signaling Technology. For background/unspecific binding, isotype controls were used in ChIP. Precipitated DNA or total DNA from input sample were subjected to qPCR with primers for the detection of the *FCER1A* promoter region [Supplemental Table I and (32)]. The relative binding of the target was calculated by the precipitated molecules of promoter region relative to promoter region input molecules. The background/unspecific binding, monitored by ChIP with the respective isotype control, was subtracted. Thus, specific relative binding of the respective TF was calculated as follows: (molecules [ChIP_TF]/molecules [input sample]) – (molecules [ChIP_isotype control]/molecules [input sample]).

Statistical analysis

Numbers of individual donors are represented by *n*. All pairwise comparisons between conditions were analyzed by the Wilcoxon rank sum test for paired samples by IBM SPSS Statistics 24 software (IBM Deutschland, Ehningen, Germany).

Results

High surface expression of FcεRI on epidermal DC from ex vivo skin of AD patients is reduced by 1,25(OH)₂-D₃ independently of maturation.

In a first approach of the study, we addressed the relevance of 1,25(OH)₂-D₃ for FcεRI in situ applying a human ex vivo skin model. We cultured ex vivo skin from HC and patients with AD in the presence or absence of 1,25(OH)₂-D₃ for 24 h and analyzed the expression of FcεRI on epidermal DC subtypes, namely

CD1a⁺Langerin⁺ LC and CD1a⁺Langerin⁻ IDEC. As described previously, HC, which lack IDEC in the epidermis, showed low expression levels of FcεRI on LC compared with LC or IDEC from AD (Fig. 1A). 1,25(OH)₂-D₃ treatment further reduced the receptor expression level in HC significantly. High expression of FcεRI on LC and IDEC from AD patients was reduced significantly by 1,25(OH)₂-D₃ in both epidermal DC subtypes, although it remained higher than in HC LC. There was no trend in the expression of maturation marker CD83 upon 1,25(OH)₂-D₃ treatment in epidermal DC from neither HC nor AD (Fig. 1B), indicating that 1,25(OH)₂-D₃ did not induce maturation of the cells. However, CD83 levels appeared to be higher in AD.

In vitro-generated IDEC express functional VDR

Our results indicate that vitamin D₃ acts on IDEC and LC. However, the main cell population in the epidermis is keratinocytes, which are responsive to vitamin D₃ (33). To exclude a putative indirect effect, we switched to *in vitro* cell models. Diverse expression of VDR in DC subtypes has been reported, and although LC express rather low levels of VDR compared with monocyte-derived DC, little is known about VDR expression and its functionality in IDEC (34). We applied an *in vitro* IDEC model and cultured monocytes isolated from HC or from AD patients as described previously (35). We refer to them as moIDEC/HC, which actually lack some IDEC hallmarks as the donors are healthy, and moIDEC/AD, respectively. The cells of both donor groups expressed similar amounts of VDR mRNA (Fig. 2A) and were highly responsive to its ligand 1,25(OH)₂-D₃ as shown by the induction of the target gene *CYP24A1* (Fig. 2B). There was no significant difference in *CYP24A1* expression after 1,25(OH)₂-D₃ treatment between the donor groups, indicating that moIDEC/HC and moIDEC/AD react similarly to 1,25(OH)₂-D₃. To our knowledge, this is the first description of the VDR being fully functional in IDEC.

1,25(OH)₂-D₃ directly reduces FcεRI at the transcriptional level in IDEC

We addressed phenotypical effects of 1,25-(OH)₂-D₃ in *in vitro* IDEC. moIDEC from both donor groups showed an immature phenotype displayed by a low expression of the maturation marker CD83. After 1,25-(OH)₂-D₃ exposure, CD83 was downregulated significantly, showing that 1,25(OH)₂-D₃ did not induce but rather inhibited maturation (Fig. 3B). Costimulatory molecules CD80 and CD86 as well as MHC class II were hardly affected by 1,25(OH)₂-D₃, and expression was low compared with LPS-activated moIDEC (Supplemental Fig. 1). moIDEC/HC showed no or low expression of FcεRI, which was further reduced significantly by 1,25(OH)₂-D₃ (Fig. 3A). In contrast, moIDEC/AD highly expressed surface FcεRI. 1,25(OH)₂-D₃ reduced FcεRI significantly to a level present in moIDEC/HC and again independently of maturation (Fig. 3A, 3B).

