25-Hydroxvitamin D$_3$ Promotes the Long-Term Effect of Specific Immunotherapy in a Murine Allergy Model

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25-Hydroxvitamin D₃ Promotes the Long-Term Effect of Specific Immunotherapy in a Murine Allergy Model

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Calcitriol (1α,25-dihydroxyvitamin D₃) is the active vitamin D metabolite and mediates immunological functions, which are relevant in allergy. Its therapeutic use is limited by hypercalcaemic toxicity. We have previously shown that the activation of the vitamin D receptor inhibits IgE expression in B cells and that B cells can synthesize calcitriol from its precursor 25-hydroxyvitamin D₃ (inactive precursor) [25(OH)D] upon antigenic stimulation. In this study, we address the impact of 25(OH)D on the development of type I sensitization and determine its role in allergen-specific immunotherapy. BALB/c mice were sensitized to OVA, under 25(OH)D-deficient or sufficient conditions. The humoral immune response over time was measured by ELISA. OVA-specific immunotherapy was established and studied in a murine model of allergic airway inflammation using lung histology, pulmonary cytokine expression analysis, and functional parameters in isolated and perfused mouse lungs. In 25(OH)D-deficient mice, OVA-specific IgE and IgG1 serum concentrations were increased compared with control mice. OVA-specific immunotherapy reduced the humoral immune reaction after OVA recall dose-dependently. Coadministration of 25(OH)D in the context of OVA-specific immunotherapy reduced the allergic airway inflammation and responsiveness upon OVA challenge. These findings were paralleled by reduced Th2 cytokine expression in the lungs. In conclusion, 25(OH)D deficiency promotes the development of type I sensitization and correction of its serum concentrations enhances the benefit of specific immunotherapy. The Journal of Immunology, 2014, 193: 000–000.

The active metabolite of vitamin D termed calcitriol (1α,25-dihydroxyvitamin D₃) mediates anti-inflammatory functions after binding to the vitamin D receptor (VDR), which then acts as a transcription factor regulating specific target gene expression (1). We have previously shown that vitamin D receptor activation inhibits IgE expression in B cells (2) via recruitment of a VDR-DNA complex to the μ-switch transcript promoter (3, 4). Furthermore, VDR activation enhances IL-10 expression in B cells after binding to its promoter (4). Accordingly, we determined that allergen-specific IgE was diminished upon VDR activation in mice (5).

Data from humans indicate that calcitriol pulse therapy induced IL-10–expressing T cells in patients with steroid-resistant allergic asthma, which was associated with an improved lung function (6). However, the long-term therapeutic use of calcitriol is limited by hypercalcemic side effects. We and others demonstrated that activated B lymphocytes (4), T cells (7), and myeloid APCs (8, 9) can synthesize biologically active calcitriol from 25-hydroxyvitamin D₃ (inactive precursor) [25(OH)D]; Supplemental Fig. 1). Thus, 25(OH)D may modulate immune reactions. Epidemiologic data from a representative cohort suggested increased serum IgE concentrations in vitamin D–deficient individuals (<50 nmol/L 25(OH) D) in contrast to vitamin D–sufficient individuals (105–115 nmol/L 25(OH)D) (10). Cord blood 25(OH)D levels were correlated with IL-10 levels and inversely with total IgE titers (11). In allergic asthma patients, vitamin D deficiency has been associated with increased requirement of inhaled corticosteroids (12) severe exacerbations, hospitalizations, and elevated IgE serum concentrations (13, 14).

To date, the only available treatment, which targets the cause of type I allergy, modifies the disease and exceeds the treatment period, is specific immunotherapy (15). By repetitive allergen exposure immune cells are targeted and modulated in their function (15). The exact cellular and molecular mechanisms of specific immunotherapy (SIT) are not fully understood, but accepted parameters include changes on the humoral level like the induction of specific IgG4 (16) in conjunction with increased frequencies of allergen-specific IL-10–secreting (17) and/or Foxp³⁺ Th cells (18). Recent data from a murine model of SIT suggested that calcitriol administered in conjunction with the allergen, further reduced allergic airway inflammation (19).

In the current study, we investigated whether and to what extent 25(OH)D might be relevant during the process of type I sensiti-
zation and desensitization. Our data show that both the sensitization and the desensitization by SIT are modulated by 25(OH)D leading to reduced humoral specific IgE and IgG1 induction and reduction of allergic airway inflammation following allergen exposure in vitamin D-deficient mice.

