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Article type : Letter to the Editor

Immunomodulation of high-dose vitamin D supplementation during allergen specific immunotherapy

Supporting Information Figures S1-S6, Table S1-S2 and Methods are in a separate data file.

To the Editor,

Allergen immunotherapy (AIT) is the only disease modifying treatment of type I allergy ¹. Although the treatment protocols improved still a reduction in treatment time and granted responder rates are warranted. Adjunct vitamin D may improve AIT effects. Signaling of the vitamin D receptor (VDR) inhibits IgE production in human B cells ², promotes IL-10 expression in several immune cell types ^{3,4} and enhances AIT efficacy in mice ⁵. However, calcitriol the bioactive form of vitamin D is not suitable for allergy therapy due to the short half-life, the narrow therapeutic range and hypercalcemic toxicity. We and others proved that endogenous calcitriol can be synthesized by activated immune cells from the precursor 25-hydroxyvitamin D (25(OH)D) ^{4,6}, and promotes long-term efficacy of AIT in an airway inflammation model ⁷. In this monocentric, prospective double-blind, randomized, placebo controlled 2-armed, parallel-design, clinical pilot trial we investigated adult patients with grass pollen-induced allergic rhinoconjunctivitis ± allergic asthma and low serum 25(OH)D. Patients received preseasonal subcutaneous grass pollen-AIT and adjunct a daily intake of 5,333 I.U./day vitamin D or placebo (ProGIT, NCT01466465; detailed Methods in the Supplementary Data, Figure S1A). Of 52 screened, 36 participants were randomized to receive vitamin D or placebo (Figure S1B). Both groups baseline characteristics were comparable (Table S1). The study was performed between November and April to exclude a

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bias from UV-derived vitamin D in three consecutive years. The serum 25(OH)D concentrations were comparably low in both study groups at baseline (vitamin D: mean 40.4 nmol/L, placebo: mean 41.7 nmol/L, Table S1). After each treatment period, the mean serum 25(OH)D concentrations increased in all participants from the vitamin D group (year 1: 118.9 nmol/L, year 2: 146.5 nmol/L, year 3: 129.2 nmol/L, n=12) and remained low in placebo group (year 1: 38.5 nmol/L, year 2: 36.2 nmol/L, year 3: 58.7 nmol/L, n=11, $P<0.001$ at each timepoint; Figure S2). The safety parameters were within the normal values throughout all timepoints and the adverse events were comparable between both groups regarding frequency and severity (data not shown). The distinct difference in the vitamin D status provides the molecular basis to determine the impact of vitamin D on AIT, which we assessed by clinical and immunologic parameters. Titrated ICT before and after the preseasonal AIT showed a significant decrease of the individual reactions in all participants over time, which was more prominent at the lower allergen doses (Figure 1A). Intergroup comparison shows a reduced intracutaneous reactivity in the vitamin D group in the 2nd year at low grass pollen allergen concentrations compared to placebo (0.5-5 SBU/ml, Figure 1A). The ICT with 500 SBU/ml served as primary outcome of this study and showed comparable results over time in all participants ($P>0.05$, Figure S3A), and between groups ($P=0.0851$, Figure S3B). The SPT reactivity decreased during the 3 years in all study participants, which was more pronounced in the vitamin D group compared to the placebo after the 1st year of AIT (Figure 1B). Titrated CPT reaction threshold increased during the 3 treatment years in all participants, which was comparable between both groups ($p=0.38$, Figure S4). At other timepoints both groups were comparable regarding intracutaneous, prick or conjunctival provocation tests (data not shown). The symptom medication scores during the first grass pollen season were comparable between both groups, and from year 2 and 3, the data were excluded from further analysis as the recovery rate was below 50% (data not shown). Investigation of immunological mechanisms showed specific IgE was induced by AIT in the placebo- but not in the vitamin D group in the first treatment year ($P<0.031$, Figure 2A). After completion of three AIT courses, grass pollen-specific IgE was reduced in both groups leading to comparable values at the end of the study (Figure S5A/B). Specific IgG4 was strongly induced by AIT in both groups ($P=0.57$, Figure 2A), with repeated increases of specific IgG4 in all individuals during the three treatment courses, which declined during each pollen season in both groups (Figure S5C, $P>0.05$, data not shown). Accordingly, the grass pollen specific IgG4/IgE ratio between both study groups showed increased values by the first AIT course ($P=0.0073$, Figure 2A), analogous to the blocked IgE response