We further analyzed the FcεRI subunits, the α-chain (encoded by *FCER1A*), and the γ-chain (encoded by *FCER1G*) to unravel underlying mechanisms of regulation. *FCER1A* was downregulated significantly by 1,25(OH)₂-D₃ in both donor groups (Fig. 3C). *FCER1G* was not altered in moIDEC/HC and rather upregulated in moIDEC/AD (Fig. 3D), indicating that 1,25(OH)₂-D₃-mediated reduction of FcεRI occurs at the transcriptional level of the receptor's α-chain. FcεRI is regulated by TLR2 ligands via the upregulation of microRNA-155 and a subsequent downregulation of *PU.1*, being a main TF for *FCER1A* in *in vitro* LC (16, 17). However, 1,25(OH)₂-D₃ treatment of IDEC rather downregulated microRNA-155 and upregulated *PU.1* (Fig. 3E, 3F). As microRNA-155 and *PU.1* are involved in DC maturation,

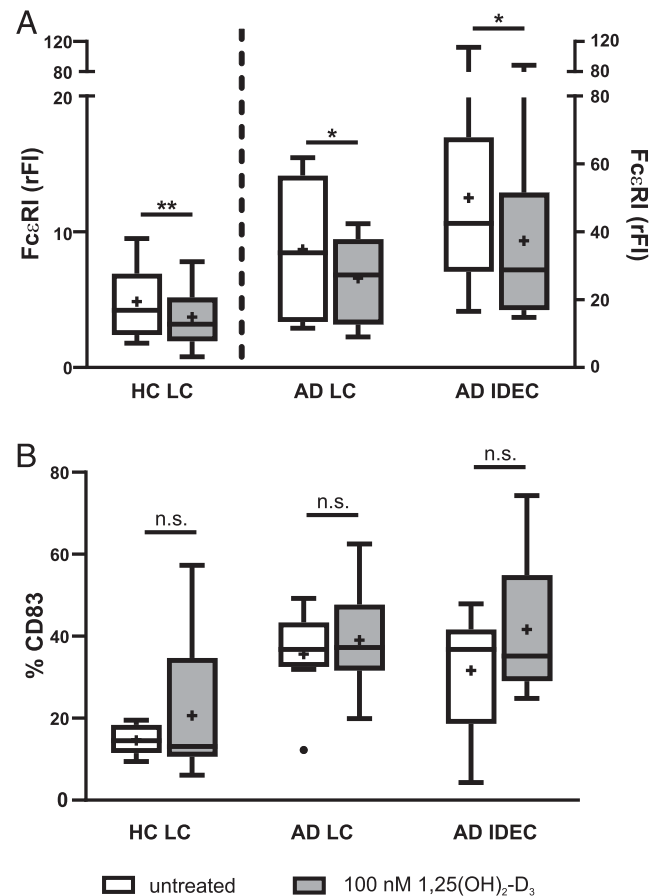


FIGURE 1. High surface expression of FcεRI on epidermal DC from ex vivo skin of AD patients is reduced by 1,25(OH)₂-D₃ independently of maturation. Ex vivo skin was prepared from healthy donors (HC) or AD patients (AD). Six-millimeter split thickness punch biopsies were cultured for 24 h in the presence (gray boxes) or absence (open boxes) of 100 nM 1,25(OH)₂-D₃. EC were isolated and stained with 7-AAD to exclude dead cells, Abs against CD1a and Langerin to discriminate LC (CD1a⁺ and Langerin⁺) from IDEC (CD1a⁺ and Langerin⁻), and with Abs against FcεRI (A) or CD83 (B) or isotype control. Cells were then analyzed by flow cytometry. Boxplots of *n* = 10 (HC) or *n* = 8 (AD) experiments are shown. In (A) (FcεRI) for HC, LC results are scaled to the left y-axis, and for AD, LC and IDEC results are scaled to the right y-axis. The median is presented as line, and the mean is presented as a plus symbol. Outliers are indicated as a black circle. Significances were calculated with SPSS applying the paired Wilcoxon rank sum test. **p* ≤ 0.05, ***p* ≤ 0.001. n.s., not significant (*p* > 0.05).

too, this further fits to our previous observation of CD83 expression (Fig. 3A). Taken together, we could not only confirm our initial observation from ex vivo skin specimen but show that 1,25(OH)₂-D₃ targets IDEC directly and reduces FcεRI expression at the transcriptional level of the α-chain. Further, 1,25(OH)₂-D₃ rather utilizes an individual, microRNA-155-PU.1-independent mechanism for this FcεRI regulation.

1,25(OH)₂-D₃-mediated reduction of FcεRI in IDEC results in impaired IgE-triggered activation

IDEC can be activated via their FcεRI receptor by IgE crosslinking. To address the functional impact of 1,25(OH)₂-D₃-mediated FcεRI reduction, we generated moIDEC/AD, left them untreated or treated them with 1,25(OH)₂-D₃ for 24 h, and then crosslinked the FcεRI with anti-IgE Abs for another 24 h. As expected, IgE crosslinking resulted in maturation (Fig. 4A, left panel and Fig. 4B) and internalization of FcεRI (Fig. 4C, left panel and