Materials and Methods

Animals and treatments

All procedures were in accordance with the local State Office of Health and Social Affairs. Female BALB/c mice (strain cByJr, Elevage Janvier, Le Genest St Isle, France) were held in a specific pathogen-free environment. Mice were fed with a vitamin D-deficient diet (Altromin, Lage or Sniff, Soest) or regular chow (control: 100 mg/kg vitamin D). Murine 25(OH)D serum concentrations were determined by EIA (IDS Hamburg) and deficiency was considered at concentrations below 50 nmol/l (1 ng/ml = 2.5 nmol/l). Ig measurement

Serum concentrations of murine total and OVA-specific IgE, IgG subclasses, IgM, and IgA were determined by ELISA as described previously (21, 23). Briefly, 96-well Maxisorb plates (Nunc, Darmstadt, Germany) were coated with anti-Ig mAbs or OVA (all 5 mg/ml from Southern Biotechnology Associates, Eching, Germany, or Sigma-Aldrich, respectively). Serum samples and standards were diluted serially in 1% skimmed milk/PBS and incubated for 2 h. The amounts of subclass-specific Ig and OVA-specific IgG1, IgG2a, IgM, and IgA were detected by a biotinylated isotype-specific Ab (Southern Biotechnology Associates) in optimal dilution followed by incubation with secondary Abs (Alexa488-labeled donkey anti-rat and Alexa555 labels donkey anti-goat; both Life Technologies, Darmstadt, Germany) and the desensitization by SIT are modulated by 25(OH)D leading to reduced humoral specific memory cells systemically without their homing primarily to the lung and to avoid interference of newly formed infiltrating cells attracted by the airway challenge. In these experiments, mice were exposed to aerosolized OVA (10 mg/ml dissolved in PBS) for 20 min/2 wk after OVA recall on days 102–104 and lung function was determined in isolated perfused and ventilated mouse lungs on day 106 (Fig. 3A).

Airway and pulmonary vascular responsiveness in isolated perfused and ventilated mouse lungs

Mature lungs were isolated as previously described (24, 25) perfused with 37°C sterile Krebs–Henseleit hydroxethyl amine lactoperoxidase buffer in a non-recirculating fashion (1 ml/min) and ventilated with constant negative pressure (-4.5 to -9 cm H2O; 90 breaths/min) in a closed chamber. Left atrial pressure was adjusted at +2.2 cm H2O. Airway resistance (resaw), dynamic lung compliance (Cdyn), tidal volume (TV), and mean pulmonary arterial pressure (Ppa mean) were monitored continuously (26). After a steady-state period of 20 min, baseline parameters were assessed and methacholine (MCh) or serotonin (Sigma-Aldrich) was administered to the perfusate for 0.5 min at 8-min intervals. Differences in resaw (Aresaw) and Ppa mean (Apapa mean) were expressed in cmH2O/ml and cmH2O, respectively. The area under curve was calculated.

Bronchoalveolar lavage fluids

Bronchoalveolar lavage was performed twice using 800 μl PBS as described previously (24, 25). Leukocytes were differentiated by means of microscopic analysis in a blinded manner (400 cells counted/individual).

The following cytokines from bronchoalveolar lavage fluid (BALF) supernatant were quantified in duplicate using a cytokine multiplex assay (Bioplex; Bio-Rad, Hercules, CA): IL-4, IL-5, IL-10, IL-12p40, and IL-13.

Collection of lung tissue and histology

Lungs were inflated through the trachea with 4% parformaldehyde at a pressure of 20 cmH2O for 15 min and embedded in paraﬁn. The tissue sections (1–2 μm) were deparaffinized and blocked after a heat-induced retrieval step with either avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) or peroxidase block (DakoCytof, Hamburg, Germany). After 1 h of incubation at room temperature, Abs against CD3 (dialysed rabbit Ab; DakoCytof), B220 (RA3-6B2; eBioscience, San Diego, CA), or cytochrome P450 subfamily 27 B polypeptide 1 (CP27B1; PC290; Binding Site, Schwetzingen, Germany) sections were blocked with 10% donkey and rabbit serum, respectively, followed by incubation with secondary Abs (all Dianova, Hamburg, Germany) for 30 min at room temperature. For detection, Streptavidin-AP kit and EnVi-

