

Vitamin D3 Regulates T Cell Immune Responses in Allergen and Rhinovirus Induced Asthma

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Abstract

Background: Serum 25(OH)-Vitamin D3 (VitD3) deficiency during infancy has been associated with asthma. The potential therapeutic role of VitD3 given in the airways and its interference with the allergen and Rhinovirus was the objective of this study.

Methods: In two cohorts of children with and without asthma, serum levels of the C-reactive protein (CRP) were correlated to Serum VitD3 and in peripheral blood T cell inhibitor marker Programmed cell death protein 1 (PD1) mRNA was analyzed. In a murine model, VitD3 was given intranasally *in vivo* and *in vitro* to lung cells with allergen and Rhinovirus.

Results: In the cohorts of pre-school age children without (control) asthma, CRP and VitD3 levels inversely correlated. In preschool asthmatic children that did not receive VitD3 supplementation as infant had more episode of asthma exacerbation associated with high CRP serum level. In peripheral blood cells from control but not asthmatic children with higher serum levels of VitD3 had lower *PD1 mRNA* levels. In murine model, OVA intranasal challenge induced Innate Lymphoid Cells type 2 (ILC2)-associated markers and Eosinophils in BALF and VitD3 inhibited lung inflammation and ILC2 markers. Furthermore, VitD3 given intranasally, induced CD4+T cells and reduced PD1, T regulatory cells in the lung. Similarly, VitD3 had a suppressive role on CD4+PD1+ T cells involved in T cell exhaustion in the airways in the absence of ST2 after Rhinovirus infection.

Conclusion: These data support an inhibitory role of VitD3 on T cell exhaustion after allergen and rhinovirus infection that is relevant for pediatric asthma.

Introduction

Allergic asthma is a chronic-inflammatory disease of the airways that affects millions of people worldwide especially children^{1,2}.

Different pathological form of hyperreaction to allergen, like allergic asthma, atopic dermatitis and food allergy are associated to allergy-induced local inflammation driven by Th2 driven immune-reaction^{3,4}. By contrast, under homeostatic conditions, the airways are kept pathogen free by the function of cells of the innate immunity⁵. Moreover, 1,25-Dihydroxyvitamin D3 (VitD3) has been suggested as a therapeutic compound for allergic asthma.⁶⁻⁸ In Germany, VitD3 is given to the infants as supplement⁹ to avoid immunological suppression especially in children which could not receive breastfeeding. Wheezing is associated with asthma in the first years of life.¹⁰ Moreover, RV is the factor that associated with wheezing in infants. In this study, we wanted to better understand the role of Vitamin D3 in pediatric asthma.

Most of the medication for asthma are given directly into the airways, we thus thought to investigate in murine model, the role of VitD3 given intranasally *in vivo*. Recent studies described the role Innate lymphoid cells (ILC2s) in the airways in models of allergic asthma.¹¹⁻¹³ When the allergen enters the airways, it interacts with epithelial cells, first. This interaction results in epithelial release of alarmins like IL-33, which activate ILC2 via ST2¹⁴. Once stimulated, ILC2s secrete IL-5, IL-9 and IL-13, classically known as Th2 cytokines.

Here we focused on the influence of VitD3 in blood cells from two pediatric cohorts with and without asthma. Moreover, we analysed the effect of VitD3 given intranasally, on allergen induced airway tolerance with focus on T regulatory cells and Innate Lymphoid Cell associated markers.¹⁵

The role of T regulatory immunosuppressive cells has been extensively studied in airway tolerance^{16,17}, by contrast, the role of ILC2 in airway tolerance is less understood.

Here we found that VitD3 inhibited key ILC2/ST2 markers like IL-33, Amphiregulin (AREG) and Ror-alpha, and PD1 and induced CD4 + T cells, *in vivo* in the airways. Similarly, VitD3 had a suppressive role on CD4 + PD1 + T cells involved in T cell exhaustion in the airways in the absence of ST2 after Rhinovirus infection.