(Figure 2A). The values of specific-IgG-subclasses, -IgA and -IgM both study groups showed comparable data (not shown). As B cells are VDR-responsive, flow cytometric analysis were performed before, during and after completion of the AIT courses. The frequency of B cells with the vitamin D-inducible surface molecule CD38 on naïve B cells increased after treatment in the vitamin D, but not in the placebo group, with CD27⁺⁺CD38⁺⁺ plasmablasts unaltered (Figure 2B, Table S2, gating in Figure S6A). The frequencies of the grass pollen-reactive B cells increased during the second year in both study groups, which was more pronounced in the vitamin D compared to the placebo group (Figure 2B, gating in Figure S6B) but not during the first treatment year (Table S2). The frequencies of IL-10⁺ B cells increased after AIT in both study groups to a comparable extent after a transient reduction in the placebo, but not the vitamin D group (Figure 2B, gating in Figure S6C). Taken together, the data of this first 3-year AIT trial with controlled high-dose vitamin D intake suggest an earlier onset of AIT-induced immunomodulation by vitamin D, as determined by ICT and SPT. This effect appears clinically relevant after the 2nd treatment year, as the symptom medication scores were comparable after treatment year 1. A beneficial immune function of vitamin D intake is supported by the increased specific IgG4/IgE ratio during initial AIT and increased VDR-inducible CD38⁺ B cells in the vitamin D group. The declining sIgE concentrations in all participants over the whole treatment time leading to comparable levels between both groups after 3 years show that vitamin D-independent IgE regulation is also effective, but requires more time or allergen stimulations, as we observed previously in a preclinical setting ⁷. Overcoming the limitation of previous studies, that 25(OH)D levels in the treatment group reach rarely values above 80 nM and differ to the placebo group significantly, our findings support data from previous observation which correlated AIT success with serum 25(OH)D concentrations above 75 nmol/L ⁸. In perspective, in a larger study, with more rapid vitamin D-updosing, considering also other AIT routes, and more sensitive allergy assessing e.g. nasal provocation, daily symptom documentation using mobile phone apps or pollen exposure chambers may clarify the impact of 25(OH)D on AIT. Here, we observed beneficial functions of vitamin D on AIT at the immunological level in the absence of potentially unwanted reactions as e.g. toxicity. The data suggest a beneficial immunomodulation by vitamin D in AIT, shortening AIT time to induce tolerance or the use of less allergen.

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Authors contributions:

M.W., A.R., and G.H. designed the study and wrote the manuscript, G.H. and N.S. (year 1) performed all clinical examinations, W.F. analyzed the clinical data, S.D. performed the authority communications and edited the manuscript, S.F. designed and performed the Ig-profile experiments, G.D. performed the flow-cytometric analysis.