sion kit (DakoCytof) were used. Nuclei were counterstained with hematoxylin and slides mounted with gelatin (Merek, Darmstadt, Germany). Negative controls were performed omitting primary Abs. Images were acquired using an AxioImager Z1 microscope (Carl Zeiss Micro-Imaging, Jena, Germany). Positive cells were quantified per high-power field (HPF = 0.237 mm2), and 5 HPF were averaged in each case. In situ detection of IgG1/IgA/B220 was performed using immunofluorescence. One to 2-μm parafﬁn sections were deparaffinized and subjected to a heat-induced retrieval step. Slides were incubated overnight at 4°C with Abs directed against IgG1 (A85-1; BD Biosciences) and IgA (polyclonal goat; Southern Biotechnology Associates) in optimal dilution followed by incubation with secondary Abs (Alexa488-labeled donkey anti-rat and Alexa555 labels donkey anti-goat; both Life Technologies, Darmstadt, Germany) for 30 min at room temperature. After rinsing, Cy5-labeled primary Ab against B220 (RA3-6B2) was applied for 30 min at room temperature. Nuclei were stained with DAPI (Sigma-Aldrich, Munich, Germany) and slides mounted with Fluoromount G (Sigma-Aldrich). Negative controls were performed omitting primary Abs. Images were acquired using an AxioImager Z1 microscope (Carl Zeiss Microimaging, Jena, Germany). Positive cells were quantified per HPF (0.237 mm2), and 5 HPF were averaged in each case. All immunohistochemical evaluations were performed in a blinded manner.

Statistical analysis

Normal distribution was tested by Kolmogorov–Smirnov test. Parametric values were tested by Student t test and non-parametric values by Mann–Whitney U test. Two-way ANOVA was used for comparison of dose-response curves (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA; SPSS19, IBM). The p values depicted as *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 were considered statistically significant.

Results

25(OH)D deficiency promotes the IgE response after sensitization

To investigate the impact of 25(OH)D on the humoral type I allergic immune reaction, we first established 25(OH)D deficiency [serum concentration < 50 nmol/l (20)] in BALB/c mice by nutritional restriction. 25(OH)D deficiency (mean 15.2 ± 0.9 nmol/l), which was achieved within 2 wk, was stable over the course of the experiment and clearly differing from control mice kept on regular chow (p ≤ 0.001) (Fig. 1A, 1B). The following OVA sensitization induced OVA-specific IgE and IgG1 serum concentrations in all mice in comparable amounts (Fig. 1C, 1D). After OVA recall on day 64 and 95, the OVA-specific IgE and IgG1 serum concentrations increased significantly in 25(OH)D-deficient mice compared with control mice leading to maximum values on day 101 (+174 and +433%; p ≤ 0.01–0.001). The sensitization-induced OVA-specific and total IgE, IgM, IgA, and IgG2a serum concentrations were comparable between all groups after OVA recall (see Supplemental Fig. 2A–E; data not shown).

Efficient specific immunotherapy in 25(OH)D-deficient mice

To investigate the role of 25(OH)D on the type I allergic immune reaction when combined with allergen-specific immunotherapy (SIT), we analyzed the specific humoral response of 25(OH)D-deficient and sufficient control mice that received 3 weekly OVA-SIT injections in increasing dosages (Fig. 2A). In SIT dosages
of ≥100 μg, serum concentrations of allergen-specific IgE were significantly reduced in 25(OH)D-deficient mice, whereas specific IgG1 was enhanced \( (p = 0.05; \text{Fig. 2B, 2C}). \) Serum OVA-IgE was comparable between 25(OH)D-deficient mice after 1 mg SIT and sufficient controls without SIT \( (P > 0.05; \text{Supplemental Fig. 2}). \) In 25(OH)D-sufficient control mice, the humoral immune response was significantly altered by SIT \( (\text{Supplemental Fig. 2}). \) The OVA-specific IgG2a, IgM, and IgA \( (\text{Supplemental Fig. 2}) \) and the polyclonal Ig-profile including IgE, IgG1, IgG2a, IgM, and IgA \( (\text{data not shown}) \) did not differ significantly among both groups. The humoral-specific IgE response is not altered significantly, especially not rescued completely, by treatment of sensitized mice with 25(OH)D without Ag \( (p = 0.11; \text{Supplemental Fig. 3}) \), and thus, this group was excluded from further analysis. These data suggest that strong Ag signaling during SIT may override 25(OH)D-mediated immunomodulation, which itself is depending on simultaneous Ag exposure. Thus, we chose a suboptimal SIT protocol using 100 μg OVA in all further experiments in 25(OH)D-deficient mice and extended the analysis to its impact on murine airway inflammation.