Our findings suggest a protective role of VitD3 in antagonizing ILC2 markers in preschool children thus providing a rationale for exploring VitD3 in immunotherapy in pediatric asthma.

Materials And Methods

All methods used in this manuscript were performed in accordance with the relevant guidelines and regulations.

HUMAN STUDIES

The present study is part of the prospective study PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases). The working package 1 (WP1) relates to studies in pre-school children with and without asthma. The study WP1 in Erlangen was approved by the local Ethics Committee of the Universitätsklinikum Friedrich-Alexander Universität Erlangen-Nürnberg (Re-No 4435) and it is registered in German Clinical Trials Register (www.germanctr.de: DRKS00004914). Parents/ guardians of all participants gave their informed consent. Two cohorts of pre-school children (age 4-6 years) with and without asthma were analysed (control children n=22 and asthma children n=24). The recruitment of the subjects, inclusion and exclusion criteria as well as the timescale for clinical visits and data collection along with the clinical aspects and characteristics were previously described^{6,18,19}.

Serum 25(OH)-Vitamin-D3 measurements.

25(OH)-Vitamin-D3 (=25(OH)VitD3) was measured in serum from baseline visits at the Dept. of Paediatrics and Adolescent Medicine of the Friedrich-Alexander Universität Erlangen-Nürnberg (Erlangen, Germany). Thereby LC-MS/MS MassChrom Kit from Chromosystems was used according to the manufacturers protocol.

Detection of C-reactive protein (CRP).

CRP values in serum samples of the children were analyzed on a Roche (Basel) Integra 800 Analyzer by turbidimetry as previously described¹ (CRPL2 reagent, interday CV 1.4% (8.1 mg/L), limit of detection 1.0mg/L).

Human RNA Isolation from Tempus Tubes and quantitative Real-Time PCR.

At Baseline visit whole blood was collected in Tempus® Blood RNA Tubes (Life Technologies™, GmbH, Darmstadt, Germany) and RNA was extracted with the MagMax for Stabilized Blood Tubes RNA Isolation Kit. Synthesis of cDNA and consequently real time-PCR were performed as described for murine cells below with the following primers and sequences: hHPRT (5'-TGA CAC TGG CAA AAC AAT GCA-3', 5'-GGT CCT TTT CAC CAG CAA GCT-3'), PD-1 (fw: 5'-CAG TTC CAA ACC CTG GTG GT-3'; rev: 5'-GGC TCC TAT TGT CCC TCG TG-3').

FEV1.

The Spirometry method for the lung function analysis was performed by using a Vyaire Bodyplethysmograph (Chicago,USA). ERS Criteria were followed during the study. The quality control was performed by an experienced technician and a physician and supported by the software. FEV1 (=Forced expiratory volume in 1 second) and FVC (=Forced vital capacity) were measured at Baseline visit (B0) by using spirometry. After a period of normal breathing the participant should inhale maximal, directly followed by maximal and fast exhalation. The volume exhaled in one second is FEV1. The total exhaled volume is FVC. The ratio FVC/FEV1 is stated as FEV1%.

MICE

This study was carried out in compliance with the ARRIVE guidelines.

All mice were maintained under specific pathogen free conditions. They had free access to food and water. The experiments were approved by the government of Mittelfranken, Bavaria (54-2532.1-2/10).

Balb/c Wt in vivo treatment with OVA / Vitamin D3.

Female Balb/c wild-type mice at the age of 8-9 weeks were treated at day 0 intranasally with 25µl PBS, PBS + Vitamin D3 (=1α,25 DihydroxyvitaminD3 from Sigma Aldrich, 10 ng), Ovalbumin (500 µg) or OVA + Vitamin D3 (500µg and 10 ng respectively, with 20 min time delay in between) according to their group. After 48h the treatment was repeated. After additional 48 h whole lung cells were isolated as described previously and cultured with anti-CD3 (10µg/ml) respectively anti-CD3(10µg/ml) and anti-CD28 (1µg/ml) antibodies¹⁷.