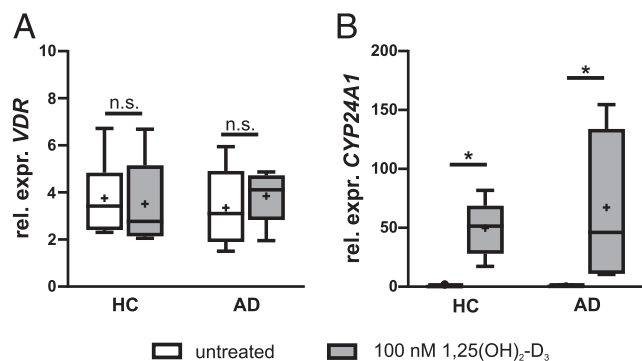


FIGURE 2. In vitro-generated IDEC express functional VDR. moIDEC were generated in vitro from monocytes from healthy donors (HC) or atopic individuals (AD) according to the protocol for IDEC generation (35) and in the presence of IgE. moIDEC were left unstimulated (open boxes) or treated with 10 nM 1,25(OH)₂-D₃ (gray boxes) for 24 h. CD1a⁺ cells were enriched by magnetic cell sorting, lysed, subjected to RNA isolation, and analyzed by qPCR for VDR [(A) $n = 5-6$] and CYP24A1 [(B) $n = 5-6$]. Results are presented as boxplots. The median is presented as line, and the mean is presented as a plus symbol. Significances were calculated applying the paired Wilcoxon rank sum test. * $p \leq 0.05$. n.s., not significant ($p > 0.05$).

Fig. 4D) of non-pretreated moIDEC/AD, whereas 1,25(OH)₂-D₃ pretreatment and subsequent absence of FcεRI (Fig. 4C, right panel and Fig. 4D) resulted in impaired maturation (Fig. 4A, right panel and Fig. 4B).

1,25(OH)₂-D₃ reduces FcεRI in in vitro-generated LC directly and independently of maturation

We wanted to confirm our observations from ex vivo skin experiments for LC, as well, and go deeper into the underlying molecular mechanisms of 1,25(OH)₂-D₃-driven FcεRI reduction. CD34LC provide a good in vitro model to address this, and TLR2-driven FcεRI downregulation is well studied, so TLR2 ligand Pam3Cys can serve as an additional control (17). First, we checked the expression of VDR in CD34LC (Fig. 5A). Compared with moIDEC, we observed a lower expression of VDR; nevertheless, VDR function and biological activity of 1,25(OH)₂-D₃ were confirmed by analysis of the target gene CYP24A1, which was induced dose dependently by 1,25(OH)₂-D₃ (Fig. 5B). Whereas Pam3Cys induced maturation, CD83 was hardly affected, and stable expressions of CD80, CD86, and MHC class II supported that 1,25(OH)₂-D₃ did not induce CD34LC activation and maturation (Fig. 5C–F). 1,25(OH)₂-D₃ downregulated FcεRI surface expression dose dependently (Fig. 5G), and mRNA levels of FCER1A and FCER1G were reduced by 1,25(OH)₂-D₃ in CD34LC dose dependently, too (Fig. 5H and data not shown). However, FcεRI reduction was not as prominent as with Pam3Cys or completely lost as observed in moIDEC. This may be due to a lower VDR expression and responsiveness in LC compared with IDEC (Fig. 2A, 2B) or monocyte-derived DC (34).

1,25(OH)₂-D₃ inhibits binding of PU.1 and YY1 to the FCER1A promotor

We analyzed microRNA-155 (Fig. 6A) and PU.1 mRNA (Fig. 6B) expression in CD34LC after 1,25(OH)₂-D₃ treatment and again observed no significant change of either one. As other TF have been described for FcεRI regulation, namely Elf-1 as an inhibitor and YY1 as an activator for FCER1A expression (36, 37), we addressed their expression. mRNA levels of both were slightly but significantly upregulated by 1,25(OH)₂-D₃ after 24 h (Supplemental Fig. 2A, 2B). Time-course experiments