The Journal of Immunology 3

Figure 1. Enhanced humoral allergic immune response in 25(OH)D-deficient mice. \( (\text{A}) \) BALB/c mice were fed with a 25(OH)D restriction (black dotted line) or regular diet (black line). Plasma 25(OH)D concentration (deficiency < 50 nmol/l, gray dotted line) were monitored over time \( (n = 32); (\text{B}) \) Experimental setup. Arrows indicate OVA sensitization \( (i.p., 10 \mu g) \) and OVA challenges \( (s.c., 10 \mu g) \) on day 64 and OVA recall \( (i.p., 10 \mu g) \) on day 95. Serum concentrations of OVA-specific IgE \( (\text{C}) \) and IgG1 \( (\text{D}) \) were measured by ELISA from the 25(OH)D restriction or regular diet group. Data show mean ± SEM, \( n = 8 \). ** \( p \leq 0.01, *** \( p \leq 0.001 \) compared with control (without SIT).

Figure 2. Dose-dependent modulation of humoral allergen-specific Igs by immunotherapy. \( (\text{A}) \) Experimental setup as described in Fig. 1A. 25(OH)D-deficient BALB/c mice were OVA sensitized. On day 42, a SIT was applied by weekly injections of increasing doses OVA \( (s.c., 10–1000 \mu g) \). Arrows indicate OVA injections. Monitoring of OVA-specific IgE \( (\text{B}) \) and IgG1 \( (\text{C}) \) serum concentrations. Data show mean ± SEM, \( n = 8 \). * \( p \leq 0.05, ** \( p \leq 0.01, *** \( p \leq 0.001 \) compared with control (without SIT).

Subsequently, we investigated the impact of 25(OH)D on the humoral immune reaction when applied in conjunction with SIT—25(OH)D in combination with SIT \( [\text{D-SIT}] \) —in 25(OH)D-deficient mice and its functional relevance in a model of allergic airway inflammation (Fig. 3A). Initial dose-response analysis indicated that three weekly administrations of \( >25 \mu g/kg \) body weight 25(OH)D were required to achieve sufficiency \( (>80 \text{ nmol/l}) \) (Fig. 3B). In all other groups, 25(OH)D deficiency remained stable over time \( (\text{mean}, 38.6 ± 0.9 \text{ nmol/l}) \) (Fig. 3B). In all sensitized groups, the allergen-specific Ig profile was comparable until day 88 \( (\text{data not shown}) \). Because of OVA recall on day 88, specific IgE and IgG1 concentrations were strongly induced in OVA-sensitized and challenged mice \( (O/O) \) but not in mice that received immunotherapy without \( (O/O+SIT) \) or with \( (25(OH)D) \) \( (O/O+D-SIT) \) \( (\text{Fig. 3C, 3D}) \). In addition, serum OVA-IgG1 levels in the D-SIT group were lower compared with SIT alone \( (\text{Fig. 3D}) \). In mice with pronounced vitamin D deficiency \( (\text{serum } 25(OH)D \text{ concentrations} < 15 \text{ nmol/l}) \), the OVA recall induced humoral-specific IgE, and IgG1 response was blocked by both SIT alone or in combination with 25(OH)D, with little stronger effects by D-SIT \( (\text{Supplemental Fig. 4}) \).
25(OH)D enhances the effects of SIT

To prove clinical efficacy of D-SIT, we investigated its impact on murine allergic lung inflammation in OVA-sensitized 25(OH)D-deficient mice following immunotherapy, OVA recall to the immune cells (i.p.) and airway exposure with aerosolized OVA (Fig. 4). Functional lung parameters at baseline levels showed decreased dynamic lung compliance (226.9 ± 4.0%) and tidal volume per body weight (225.6% ± 4.0) in lungs of O/O compared with lungs of nonsensitized and OVA-challenged control mice (PBS/O, p # 0.01; Fig. 4A, 4B). Although only marginally influenced by SIT (O/O+SIT), D-SIT (O/O+D-SIT) significantly improved dynamic lung compliance and tidal volume per body weight compared with OVA-sensitized and challenged mice without SIT (O/O, p # 0.01–0.05) to an extent comparable with nonsensitized control mice (PBS/O, both p = 0.4; Fig. 4A, 4B). No difference in baseline airway resistance was observed between the groups (data not shown).