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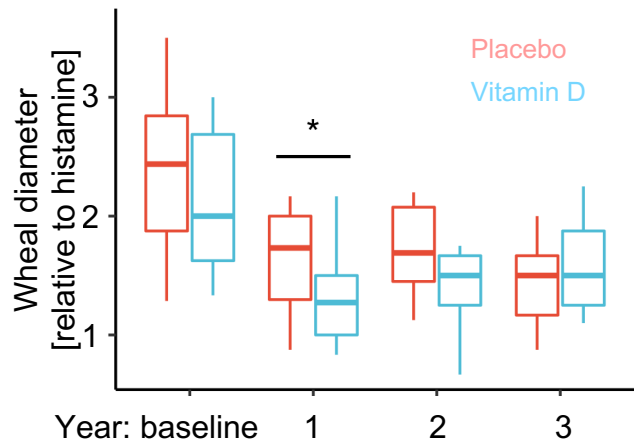
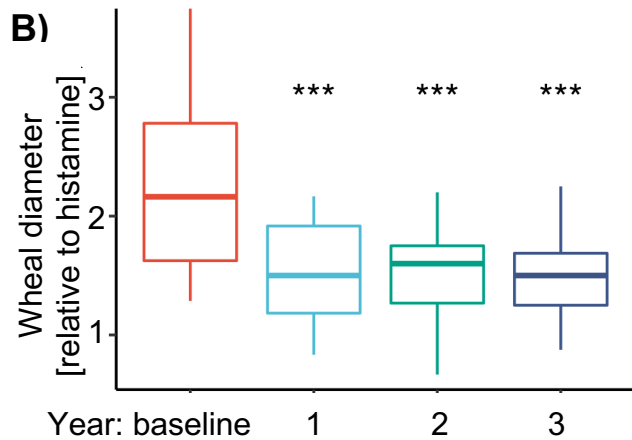
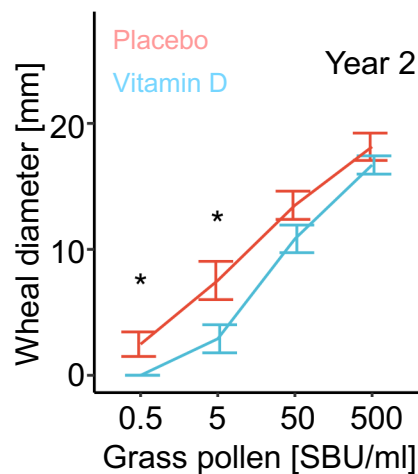
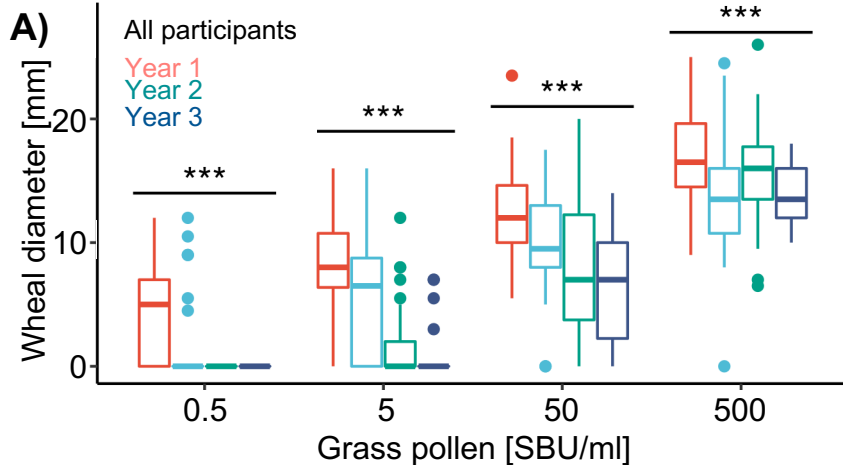
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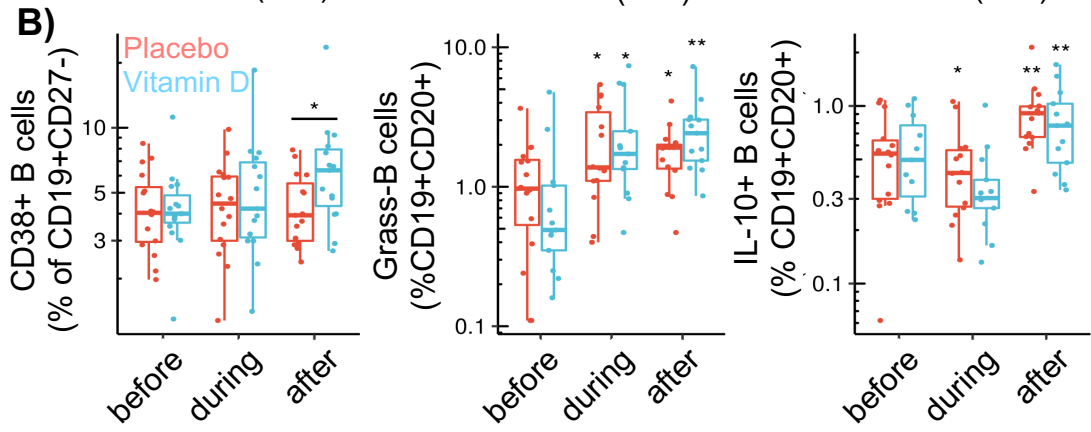
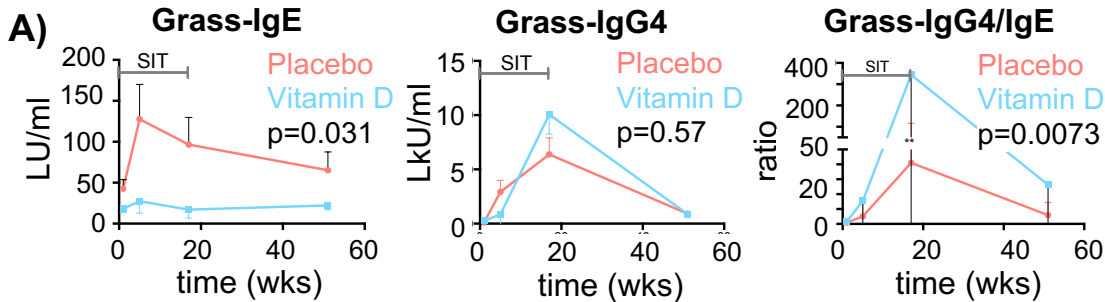
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Figure legends

Figure 1. Reduced skin test reactivity during AIT is promoted by vitamin D. Grass pollen-AIT was performed preseasonal in 3 consecutive years during the low UV winter months together with daily 5,333 I.U. vitamin D or placebo. The grass pollen-specific A) titrated intracutaneous test (ICT), B) skin prick test (SPT) was determined at baseline and after each therapy course in all participants (left diagrams) and analyzed regarding both study group (right diagrams). The points represent the box plot outliers. * $P < 0.05$, ** $P < 0.01$, Kruskal-Wallis-Test or Dunn's-test for multiple independent samples, intergroup differences by Mann-Whitney U test.

Figure 2. The regulation of the humoral and B lymphocytes by grass pollen-specific immunotherapy in the presence of vitamin D. Before, during and after each AIT course the immune response was assessed. A) grass pollen-specific ELISA in year 1. P-values represent grouped 2-way ANOVA. B) B cell flow cytometric analysis of vitamin D-responsive CD38+ cells, grass pollen-specific and IL-10+ B cells in year 2. The data shown represent the mean values and interquartile ranges. Statistical analysis was performed applying the Mann-Whitney U test comparing with before baseline values unless stated otherwise * $P < 0.05$, ** $P < 0.01$.