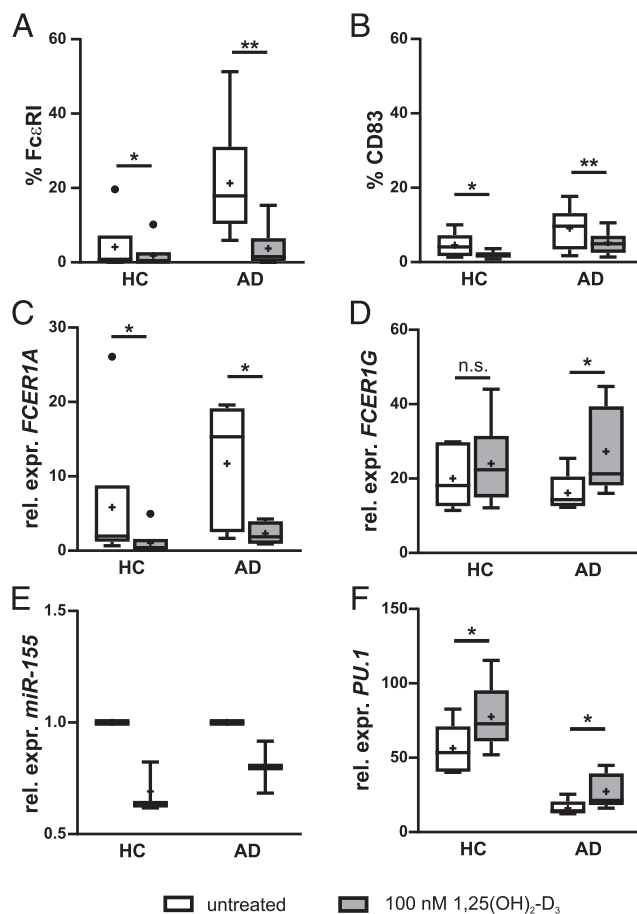


FIGURE 3. 1,25(OH)₂-D₃ directly reduces FcεRI at the transcriptional level in IDEC. moIDEC from healthy donors (HC) or atopic individuals (AD) left unstimulated (open boxes) or treated with 10 nM 1,25(OH)₂-D₃ (gray boxes) for 24 h. (A and B) Cells were stained with Abs against FcεRI (A), CD83 (B), or isotype control; CD14 and CD1a for gating; and 7-AAD for exclusion of dead cells and analyzed by flow cytometry. Boxplots of $n = 6$ (HC) or $n = 14$ (AD) experiments are shown. (C–F) CD1a⁺ cells were enriched by magnetic cell sorting, and RNA levels were analyzed by qPCR for FCER1A [(C) $n = 5-6$], FCER1G [(D) $n = 5-6$], and PU.1 [(F) $n = 5-6$] or by TaqMan microRNA assay for microRNA-155 [(E) $n = 2-3$]. Results are presented as boxplots. The median is presented as line, and the mean is presented as a plus symbol. Outliers are indicated by a black circle. Significances were calculated applying the paired Wilcoxon rank sum test. * $p \leq 0.05$, ** $p \leq 0.001$. n.s., not significant ($p > 0.05$).

revealed that FCER1A had already been downregulated within the first 6 h (Fig. 6C, 6D, Supplemental Fig. 2C–E), whereas ELF-1 (Fig. 6C) and YY1 (Fig. 6D) were regulated at later time points. PU.1 mRNA was not altered at any time point (data not shown). Other positive transcription regulators have been described for FcεRI expression. We checked expression levels of HMGB1, HMGB2, and SPI and found an upregulation rather at late time points (Supplemental Fig. 2C–E). This would not explain the rapid downregulation of FcεRI. We wondered, if not the TF expression levels, then maybe their binding profile to the promotor is involved in 1,25(OH)₂-D₃-induced reduction of FcεRI. We applied ChIP and subsequent qPCR with primers for the promotor region of FCER1A to analyze the binding of the known TF for the FcεRI α-chain. We found robust binding of PU.1 as well as YY1 in untreated cells (Fig. 6E open boxes). This binding was reduced significantly for both TF after 1,25(OH)₂-D₃ treatment (Fig. 6E, gray boxes). Binding of transcription inhibitor Elf-1 and VDR itself was not detected under both conditions, excluding a competitive-binding