Collection and analysis of the BAL

Bronchoalveolar lavage was performed 24h after the last allergen challenge, by intratracheally injecting and aspirating 0.8 ml saline twice. After its collection the BALF was centrifuged for 5 min at 1500 rpm.

The cell pellets were resuspended in 1 ml PBS and an aliquot was stained with trypan blue solution and cells were counted using a Neubauer chamber. Eosinophils and neutrophils were detected by fluorescence-activated cell sorting (FACS) analysis. The cell surface staining was performed with antibodies against CD3 (eBioscience, Frankfurt, Germany), GR-1 (BD Bioscience, Heidelberg, Germany), CD45R (eBioscience, Frankfurt, Germany) and CCR3 (BD Bioscience, Heidelberg, Germany) for 30 min at 4°C.

Histological analysis

Lung tissues were analyzed by using paraffin-embedded tissue slices for histology. After staining with Giemsa staining, peribronchial and perivascular inflammation was assessed by a pathologist blinded to the experimental group assignments of the individual lungs. Inflammation was graded by using a semi-quantitative scoring system with a range pending between 1 (mild) and 4 (severe) as described before¹⁷.

Bl6/C57 WT, ST2-/- in vitro treatment with Rhinovirus, OVA / Vitamin D3.

We analyzed lung cells from Wild-type and ST2-/- mice at the age of 7-9 weeks. All mice were based on a Bl6/C57 genetic background. Whole lung cells were isolated from the mice on day 0 and partly incubated with Rhinovirus (250µl RV/106 cells) for 1 hour at room temperature on a horizontal shaker. Afterwards OVA (500 mg/ml) and/or Vitamin D3 (10nM) were added to the cells infected with RV as well as to those not infected. Without further stimulation, cells were then cultured for 24h at 37° and 5% CO2.

RNA isolation and quantitative real time-PCR

To extract RNA from murine lung cells we used PeqGold RNA Pure according to the manufacturer's protocol (PeqLab, Erlangen, Germany). For reverse transcription of RNA (1µg), we used the first strand cDNA synthesis kit for RT-PCR (MBI Fermentas, Sat. Leon-Rot, Germany) followed by the amplification by quantitative real-time PCR (qPCR) using SoFast EvaGreen Supermix (Bio-Rad Laboratories, München, Germany). The qPCR itself was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with a cycle of 2 min 98°C, 50 cycles at 5 s 95°C, 10 s 60°C, followed by 5 s 65°C and 5 s 95°C.

The primers and sequences used for mouse were: mHPRT (5'-GCC CCA AAA TGG TTA AGG TT-3', 5'-TTG CGC TCA TCT TAG GCT TT-3'), mICOS (5'-GTG CAG CTT TCG TTG TGG TA -3', 5'-TCA GGG GAA CTA GTC CAT GC -3'), mL33 (5'-CCT CCC TGA GTA CAT ACA ATG ACC- '3, 5'-GTA GTA GCA CCT GGT CTT GCT CTT -3'), mFoxp3 (5'-AGA GCC CTC ACA ACC AGC TA -3', 5'-CCA GAT GTT GTG GGT GAG TG -3'), mL10 (5'-CCA AGC CTT ATC GGA AAT GA -3', 5'-TTT TCA CAG GGG AGA AAT CG -3'), mRora (5'-TCT CCC TGC GCT CTC CGC AC-3', 5'-TCC ACA GAT CTT GCA TGG A -3'), mST2 (5'-GCG GAG AAT GGA ACC AAC TA-3, 5'-AAG CAA GCT GAA CAG GCA AT -3'), mAREGB (5'-AGC TGA GGA CAA TGC AGG GTA-3, 5'-AGT GAC AAC TGG GCATCT GG-3), mPD1 (5'-TCA AGG CATGGT CAT TGG TA-3, 5'-TAG GCC ACA CTA GGG ACA GG-3).