Airway hyperresponsiveness (AHR) upon MCh provocation was detected in all OVA-sensitized groups (O/O, O/O+SIT, and O/O+D-SIT) compared with nonsensitized mice (PBS/O, all p # 0.01 and p # 0.05) (Fig. 4C). Reflecting our findings on the baseline parameters, D-SIT, in contrast to SIT alone, decreased AHR compared with OVA-sensitized mice without SIT (O/O). In OVA-sensitized mice with pronounced 25(OH)D deficiency (<15 nmol/l), distinct AHR was observed and the impact of D-SIT was pronounced compared with SIT alone as reflected by significantly diminished AHR (Supplemental Fig. 4D).

Immune cell infiltration and cytokine expression is reduced in the lungs following D-SIT but not SIT alone

We investigated the impact of D-SIT on the pulmonary immune reaction by analyzing cellular subsets and cytokine expression in the BALF from mice treated as described in Fig. 3. The immune cell counts were 8-fold higher in OVA-sensitized and challenged mice (O/O) compared with nonsensitized mice (PBS/O, p # 0.001) (Fig. 5A). SIT and D-SIT strongly reduced the immune cell influx (p # 0.05), mainly of eosinophils. BALF concentrations of IL-4, IL-12p40, and IL-13 were strongly in-
increased in sensitized mice (O/O) compared with nonsensitized mice (PBS/O). Paralleling the lung function parameters, these Th2 cytokines were reduced by D-SIT, but not by SIT alone, compared with sensitized mice without SIT (O/O) (all p ≤ 0.05, IL-12p40 p = 0.054; Fig. 5B–D), BALF concentrations of IL-5 and IL-10 were comparable between all sensitized mice (O/O, O/O+SIT, and O/O+D-SIT; data not shown).

Histological analysis showed a strong pulmonary CD3⁺ T cell infiltration in OVA-sensitized mice (O/O, O/O+SIT, and O/O+D-SIT) compared with nonsensitized mice (PBS/O) (Fig. 6A). Mean CD3⁺ cell infiltration was 15% lower after D-SIT compared with SIT alone (p > 0.05). Interestingly, B cell influx in OVA-sensitized mice (O/O) was increased after D-SIT (p ≤ 0.05), but not after SIT alone (O/O+SIT), reflecting reduced lung inflammation (Fig. 6B). The ratio of IgG1⁺/IgA⁺ infiltrating B220⁺ B cells in sensitized mice (O/O) was increased by SIT and D-SIT but failed statistical significance (from 0.4 to 1.0 and 1.5; p = 0.31 and 0.28; data not shown).

Remarkably, cytochrome P450 subfamily 27 B polypeptide 1, also referred to as 25-hydroxyvitamin D₃-1α-hydroxylase (CYP27B1), the essential enzyme to convert 25(OH)D to active calcitriol, is expressed in myeloid and lymphoid immune cells infiltrating the lungs of sensitized mice (Fig. 6C). The number of CYP27B1⁺ cells was decreased by D-SIT compared with SIT alone or without immunotherapy (D-SIT: mean 25.7; SIT: 61.5; O/O: 58 cells per 5 HPFs). These data show that allergen-driven inflammation facilitates the local synthesis of calcitriol from 25(OH)D by immune cells.

**Discussion**

We show that 25(OH)D deficiency results in enhanced humoral IgE and IgG1 responses following type I sensitization. Furthermore, we demonstrate that coadministration of 25(OH)D enhances the effect of specific immunotherapy in a murine model of allergic airway inflammation. Importantly, these data suggest that 25(OH)D, the precursor of calcitriol, exerts beneficial immunomodulatory functions without toxic side effects and is therefore superior to calcitriol in a therapeutic context.