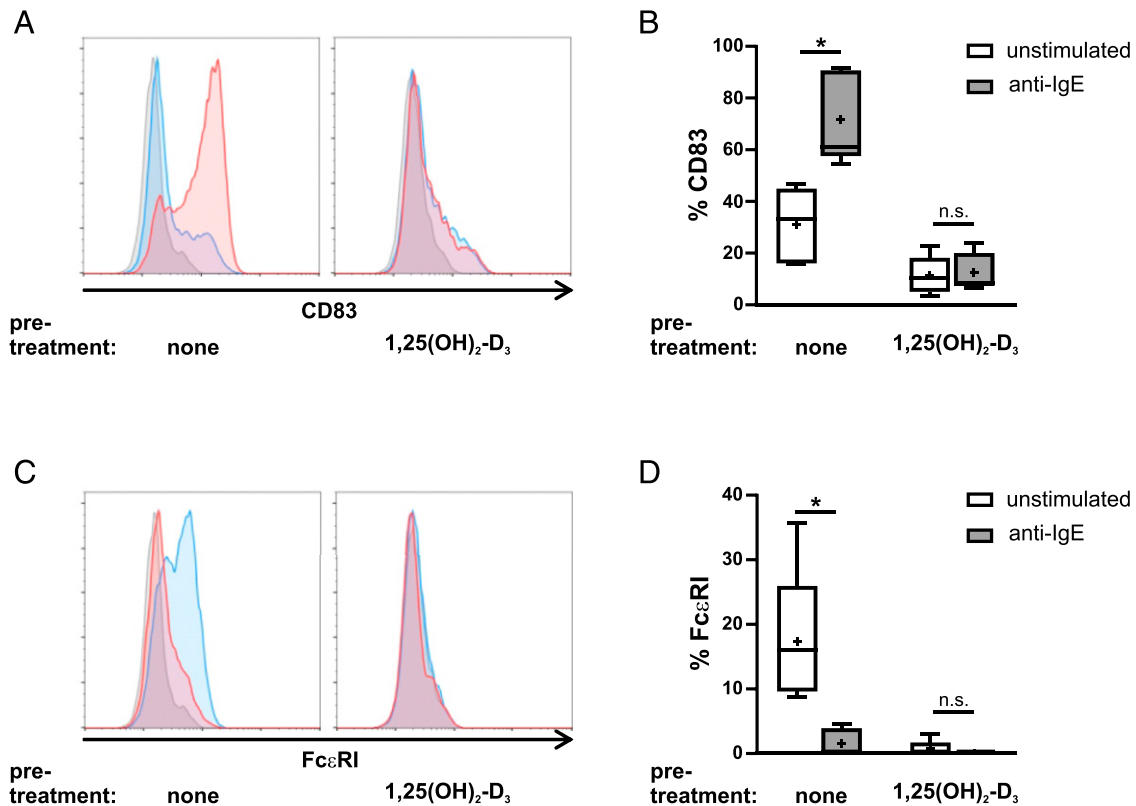


FIGURE 4. 1,25(OH)₂-D₃-mediated downregulation of FcεRI on moIDEC results in impaired IgE-mediated activation. moIDEC from atopic individuals were either left untreated (none; left panel) or pretreated with 10 nM 1,25(OH)₂-D₃ (right panel) for 24 h. Cells were then left unstimulated (blue lines or open boxes) or stimulated with anti-human IgE Abs (anti-IgE, red lines or gray boxes) for another 24 h. Cells were then analyzed by flow cytometry after staining with Abs against CD83 (A and B) or FcεRI (C and D) or isotype control [gray in (A) and (C)] and CD14 and CD1a for gating on DC. Dead cells were excluded from analysis by 7-AAD staining. (A and C) Results of one representative experiment; (B and D) results of *n* = 5 experiments presented as boxplots. The median is presented as line, and the mean is presented as a plus symbol. Significances were calculated with SPSS applying the paired Wilcoxon rank sum test. **p* ≤ 0.05. n.s., not significant (*p* > 0.05).

mechanism. Taken together, 1,25(OH)₂-D₃ prevents PU.1 and YY1 binding to the *FCER1A* promoter, resulting in the reduction of FcεRI.

Discussion

It is well known that sunlight and UVB exposure have a beneficial role in AD. UVB treatment of healthy individuals and psoriasis and AD patients results in higher vitamin D₃ levels (summarized in Ref. 38). Thus, in the past years, several studies addressed the role of vitamin D₃ in AD concerning the severity of the disease and IgE serum levels but with inconsistent results (reviewed in Ref. 19, 20). Although topical treatment is not recommended and may change for the worse, oral supplementation can improve the clinical situation at least in a subgroup of AD patients, namely those with allergic sensitization (28, 29). In our study, we suggest that this results from a direct impact of vitamin D₃ on the expression of the high-affinity receptor for IgE, FcεRI (the major sensor for allergens), on DC. We show a reduction of FcεRI by active vitamin D₃ in situ on LC and IDEC. Indeed, a reduction of FcεRI⁺ EC upon UVB phototherapy in AD has been reported (39). Our data support the assumption that the loss of FcεRI⁺ cells after UV treatment in AD may result from vitamin D₃-dependent FcεRI reduction on the cell surface itself instead of or in addition to emigration of certain cell subsets from the epidermis. A recent study about children with AD shows lower FcεRI expression on blood DC in vivo after oral vitamin D₃ treatment and in vitro after treatment of whole blood (40). Our data expand this knowledge not only to skin DC but show further that vitamin D₃ acts directly

on LC and IDEC. We showed VDR expression and function in IDEC, which is more prominent compared with LC. Moreover, 1,25(OH)₂-D₃-treated in vitro IDEC could not be activated by FcεRI crosslinking. Most likely, this impaired IgE-mediated function is a result of the diminished receptor expression. Indeed, it was reported previously that CD1a⁺ EC from patients treated with tacrolimus showed reduced FcεRI expression accompanied by inhibited capability to induce T cell response (41). Nevertheless, additional signaling mechanisms may be involved, similar to c-kit downregulation by vitamin D₃ in mouse mast cells and its association with DC development and maturation (42). Vitamin D₃ inhibits mast cell activation by increased VDR expression, which then in turn binds to Lyn and inhibits complex formation with FcεRI-β and Syk as well as downstream phosphorylation events, including those of FcεRI-β, Syk, MAPK, and NF-κB (43). However, we observed no difference in VDR expression levels after vitamin D₃ treatment in IDEC (Fig. 2A), and the β-chain of the receptor complex is absent in APC (15, 44). In our study, the regulatory mechanisms in IDEC are rather based on the reduction of the α-chain (Fig. 3C).

Even though the in vitro IDEC and LC are capable to mature after different stimuli in general, 1,25(OH)₂-D₃ alone did not induce maturation, as we show by expression analyses of the maturation marker CD83 and costimulatory molecules. Thus, in contrast to the regulation of FcεRI by TLR2 ligands we showed previously (16, 17), 1,25(OH)₂-D₃ can regulate FcεRI expression without induction of maturation. In line, we show that 1,25(OH)₂-D₃ directly regulates FcεRI expression at the transcriptional