Immunomodulation of high-dose vitamin D supplementation during aller-gen specific immunotherapy

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Synopsis Provitamin D and Grass-pollen ImmunoTherapy (ProGIT)

- Approved by the local ethics committee (LaGeSo Berlin, No. 10-0383), and Federal authorities Bundesamt für Arzneimittel und Medizinprodukte and Paul-Ehrlich-Institut (No. 1177-03).
- Registered at Eudra-CT No. 2010-021775-80, clinicaltrials.gov (NCT01466465),
- Conducted in accordance with the national drug laws (AMG), ICH-GCP, and the Declaration of Helsinki.
- Adult patients with clinically relevant grass pollen-induced allergic rhinoconjunctivitis with or without allergic asthma and low serum 25(OH)D were randomized to receive 5,333 I.U./day vitamin D or placebo during the low UV radiation winter months. All participants received at the same time a preseasonal grass pollen-specific subcutaneous immunotherapy.

Participant selection

Inclusion criteria: written informed consent, adults (18 - 55 years), clinically relevant grass pollen-allergy > 2 years, positive grass pollen-Prick test (>3 mm), *P.pratensae*-specific IgE ≥ CAP class 2 (0.7 kU/L, Thermo Fisher), positive intracutaneous test (ICT, ≥10 mm at 500 SBU/ml), lung FEV1 >70%, retrospective symptom score (rSS) >12, serum 25(OH)D <80 nmol/L, clinically relevant grass pollen-induced allergic rhinoconjunctivitis ± asthma ≥ 2 years, women in childbearing age: effective contraception.

Exclusion criteria: Sensitivity against excipients of Vigantol® Öl or Allergovit® Gräser, contraindication against specific immunotherapy, started, current or complete grass-AIT within the past 2 years, vaccination 7 days before AIT, instable asthma (FEV1<70%), serum 25OHD >80 nmol/L, planned UV-exposure (tanning, traveling to regions with UV-index/day >5), abnormal serum calcium, phosphate or kreatinine values or clinically relevant abnormal blood counts, treatment with vitamin A-derivatives, hypercalcaemia, hypercalciuria, kidney stones, sarcoidosis, pseudohyperparathyroidism, pregnancy or lactation, prohibited concomitant drugs including: phenytoin, barbiturates, thiazides, glycosides, tricyclic antidepressants, neuroleptics (the latter until 2 weeks before screening), symptomatic treatment of rhinoconjunctivitis- or asthma except beta-2-agonists or antihistamines, beta receptor blockers, immunosuppressants or -modulators, incl. corticosteroids

Randomization and power calculation

The participants were randomized to receive cholecalciferol (oral form of vitamin D) or placebo in a 1:1 ratio using a randomization list generated by the Charité Pharmacy Department.

The power calculation was based on published data¹ and followed the assumption of differences in the grass-ICT mean wheal size to 500 SBU/ml (estimated 11 +/- 2.15 mm) and a decline in the vitamin D group to be 20% more than in the placebo group, an alpha error of 0.05 and power of 80% resulting on published data in a group size of n=17 (PASS 2008 software, Utah, USA). Given a calculated dropout rate of 5% in the 1st year, we included 18 probands per group.

Supplementary Results

Group characteristics

The baseline patient characteristics are comparable between both groups regarding sex, age, height, weight, body-mass-index, allergy history, total and specific serum IgE-concentrations and vitamin D status (Table S1). The drop-outs were independent of the baseline or study parameters and not treatment associated (data not shown).

Interventions

All participants received a pre-seasonal grass pollen-specific subcutaneous immunotherapy according to the manufacturer's instructions (AIT, Allergovit 100% Gräser®, Allergopharma, Reinbek, Germany, Figure S1). Briefly, updosing was performed in weekly intervals with 1.6%, Visit 1 (V1), 3.2% (V2), 6.5% (V3), 13% (V4), 25% (V5), 50% (V6), 100% (i.e. 6000 therapeutic entities (TE) grass pollen, V7) followed by 3 maintenance doses after 2 wks (V8) and 4 week intervals (V9, V10). Cellular and serum antigen-specific responses were determined on before AIT-course (visit 1; V1), during up dosing at V5 (1 week after 4th dose with 13%, before injection of the following 25% dose) and 1 week after 4th 100% dose at V11. Randomized patients received either vitamin D (5,333 I.U./day, 8 drops Vigantol®) or placebo (neutral carrier oil, Migliol®, both Merck, Germany). The vitamin D dose was recommended by the manufacturer for the treatment of vitamin D deficiency and confirmed in previous own studies to achieve 25(OH)D blood concentrations exceeding 75 nmol/L^{2,3}. The study was performed in 3 consecutive years between November-April when UV radiation was low (UV-Index <1; UVI; details see Bundesamt für Strahlenschutz, www.bfs.de biosynthesis and^{4,5}). At this time of year, we and others demonstrated a high frequency of vitamin D deficiency in healthy and allergic individuals in Berlin^{2,3,6}, due to lacking UV-biosynthesis and low vitamin D intake in Germany^{4,7}. The first patient was randomized on 24th Nov 2011 and last patient's follow up was on 13th Jan 2015.