Flow cytometric analysis and intracellular staining

For intracellular staining total lung cells from Balb/c wild-type mice were incubated over night with anti-CD3 antiCD28 antibodies and then stimulated for 4h with PMA/ Ionomicin and a protein transport inhibitor according to the manufacturer's protocol. Cells were harvested, washed and stained with anti-CD4, anti-CD8 and anti-CD25 antibodies for 30 min at 4°C and then stained with anti-Foxp3, anti IFN-gamma and anti-IL10 antibodies in staining buffer (BD Biosciences, Heidelberg) for 35 min at 4° C. Afterwards cells were washed again and resuspended in stain buffer. The following anti-mouse antibodies were used: CD4 FITC (BD Biosciences), CD4 PerCP (BD), CD25 PerCP (BD), Foxp3 APC (Miltenyi Biotec, Bergisch Gladbach, Germany).

Lung cell culture

WT Bl6/C57 and ST2^{-/-} total lung cells were incubated in the presence of Vitamin D3, OVA and with or without RV infection at 37°C. After 24h cells were harvested, washed and stained with anti-CD4 and anti PD-1 antibodies in staining buffer (BD Biosciences, Heidelberg) for 30 min at 4°C. Marked cells were acquired by using FACS-Calibur (BD Biosciences, Heidelberg) and analyzed with FlowJo.

Enzyme linked immunosorbent assay (ELISA).

Murine IL-5, IL10 and IL13 were detected in cell culture supernatants by DuoSet Elisa kit from R&D (Hamburg,Germany), according to the manufacturer protocol.

Statistical analysis

Differences were evaluated for significance (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$) by using the non-parametric one-tailed Mann-Whitney-test (Prism version 7 for Windows; GraphPad, La Jolla, CA, USA) and a two tailed Student t-test was used (Prism version 7 for Windows; GraphPad, La Jolla, CA, USA). Data are shown as mean values + s.e.m. For correlation of two parameters GraphPad Prism software (version 7 for Windows) was used.

Results

Clinical outcome of the cohorts of children analyzed in this study

The clinical data of these cohorts of children are reported in Table 1. Further, clinical details of the cohort were recently described^{6,18-20}. In Germany, Vitamin D3 is given to the infants as supplement⁹ to avoid immunological suppression especially in children which could not receive breastfeeding. Wheezing is associated with asthma in the first years of life¹⁰ moreover, RV is the factor that associated with wheezing in infants. In this study, we wanted to better understand the role of Vitamin D3 in pediatric asthma.

Serum-C-reactive protein (CRP) and 25 (OH)-VitD3 levels inversely correlated in control but not in asthmatic pre-school children

C-reactive protein (CRP) is a plasma protein, whose levels rise in response to inflammation and infections. It is an hepatic acute-phase protein that increases following interleukin-6 secretion by macrophages and T cells. Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) promoting phagocytosis by macrophages, which clears necrotic and apoptotic cells and bacteria. In healthy adults, the normal concentrations of CRP varies between 0.8 mg/L to 3.0 mg/L. However, some healthy adults show elevated CRP at 10 mg/L. The plasma half-life of CRP is 19 hours.²¹ We next asked if serum CRP level at recruitment in our cohort of children correlated with their serum level of 25(OH)VitD3.

The design of the study is reported in Fig 1a and the levels of CRP of the cohorts are reported in Table 1. We noticed that both control and asthmatic children group had one child with low 25(OH)VitD3 (less than 20 ng/ml) and very high CRP. We next correlated 25(OH)VitD3 and serum CRP levels without this very high CRP value in both cohorts (Fig 1b,c and d,e) and next in asthma we analyzed only data with CRP data lower than 2.5mg/ml, to be in the CRP range of controls (Fig 1f) and looked at the correlation with serum VitD3 (Table 2). Here we found that, control but not asthmatic children had an inverse correlation between their serum CRP value and serum 25(OH)VitD3 levels (Fig1c). Considering the CRP value in asthma, we noticed they were higher as compared to control children, indicating an ongoing infection, inflammation in these asthmatic children (Fig 1e).