In this study, 25(OH)D deficiency was achieved by nutritional restriction within 2 wk. This is attributed to its large volume of distribution [e.g., in the serum, muscles, and fat tissue (28)]. D-SIT-induced 25(OH)D serum concentrations are in a physiological range also observed after UV exposure in humans (20, 29). Direct VDR activation by 25(OH)D requires concentrations > 400 nM in vivo and in vitro (30–32), which is 1.5-fold more than observed using D-SIT in this study. Thus, direct functions of 25(OH)D alone on immune cells are unlikely, as confirmed by the observed lack of specific IgE regulation by 25(OH)D alone without the Ag. To exert functions on immune cells, 25(OH)D requires enzymatic conversion to calcitriol, which is expressed in activated B cells (4), T cells (7), and myeloid APCs (8, 9). Our data support the concept that allergen-specific immune cells are activated by the SIT allergen, express CYP27B1, and synthesize calcitriol from 25(OH)D endogenously. Indeed, we were able to detect CYP27B1 expression in the lung after allergen challenge.

On the humoral level, OVA-specific IgE and IgG1 serum concentrations are reduced in the presence 25(OH)D following Ag challenge in sensitized mice compared with the deficient control. These data are in line with previous data from mice treated with a synthetic low-calcemic calcitriol-derivative (5) or vdr knockout mice (33). As 25(OH)D alone the absence of Ag is inactive in the concentrations determined, the reduced Ig response in the presence of 25(OH)D following Ag challenge supports the hypothesis that endogenous calcitriol signaling is dependent on CYP27B1 expression in activated immune cells, which leads to reduced IgE expression, as we have shown in human B cells (3, 4). In 25(OH)D-deficient mice, the OVA-specific serum IgE and IgG1 concentrations were comparable before the final OVA recall between

**FIGURE 5.** D-SIT reduced lung infiltration and inflammatory cytokine expression. Experimental setup as shown in Fig. 3A. BALFs were obtained on day 106. Cellular differentiation (A), IL-13 (B), IL-4 (C), and IL-12p40 (D) concentrations. Dotted line indicates sensitivity threshold. Data are shown as mean fold induction (n = 7). *p ≤ 0.05, **p ≤ 0.001.

**FIGURE 6.** D-SIT reduced the influx of inflammatory cells into the lung. Experimental setup and groups as described in the legend of Fig. 3A. Lung histology was determined 2 d after OVA exposure on day 106 regarding expression of CD3 (A), B220 (B cells) (B), and CYP27B1 (endogenous calcitriol-synthesis) (C). Representative stainings from one mouse of the O/O group are shown in ×100 original magnification (inset, ×400). Data are shown as mean cells per 5 HPFs ± SEM; n = 7. *p ≤ 0.05, **p ≤ 0.01.
sensitized mice without SIT, following SIT or D-SIT. However, OVA recall strongly induced both, specific IgE and IgG1, in sensitized mice, but not following SIT or D-SIT. These findings indicate that the OVA recall–induced Ig induction is attributable to the newly generated plasmablasts, which depend on memory B cells and modulation by SIT. The lack of specific Ig induction after final OVA recall indicates that the specific memory B and T cells are most likely suppressed by immunological tolerance. The limited difference between D-SIT compared with SIT alone in the specific Ig regulation may result from continuous secretion of specific Abs by long-lived plasma cells, which were induced during sensitization and resistant to modulation of B or T cells (34), and/or the relatively high efficacy of SIT. Also, a delayed action of immunotherapy on Ig-secreting cells must be considered. More importantly, our data suggest that 25(OH)D is enhancing SIT-mediated humoral tolerance mechanisms and is still effective 39 d after completion, suggesting a long-term immune modulatory effect on type I allergy.

Besides airway infiltration and BALF cytokine levels, lung function parameters in 25(OH)D-deficient mice (mean, 39 nmol/l) were significantly improved by D-SIT, but not SIT alone, as shown by significantly improved baseline dynamic lung compliance, reduced vascular and AHR. In addition, we observed enhanced clinical efficacy of D-SIT compared with SIT alone, because the impact on lung function and inflammation parameters by SIT alone was comparable to not statistically different from sensitized mice (O/O). Remarkably, the lung function was comparable between the D-SIT and nonsensitized control group. The further enhanced D-SIT efficacy regarding the reduction of AHR in mice with pronounced 25(OH)D deficiency (<15 nmol/l) again demonstrates a functional relevance of endogenous calcitriol synthesis. This is most likely related to an altered memory immune response to the OVA inhalation and not to acute vitamin D–mediated effects on lung cells because 25(OH)D concentrations > 400 nmol/l are required for direct VDR activation in mice (30) (in this study: 238 ± 13 nmol/l). Furthermore, lung cells do not have the capability to endogenously produce calcitriol as CYP27B1 is absent (35), which is confirmed by our histological analysis. Also, the specific IgE response of sensitized mice remained unchanged following 25(OH) D treatment without simultaneous Ag. Finally, D-SIT efficacy was present when serum 25(OH)D concentrations were back to baseline and comparable between all groups.