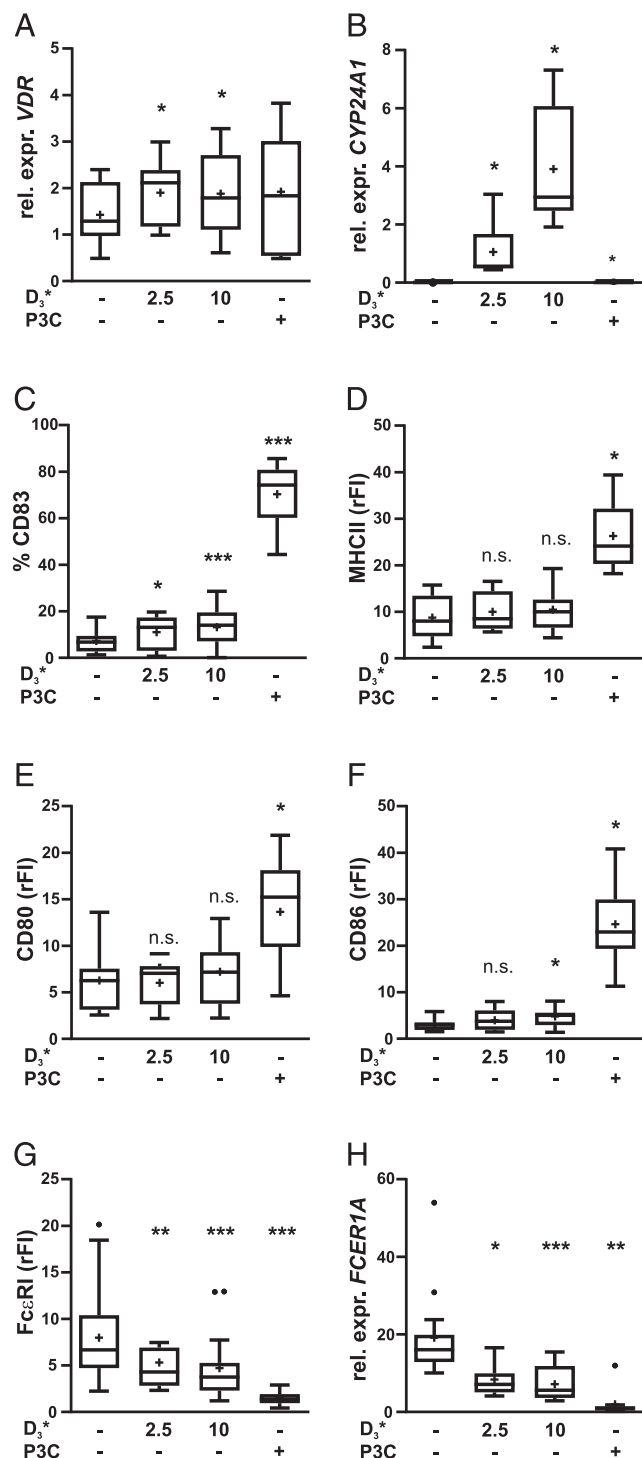


FIGURE 5. 1,25(OH)₂-D₃ reduces FcεRI in in vitro-generated LC directly and independently of maturation. CD34⁺LC were generated in vitro from hematopoietic stem cells and left unstimulated (minus symbol) or stimulated for 24 h with indicated concentrations of 1,25(OH)₂-D₃ (D₃*; in nM) or 1 μg/ml TLR2 ligand Pam3Cys (P3C; plus symbol) and subjected to flow cytometry (C–G) or magnetic cell sorting for CD1a⁺ cells followed by RNA preparation and qPCR (A, B, and H). Cells were stained with Abs against CD83 (C), MHC class II (D), CD80 (E), CD86 (F), and FcεRI (G) or isotype control and counterstained with CD1a and CD14 Abs. Dead cells were excluded from analysis by 7-AAD staining. mRNA levels of *VDR* (A), *CYP24A1* (B), and *FCER1A* (H) were analyzed by qPCR. Results are presented as boxplots. The median is presented as a line, and the mean is presented as a plus symbol. Outliers are indicated by a black circle. Significances were calculated with SPSS applying the paired Wilcoxon rank sum test. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001. n.s., not significant (*p* > 0.05).

level of its α-chain in epidermal DC but independently of the microRNA-155-PU.1-axis. Expression profiles of known transcriptional regulators could not explain this phenomenon. Vitamin D₃ has mainly been described and analyzed as gene activator. It binds to its receptor VDR, which in turn binds as a heterodimer with retinoid X receptor RXR to vitamin D response elements and activates gene transcriptions. In contrast, little is known about the mechanisms by which vitamin D₃ acts suppressively on gene transcription. Several explanations, including competition with TF for DNA binding or coreceptor binding and chromatin arrangement, have been proposed (45). The suppressive effect is dependent on natural promotor and chromatin context, but isolated suppressive vitamin D responsive elements act as positive, activating elements (45). Seuter et al. (46) analyzed the cistrome of PU.1 and VDR and found a significant modification of the PU.1 cistrome by 1,25(OH)₂-D₃. Most notably, PU.1 binding to genomic DNA was increased by 1,25(OH)₂-D₃. However, 11.6% of the loci still showed reduced binding of PU.1 after treatment with 1,25(OH)₂-D₃. Whereas increased PU.1 binding correlated strongly with an overlap of PU.1 and VDR/DR3 loci, this was not the case for decreased binding. We investigated the binding of TF to the *FCER1A* promotor and found that 1,25(OH)₂-D₃ treatment results in dissociation of PU.1 and YY1 from the promotor of *FCER1A*. As assumed from in silico analysis and in line with the reported lack of PU.1-VDR/DR3 loci overlap for reduced PU.1 binding, VDR itself did not bind to the *FCER1A* promotor. Elf-1 can compete with TF PU.1 and YY1 for *FCER1A* promotor binding (37). However, we observed no binding of Elf-1 to the *FCER1A* promotor, indicating Elf-1 is not involved in vitamin D₃-mediated FcεRI regulation. In other allergic diseases and asthma, the key roles of IgE and FcεRI have been investigated mainly in mast cells and basophils. 25(OH)-hydroxyvitamin D₃ and 1,25(OH)₂-D₃ repress FcεRI-mediated activation in mast cells, shown, for example, by impaired histamine release (47). To our knowledge, the influence of vitamin D₃ on the expression level of the receptor itself has not been addressed, and one could speculate about a general mechanism how vitamin D₃ may regulate several IgE-mediated diseases.