VitD3 supplementation in infancy is associated with reduced asthma exacerbations

To analyze the role of VitD3 supplementation during infancy on asthma exacerbations, we then analyzed the number of asthma exacerbations and asked if VitD3 supplementation during infancy would associate with less disease exacerbations. Here we found that asthmatic children without VitD3 supplementation had more episodes of asthma exacerbations (Fig 2a) that were associated with induced CRP (Fig 2b), a factor associated with increased airway hyperresponsiveness (Fig 2c). In conclusion, increased asthma exacerbations associated with CRP in the absence of VitD3 supplementation, indicating a possible protective role of VitD3 during asthma exacerbations.

PD1 is decreased in blood cells of control children with higher serum levels of 1,25 (OH) Vitamin D3

We next wanted to analyze the role of 25(OH) VitD3 on Programmed cell death protein 1 (PD-1) in the cohort of preschool children. PD-1 is a protein on the surface of immune competent T cells that down-regulates T cell inflammatory activity.^{22,23} Considering VitD3 serum levels lower than 20 ng/ml as low, we found that high VitD3 associated with decreased PD1 mRNA levels in blood cells of control but not asthmatic children (Fig 2d, e). We also saw a trend towards *PD1 mRNA* induction in asthmatic children with higher serum VitD3 levels.

VitD3 given intranasally reduced lung inflammation

We next wanted to analyze the effect of intranasal treatment of Vitamin D3 in the airways in a murine model of airway tolerance (Fig 3a). To this aim, we treated mice intranasally with VitD3 and looked at airways inflammation (Fig 3b). Here we found that allergen challenge, without allergen sensitization, was accompanied by induction of local inflammation (Fig 3a,b). By contrast, intranasal VitD3 treatment, reduced lung inflammation (Fig 3b). We then asked if Eosinophils in bronchoalveolar lavage fluid were involved in regulating airway tolerance after allergen challenges. Here we found that allergen challenge, without allergen sensitization, was accompanied by induction of local inflammation and induced eosinophils in BALF (Fig 3c). By contrast, intranasal VitD3 treatment, reduced lung inflammation (Fig 3c). Altogether these data indicate an anti-inflammatory effect of VitD3 given intranasally in a model of airway tolerance.

1,25(OH)VitD3 inhibited IL33 and Ror-alpha in the airways

We then asked if ILC2 were upregulated in our model of airway tolerance after intranasal treatment with VitD3 and allergen challenge (Fig 4a).²⁴

Here we found that, intranasal delivery of VitD3, reduced Amphiregulin (AREG), which is a member of the epidermal growth factor family (Fig 4b), as well as IL-33, an alarmin and ligand of ST2, a receptor on Innate lymphoid cells⁶(Fig 4c). Intranasal delivery of VitD3 did not regulate the ILC2 markers ST2 nor ICOS (Fig 4 d, e) but reduced Ror-alpha, an ILC2 signature transcription factor (Fig 4f)²⁵. Thus airway tolerance induced by VitD3 and allergen given intranasally did not suppress completely ILC2 as confirmed by residual ILC2 cytokine IL-5 and IL-13 in the airways of treated mice (Fig 4 g,h).

Airway tolerance induced T regulatory cells and with 1,25 (OH)VitD3 reduced PD1 in the airways of OVA challenged mice

To further analyze the underlined mechanism of VitD3 in airway tolerance, in the same model (Fig 5a), VitD3 induced CD4+ T cells and reduced CD4+CD25+Foxp-3+ T regulatory cells in the airways (Fig 5b-d)¹⁶. Moreover, allergen challenge induced of IL-10 production, was kept upregulated by VitD3 treatment after anti-CD3 antibody challenge (Fig 5e). Finally, treatment of Vitamin D3 with allergen, reduced significantly PD1 mRNA in the airways (Fig 5f).

Vit D3rescued CD4+ T cells after RV infection *in vitro* in the absence of ST2 in lung cells

Murine IL-C2 in the lung were described in a model of influenza, where they promote a pathologic effect on airway hyperresponsiveness as well as a protective role in epithelial repair. To understand the effect of ILC2 in RV infection present during allergen challenge and the protective effect of VitD3 on this axis, we challenged *in vitro* lung cells from wt and ST2^{-/-} mice with OVA and infected them with RV and challenged them with VitD3 (Fig. 6a). Here we found that, Vit D3 induced the number of CD4+ T cells in RV infected lung cells in the absence of ST2 (Fig 6b). These experiments further support a CD4+ T cells inducing role of VitD3 in the airways during allergen challenge only in the absence of IL-33/ST2. Thus, IL33/ST2 axis is involved in T cell death induced by rhinovirus.