Efficient vitamin D supplementation in the study cohort

Most vitamin D is derived from UV-biosynthesis or oral intake⁴. Here, daily 5,333 IU/day vitamin D increased serum 25(OH)D in all participants leading to a highly significant difference compared to placebo at the end of each supplementation period ($p < 0.001$ at all timepoints; Supplementary Figure S2). Toxic levels were not observed in any participant ($>350\text{--}450$ nmol/L^{5,8}, Figure S2), supporting meta analyses showing safety 10,000 I.U. vitamin D daily⁸ and toxicity associated serum 25(OH)D above 535 nmol/L⁸, i.e. 2.4-fold higher than the maximal, and 4.1-fold higher than the median concentration observed here. We observed no vitamin D associated adverse events as and the quality of adverse events did not differ between both groups (data not shown). Also maintained low 25(OH)D values in the placebo group show lacking UV-bias during the season with low UV radiation.

Clinical assessment and study outcomes

All clinical assessments were performed and analyzed in a double-blinded manner according to international guidelines. Titrated grass-pollen specific intracutaneous test (ICT, 0.5-5-50-500 SBU/ml, Allergopharma; Histamin was strongly positive in all donors but showed variability, most likely due to the anatomical site at the wrist region), titrated conjunctival provocation test (CPT, 330-1,000-3,300-10,000-33,000-100,000 SQU/ml, ALK-Abello, Hamburg, Germany), skin prick test (SPT, Allergopharma) were performed according to international guidelines⁹ before and after preseasonal AIT courses (ICT: V1 and V11, SPT and CPT: V1 and V10) and at the FU visit.

During the grass pollen-season, the patient recorded symptoms and drug requirements at a daily basis (symptom-medication-score; SMS,¹⁰) using guided forms (paper, Excel). The symptom score was added to the scored medication, used in the following order: topical antihistamine, systemic antihistamines, prednisolone; bronchial symptoms with salbutamol or budesonide according to the GINA guidelines.

In a retrospective symptom score, the patient-reported symptoms during the previous grass pollen-season were assessed before each treatment course as published previously¹¹ resulting in a sum between 0-30 points. The global symptom score was assessed after each AIT-course and evaluates the symptoms compared to the previous season by a 5-point scale (1=much better, 2=better, 3=unchanged, 4=worse, 5=much worse). The data show after the 3 AIT courses, the retrospective symptom scores and seasonal global symptoms were reduced compared to baseline without intergroup difference (data not shown).

The treatment tolerability was recorded at a daily basis during the treatment period in a medication diary using a 5-point scale (1=very good, 2=good, 3=average, 4=poor, 5=very poor). The safety parameters (differential blood count, serum concentrations of calcium, phosphorus and 25(OH)D were determined by the clinics routine laboratory (Labor Berlin, using GC-MS and electrochemiluminescence immunoassay (IDS), respectively). All data were monitored from an independent, external data manager and locked before unblinding.

Immunologic parameters

Antigen-specific cellular and serologic parameters were determined at visit 1 (V1; before AIT-course, V5 (during up-dosing) and at V11 (1 week after 4th maintenance dose). Grass-specific IgE and IgG4 were analyzed by ELISA, Ig isotype profiling by bead-based multiplex assays and *ex vivo* flow-cytometry.

Grass-specific IgE serum concentrations were quantified at the baseline by the clinical routine laboratory (CAP-System, CAP class 2 >0.7 kU/L was considered positive, Thermo Fisher). During study, Grass-specific ELISA were performed as described previously¹². Briefly, recombinant Phleum pratense 5-antigen (5 µg/ml bicarbonate buffer, kindly provided by Allergopharma) was coated on 96-well plates (maxisorb; NUNC, Roskilde, DK) overnight, after blocking using 3% BSA in 0.05% Tween 20/TBS, diluted serum incubation for 2 hrs. Detection of specific Ig was performed using biotinylated anti-IgE (HP6029, Southern Biotech) and Streptavidin–HRP (Sigma-Aldrich) or HRP labeled anti-IgG4 (BD Biosciences) followed a colorimetric reaction using TMB substrate (Sigma-Aldrich) quantified in a plate reader at 450 nm. A reference diluted standard serum with high grass-IgE and –IgG4 from a patient with successful immunotherapy was used to calculate standard laboratory units (L.U.) applying a 4-point standard curve function.

The complete grass pollen-specific Ig-profile of all isotypes over time was determined using a multiplex bead array (xMAP technology). Bead-conjugated isotype specific antibodies (Human antibody isotyping kit, Thermo Fisher) were incubated with participants serum followed by incubation with biotin conjugated grass pollen (ALK). After incubation with Streptavidin –PE the amount of bound Ig was quantified by measuring the fluorescence intensity of PE bound to bead-conjugated isotype specific antibodies (human Ig-quantification kit, Affymetrix, Thermo Fischer) using the Bioplex 200 device (BIO-RAD). The data was analyzed using the Bioplex manager software. This assay was applied for all specific-Ig data from year 2-3. Regarding IgG2 all values were below the detection threshold. The laboratory units (L.U.) were calculated applying a 4-point standard curve function from diluted pooled sera of positive donors.