To our knowledge, this is the first report on the impact of repetitive Ag (SIT model) on long-term immunological memory function in mice (see Fig. 2). In previous reports on murine models, SIT was started on days 2–14 after sensitization (in this study: day 21) and performed daily or alternating days (in this study: weekly intervals). Also, lung function analysis was determined 1–2 wk after SIT (19, 36) and thus earlier than in this study (6 wk) (Fig. 3A). Given the short intervals between initiation of SIT and lung function analysis, activation-induced cell death or anergy may bias the data. This is of importance because the long-term function (e.g., through modulation of specific memory cells and not rapid anergy) is of importance in the treatment of seasonal pollen-induced rhinoconjunctivitis (15). Finally, a beneficial impact of D-SIT on AHR compared with SIT alone was observed in this study. Regarding the outcome on AHR, our data are consistent with a previous report by Agrawal et al. (37) in which the impact of daily high dose oral vitamin D3 supplementation was investigated in a short-term protocol. However, in our experimental setting, all animals were 25(OH)D-deficient at the time of lung function analyses, which suggests a long-term action on the D-SIT–induced memory cells, rather than acute 25(OH)D function on the lung challenge–activated infiltrating effector cells. Thus, D-SIT may be therapeutically superior to vitamin D3 alone by induction of a long-lasting immune modulation of memory cells and by reduction the risk of potential vitamin D–associated side effects by long-term use (e.g., kidney stones).

However, our findings are consistent with previous reports in mice showing the effects of SIT in the presence of calcitriol including the reduction of the Th2 cytokine IL-13 in the BALF (19, 36). Because IL-13 expression is critical for the induction of AHR (38), the reduced IL-13 levels might at least partly explain the ameliorated AHR in D-SIT–treated mice. There is no consensus on the impact of VDR on purified T cells in allergy [e.g., naïve T cells differentiated into GATA3+ Th2 cells (39) or into IL-10–secreting Tr1 cells (40)]. In this study, we determined reduced OVA-specific Ig serum concentrations of the Th2-induced isotypes IgE and IgG1 after D-SIT compared with SIT alone. In addition, the Th2 cell cytokine expression was reduced after OVA airway challenge. In line, VDR activation reduced the infiltration of Th2 cells into inflamed tissue in a murine model of inflammatory bowel disease (41). Taken together, our data suggest that the specific Th2 response is decreased by 25(OH)D coadministered with SIT.

Pulmonary vascular hyperresponsiveness has been described earlier in OVA-sensitized and challenged mice (25) and correlates with pulmonary arterial remodeling (27) while dissociated from AHR (27, 42). To date, its pathogenic role in allergic lung inflammation is not fully understood, but it might be associated with pulmonary arterial remodeling observed in severe asthmatic patients (43). By diminishing pulmonary vascular hyperresponsiveness, D-SIT could additionally provide protective effects on pulmonary vasculature. Inconsistent with our findings, less IL-5 and eosinophils were previously observed upon calcitriol treatment by Taher et al. (19). However, in VDR knockout mice, VDR signaling was positively associated with allergen-induced airway inflammation, splenic Th2 cytokine expression, and influx of eosinophils into the lung (33). Consistently, we observed a marginally higher eosinophilic infiltration of the lungs after D-SIT compared with SIT alone (p = 0.20), which was significantly decreased compared with OVA-sensitized mice without SIT (p < 0.05).

Taken together, our study provides evidence that 25(OH)D deficiency enhances type I sensitization and that applying 25(OH)D promotes the effects of allergen-specific immunotherapy in deficient mice suggesting enhanced immune tolerance induction. This function of 25(OH)D in additional sensitization routes and Ag models (e.g., intranasal dust mite allergen) is not known yet, but it provides an interesting topic for future investigations. Because 25(OH)D deficiency (<50 nmol/l as investigated in this study) is frequent in healthy individuals and type I allergic patients (29, 44, 45) and during that period, pollen-specific SIT is regularly initiated as seasonal pollen are absent, the data of this study suggest that correction of 25(OH)D deficiency may enhance the efficacy of SIT.

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