Vit D3 reduced lung CD4+PD1+ CD4+ T lymphocytes independently from ST2 deficient cells

We next asked if, in this *in vitro* system VitD3 would rescue T cells from cell death by inhibiting PD1 a marker of T cell exhaustion.²⁶ In addition, we wanted to better understand the role of ST2 in T cell survival. Here we found that, in the same experimental conditions depicted in Fig 7, CD4+PD1+ T cells were inhibited by VitD3 in the presence of allergen challenge dependently from ST2 (Fig 6c). In summary, VitD3, has a suppressive role on CD4+PD1+ T cells involved in T cell exhaustion in the airways in the absence of ST2.

Discussion

VitD3 influences immune system in asthma.⁶⁻⁸ Here we found that in healthy, but not in asthmatic preschool children serum 25(OH)VitD3 inversely correlated with CRP, a protein upregulated during ongoing inflammation and infections.²⁷ Furthermore, we found that, asthmatic children had higher CRP serum level, indicating induced ongoing inflammation. Finally, asthmatic pre-school children which did not receive VitD3 supplementation in infancy had more episodes of asthma exacerbations associated with high serum CRP levels indicating a limiting effect of VitD3 on infection also in preschool children with asthma.

Next, we wanted to see how Vitamin D3 affects T cells activation *in vivo* in asthma. Therefore we decided to look at PD1, a marker for downregulation of T cell mediated immune responses and also a marker for T cell exhaustion²². We found that control children with high Vitamin D3 serum levels have less PD1 mRNA in their blood cells than those with low VitD3 levels. This was not seen in asthmatic children, indicating the presence of a PD1 inducing pathway in asthma.

To look for a new molecular therapy for allergic asthma, we treated mice intranasally with VitD3 within 20 minutes after OVA allergen challenge. Here, VitD3 induced CD4 + T cells and down-regulated Foxp-3 T regulatory cells and PD1 in the lung.

We further demonstrated that VitD3 induced CD4 + T cells after OVA challenge. CD25, Foxp3 and IL10, all markers for T regulatory cells^{28,29}, were increased by OVA and downregulated by VitD3.

Furthermore, some ILC2 inducing markers like IL-33 were reduced by Vitamin D3 combined with allergen, indicating an homeostatic function in the airways of treated mice³⁰. It would be very interesting to know if Vitamin D3 could show a direct prophylactic effect.

Our data show a protective homeostatic effect of VitD3 *in vivo* and *in vitro*. Further, considering its limiting effect on lung ILC2, a cell type persistent in the lung of asthmatics, VitD3 given intranasally represent a new avenue for the treatment of asthma especially in asthmatic children.

Here we further found that, Vit D3 induced the number of CD4 + T cells in RV infected lung cells in the absence of ST2. These experiments further support a CD4 + T cells inducing role of VitD3 in the airways during allergen challenge only in the absence of IL-33/ST2. Thus, IL33/ST2 axis is involved in T cell death induced by rhinovirus.

In addition we found that VitD3, has a suppressive role on CD4 + PD1 + T cells involved in T cell exhaustion in the airways in the absence of ST2. These data support an interactive role of VitD3 and ILC2 in the lung that can be used for the treatment of allergic diseases and rhinovirus exacerbations seen in pediatric asthma.⁶

Declarations

Author Contributions.

P.H. generated the results for Figure1, 2, 6 and analysed all the data present in this manuscript. D.A. generated data for figures 3-5. K.A. is the paediatrician responsible of this study in Erlangen, M.R. measured the VitD3 and CRP levels in the serum of the children, N.L. further analyzed the results for Figure 1. L.G. helped with Figures 3-5. S.W. helped with ST2 ko mice. N.G.P. is the coordinator of Predicta. S.F. supervised the whole work and wrote the manuscript with PH. All authors reviewed the manuscript.