Flow cytometric analysis of peripheral B cell populations was performed according standard protocols considering CD27-CD19+ naïve B cells, CD27+CD19+ memory B cells and CD27++CD38++CD19+ plasma cells and IL-10+ B cells¹³. Grass-pollen-specificity was determined regarding B cells by fluorescently labeled allergen as described in¹⁴ by *ex vivo* stained by FITC-labelled grass pollen (ALK), by technical limitations a second equimolar grass pollen-staining to exclude rare fluorescence specific cells was not performed; IL-10-expression was determined after 4 hrs PMA/Ionomycin-restimulation including a Golgi block for 3h with 2 µg/ml Brefeldin A (Sigma Aldrich), fixation and permeabilization (Perm2, BD Biotech) staining using a fluorescent-labelled antibodies against CD19 (clone 4G7), CD20 (clone REA780), CD27 (M-T271), CD69 (FN50), IL-10 (JES3-19F1), CD38 (IB7), IgD (REA 740), CD14 (all from Miltenyi Biotech or BD Pharmingen) and analyzed on a MACSquant cytometer (8 colors, Miltenyi Biotech) using FlowJo-Software (Treestar).

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Supplementary Figures and Tables 1

Table S1. Baseline group characteristics. Data shown as mean $\pm$ SD or median [IQR], 25(OH)D in nmol/L; n.a.=not applicable. *P*-value (1=Chisquare, 2=T-Test, 3=Mann-Whitney U test, significant *P*<0.05).

Variable	Vitamin D	Placebo	<i>P</i> -value
Number (n)	18	18	n.a.
Sex (f/m)	8 / 10	6 / 12	0.49 ¹
Age (years)	32.3 $\pm$ 9.0	31.6 $\pm$ 9.7	0.41 ²
Height (cm)	174 $\pm$ 10.3	177 $\pm$ 9.3	0.37 ²
Weight (kg)	72 $\pm$ 14.3	77 $\pm$ 15.74	0.93 ²
Body Mass Index (BMI)	23.7 $\pm$ 4.2	23.0 $\pm$ 3.7	0.57 ²
total IgE (IU/L serum)	195 $\pm$ 48.3	288 $\pm$ 96.8	0.39 ²
grass-IgE (IU/L serum)	14.3 [3.6-29.2]	20.6 [7.2-32.4]	0.47 ³
grass-IgE (CAP class)	3.4 $\pm$ 0.3	3.7 $\pm$ 0.3	0.40 ¹
25(OH)D (baseline)	40.4 $\pm$ 3.8	41.7 $\pm$ 3.4	0.80 ¹

Supplementary Table S2: Immunological monitoring of B lymphocytes. During the first year of the study, PBMC were investigated by flow cytometry as described the methods section. B cells were defined as CD19+CD20+, excluding CD3+CD4+ T cells, CD14+ Monocytes and 7AAD+ dead cells. Data represent mean values and p-values calculated by Mann Whitney *U* Test.

Subset	V1		V5		V11		
	placebo	vitamin D	placebo	vitamin D	placebo	vitamin D	
%CD19 (PBMC)	5.93	7.60	6.97	8.17	5.91	5.84	mean
	0.09		0.20		0.74		p.val
%CD27 (B cells)	19.10	14.10	18.80	14.60	22.80	15.60	mean
	0.02		0.03		0.01		p.val
%CD38+ (CD27++)	1.72	1.66	1.77	1.26	2.34	2.63	mean
	0.98		0.09		0.58		p.val
%CD38+ (CD27+/-)	4.43	4.50	4.68	5.58	4.44	7.08	mean
	0.86		0.64		0.04		p.val
%Grass+CD19+ B cells	0.24	0.18	0.29	0.31	0.25	0.23	mean
	0.40		0.62		0.74		p.val