Vitamin D₃ can induce tolerogenic DC in general, which are inhibited in their activation capacity, express tolerogenic molecules, and induce regulatory T cells (48, 49). Subsequently, the inflammatory outcome of an induced immune response is dramatically changed in the presence of vitamin D₃. Ig-like transcript (ILT) 2 and ILT3 both are negative regulators in DC bearing ITIM and inducing or supporting tolerance (50, 51). Indeed, in the presence of 1,25(OH)₂-D₃, ILT2 and ILT3 are increased and IgE-mediated downregulation of both ILT2 and ILT3 is inhibited in IDEC (data not shown). These mechanisms may contribute to a putative beneficial role of vitamin D₃ in AD. However, Akan et al. (28) showed that serum vitamin D₃ correlates negatively with SCORAD in AD only in patients with IgE-mediated allergic sensitization. Others emphasized the negative correlation of vitamin D₃ and AD severity in individuals with food allergy (29). Both classified the patients by analyses of specific IgE rather than total IgE. Together with our results, this association may explain different reports regarding the correlation of vitamin D₃ serum levels and AD severity and connect it to an IgE-dependent process. Indeed, the diseases' phenotype can differ dramatically in total IgE levels. In contrast, some AD patients may show or develop allergies and/or asthma, whereas others do not. Thus, vitamin D₃ may tolerize epidermal DC specifically to FcεRI engagement by the receptor reduction we show in this article and general tolerogenic mechanisms described above. Taken together, it is not surprising that the vitamin D₃ effect has different