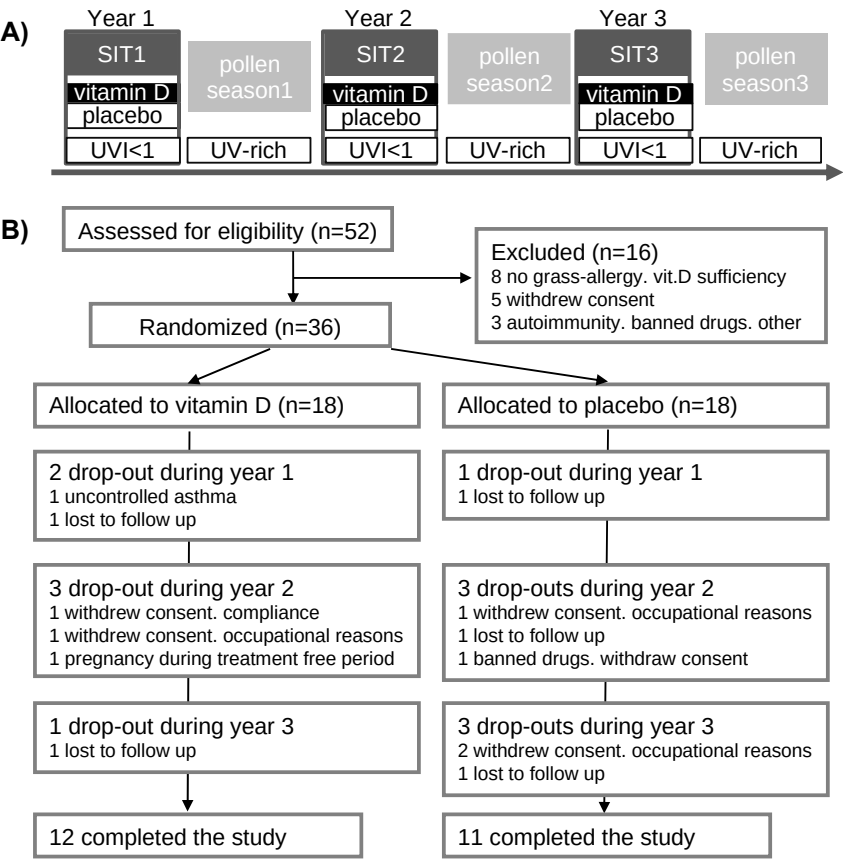


Figure S1. A) Study flow diagram. UVI=UV-index. B) Patient disposition throughout the study.

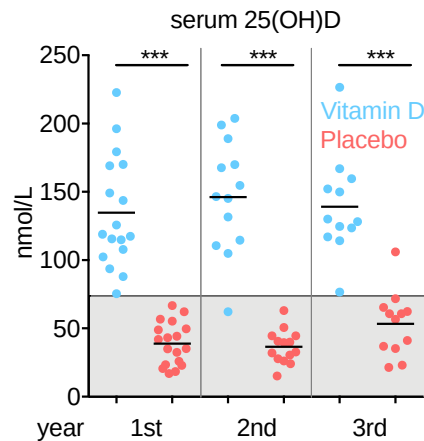


Figure S2. Efficient vitamin D supplementation. The serum concentrations of the vitamin D storage metabolite 25(OH)D were determined at the end of the preseasonal AIT courses for 3 consecutive years. The data show the individual values of the participants randomized to receive vitamin D or placebo at the same time. Bars represent the mean values, *** $P < 0.001$, Mann Whitney U test.

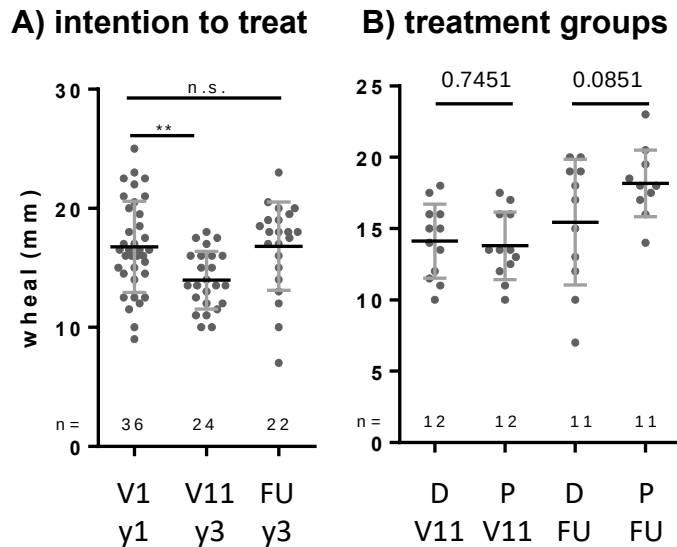
Intracutaneous test reaction to 500 SBU/ml

Figure S3. The intracutaneous test reaction to 500 SBU/ml grass pollen. A) Response of all donors of the intention to treat group. B) Intergroup comparison in year 3 after the final AIT treatment course and at the follow up visit. V1=before, V11=after AIT course, FU=follow up, y1=year1, y3=year3, D=vitamin D, P=placebo group. Data shown as individual values, the bar indicates the mean value, *T*-Test $**P < 0.01$.

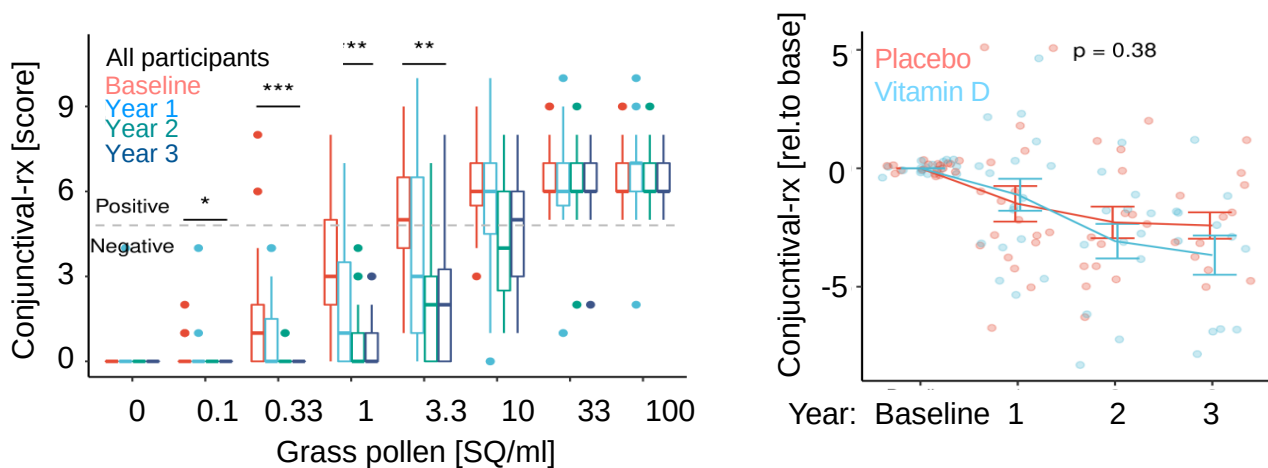
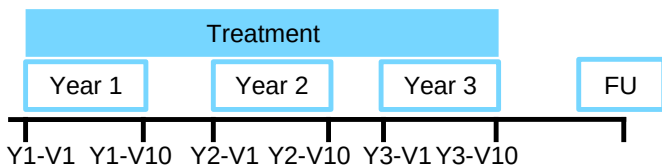
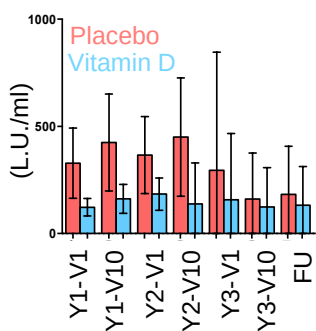


Figure S4. Conjunctival reactivity is declining during AIT. Details see Figure 1 legend, data shown from all participants (left) and the study groups (right). Titrated CPT was positive and stopped upon a score of 5 (grey line). The change from baseline was calculated according to the individual positive threshold concentrations. The points indicate outliers of the boxplots. $**P < 0.01$, $***P < 0.001$, Mann-Whitney *U* test.

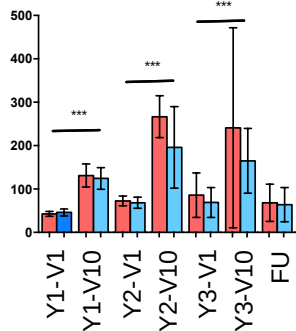
A) Study scheme



B) grass-IgE

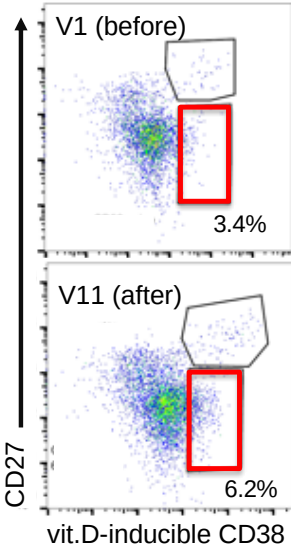


C) grass-IgG4

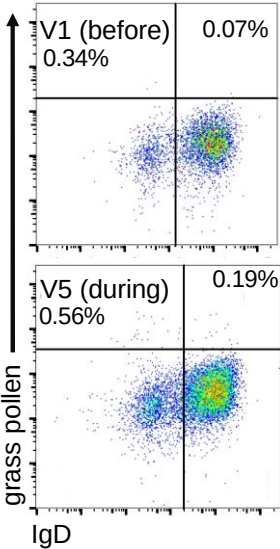


Supplementary Figure S5. The grass pollen-specific immunoglobulin profile over time. A) The grass pollen-specific immunoglobulins serum concentrations were determined before and after the 3 consecutive treatment courses regarding B) IgE- and C) IgG4-isotype. Y=year, V1=visit before/ V10=visit after AIT course, FU=follow-up). Columns represent mean $\pm$ SEM, * P <0.05, ** P <0.01, *** P <0.001, Students' T Test.

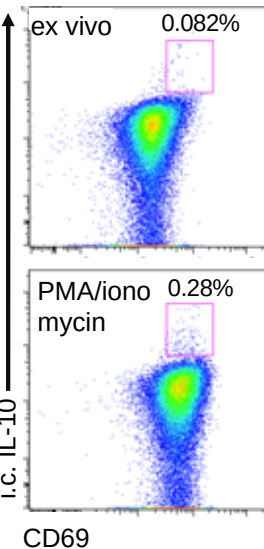
A) CD38⁺ B cells



B) grass-B cells



C) IL-10 B cells



Supplementary Figure S6: B cells gating strategy. PBMCs, gated on lymphoblasts and CD19⁺CD20⁺ B cells, excluding CD14⁺ and 7AAD⁺ positive cells and A) CD38⁺ B cells were determined from the CD27⁺/⁻ B cells (red box) and CD27⁺ plasma blasts (black box). B) Grass pollen-specific B cells were determined by direct binding of fluorescent binding of labelled allergen to the antigen receptor. C) Expression of intracellular IL-10 expression was determined after restimulation with 4 h PMA/Ionomycin including 3h Brefeldin A-Golgi block. The dot plots show representative data.