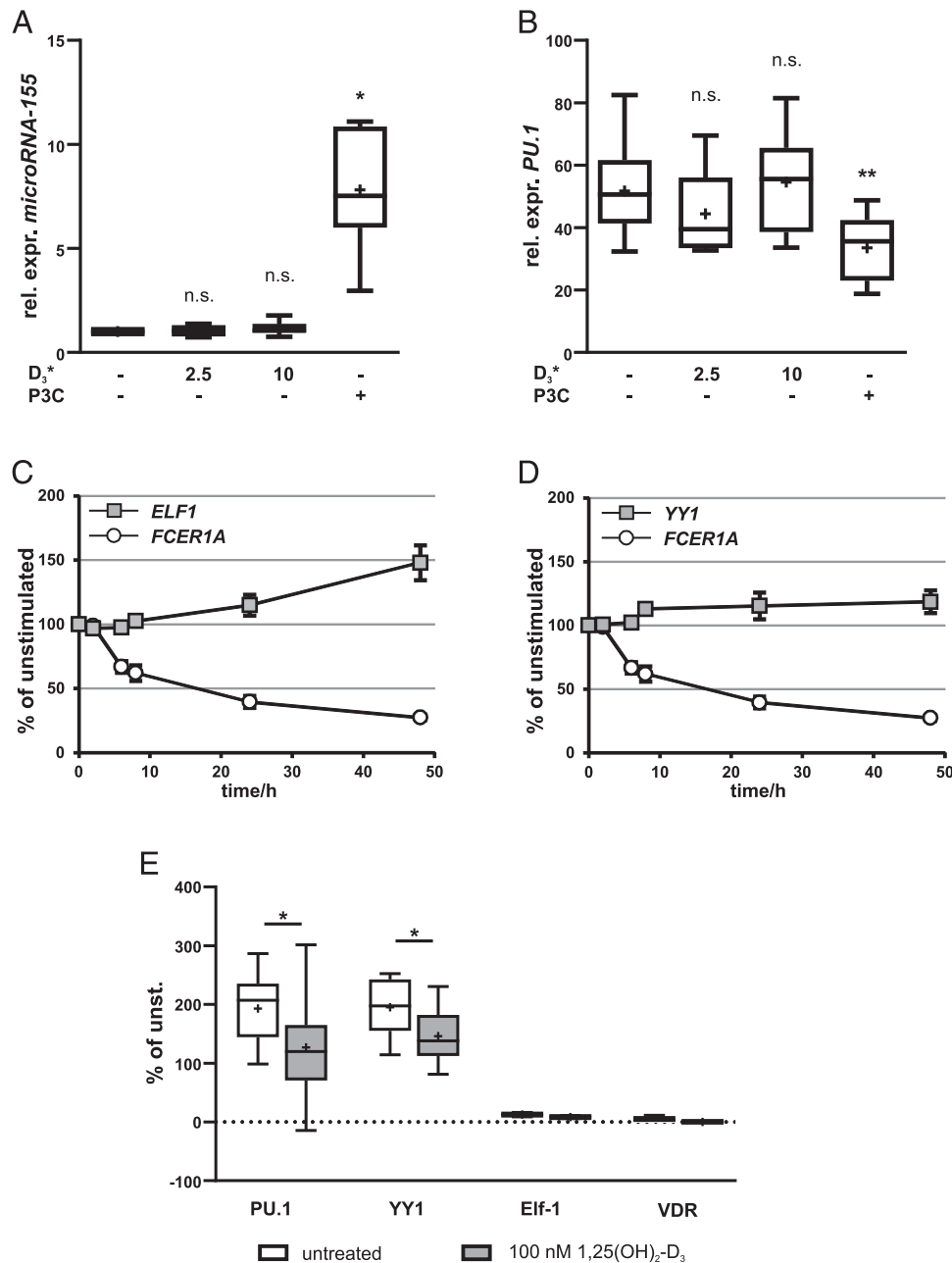


FIGURE 6. 1,25(OH)₂-D₃ inhibits binding of PU.1 and YY1 to the *FCER1A* promoter. (**A** and **B**) CD34⁺ cells were left unstimulated (minus symbol) or stimulated for 24 h with indicated concentrations of 1,25(OH)₂-D₃ (D₃*; in nM) or 1 μg/ml TLR2 ligand Pam3Cys (P3C; plus symbol). CD1a⁺ cells were enriched and subjected to TaqMan microRNA Assay for *microRNA-155* (**A**) or qPCR for *PU.1* (**B**). Results are presented as boxplots. Significances were calculated with SPSS applying the paired Wilcoxon rank sum test. (**C** and **D**) CD34⁺ cells were left unstimulated or treated with 10 nM 1,25(OH)₂-D₃ for the indicated period of time, and CD1a⁺ cells were enriched and subjected to qPCR of the respective target. Relative expression levels were calculated, and mean values of $n = 5$ experiments are presented as percentage of expression in unstimulated cells at the same timepoint. (**E**) CD34⁺ cells were left unstimulated or stimulated with 10 nM 1,25(OH)₂-D₃ for 24 h, harvested, and lysed. Lysates were kept as input sample or subjected to ChIP with Abs against PU.1, YY1, Elf-1, and VDR or the respective isotype control for background analysis. Precipitates and input samples were analyzed by qPCR with primers for the proximal promoter for *FCER1A*. Specific relative binding of the respective TF was calculated from precipitated promoter region as follows: (molecules [ChIP_TF]/molecules [input sample]) – (molecules [ChIP_isotype control]/molecules [input sample]). Results are shown as boxplot for $n = 9$ (PU.1), $n = 6$ (YY1), $n = 2$ (Elf-1), or $n = 4$ (VDR) experiments. The median is presented as line, and the mean is presented as a plus symbol. Significances were calculated with SPSS applying the paired Wilcoxon rank sum test. * $p \leq 0.05$, ** $p \leq 0.01$. n.s., not significant ($p > 0.05$).

outcomes in different patients, suggesting that individual rather than general therapeutic strategies may involve vitamin D₃.

Applying ex vivo skin, we could further show a significant vitamin D₃-mediated downregulation of FcεRI in situ. However, the high expression of the receptor on LC and IDEC in AD skin could not be reduced to a level found in HC LC, where FcεRI is expressed weakly. The so-far-unknown mechanisms in AD skin,

which are responsible for the high surface expression of FcεRI in epidermal DC, may counteract the vitamin D₃ effect in situ, so a diminished but not an abolished surface expression is reached. Nevertheless, IDEC seem to be slightly more responsive to 1,25(OH)₂-D₃ than LC, reflecting our in vitro results discussed above.

Recently, it has been reported that DC in psoriatic lesions express FcεRI, too (52). Treatment strategies of psoriasis include, besides

phototherapy, topical treatment with vitamin D₃ derivatives like calcipotriol, directly targeting keratinocytes but also modulating DC cytokine profiles (53–55). Our data suggest that vitamin D₃ might also act directly at the DC level by reducing FcεRI expression. However, the role of FcεRI in psoriasis remains to be investigated.

Taken together, we provide evidence for a new mechanism of action of vitamin D₃ in AD by virtue of the direct regulation of FcεRI on DC. This may explain the connection of the vitamin D₃ impact in AD to allergic sensitization and the conflicting results of different studies and reflects the complexity of the disease. Further investigations are needed to understand the vitamin D₃ paradigm to open new avenues in the development of therapeutic approaches in a broad range of IgE-related diseases like AD and allergies.

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Disclosures

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