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Data availability statement.

All datasets [GENERATED/ANALYZED] for this study are included in the manuscript.

Disclosure of potential conflict of interest.

The authors declare no conflict of interest on the matter described in this manuscript.

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Ethical Statement.

All human and murine studies performed in this study received ethical approval from local institutions.

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Tables

Table 1. Clinical data of children at baseline visit

Control	Age	Gender	Skin Prick Test	FEV1%	VitD Supplement*
208	6	m	/	77	No
211	6	f	/	121	No
214	5	m	/	110	No
215	4	m	/	118	No
218	4	f	/	111	Yes
219	5	f	/	107	Yes
220	5	f	-	84	No
221	3	m	/	/	Yes
222	6	m	/	105	Yes
226	4	f	/	109	No
227	6	m	-	87	no
232	4	m	-	100	no
233	5	f	/	112	no
234	5	f	+	119	yes
235	4	m	/	113	no
236	5	m	-	111	yes
237	4	m	-	109	yes
240	4	f	-	92	no
241	5	m	-	123	no
244	5	f	-	107	no
245	4	m	-	121	yes
246	5	m	/	109	no
Mean	4,73	f=41%,m=59%	neg=90% pos=10%	106,9	Yes=36,4%; No=63,6%
SEM	0,18			2,67	

Asthma	Age	Gender	Asthma* Severity	Phenotype**	Skin Prick Test	FEV1%	Treatment	VitD3*** Prophylaxis	Level of VitD3	Number of exacerbations in the last 3 month	Number of exacerbations in the last 12 month
201	6	m	I	V	+	126	Steroid	no	9,69	0	0
202	6	m	I	U	+	111	Steroid	no		3	12
203	5	f	II	U	+	95	Steroid	no	6,72	0	0
204	6	m	II	A	+	128	Steroid	no	9,85	1	2
205	5	m	I	U	/	102	Steroid	no	11	4	5
206	5	f	I	U	+	129	Steroid	no	16,8	1	2
207	5	m	I	V	+	143	Steroid	no	17,6	4	10
209	4	f	II	v,a	+	115	Steroid	yes		3-4	7-8
210	6	f	I	V	+	98	Non-Steroid	no	11,3	3	10
212	5	m	II	e,v	-	96	Steroid	no		3	12
213	4	m	III	e	-	115	Steroid	no	17,8	3	12
216	5	f	III	a,v	+	92	Steroid	no	19,9	15	20
217	6	f	I	a,e,v	+	111	Steroid	no	15,8	2	5
223	5	m	I	V	+	99	Steroid	no	37,8	0	4
224	4	f	I	V	-	135	Steroid	yes	24,3	4	5
225	4	m	I	V	/	99	Steroid	yes	33,5	4	10
228	5	m	I	V	+	88	Non-Steroid	yes	33,5	2	4
229	4	m	I	V	+	87	Non-Steroid	no		1	3
230	5	m	I	V	+	101	Non-Steroid	no	28,7	2	4
231	4	m	I	V	+	71	Steroid	no	11,8	3	8
238	4	m	I	V	+	77	Steroid	no	8,5	3	9
239	5	f	I	E	/	98	Non-Steroid	yes	13,1	1	~200
242	5	m	II	a,e,v	/	81	Steroid	no	19,2	3-5	12
243	5	f	II	V	+	69	Steroid	no	30,6	20	120
Mean	4,92	f=37,5%; m=62,5%	I=62,5%; II=29,2%	v=50% u=16,7%	neg=15% pos=85%	102,75	Non Steroid=20,9% Steroid 79,1%	Yes 20,8 No=79,2			
SEM	0,15					4,04					

Table 2. Analysed data of children at baseline visit (B0)

Control	Virus Swab**	25(OH)VitD3***
208	-	/
211	RV ++	16,9
214	RV ++	13,4
215	-	26
218	RV +++	17,4
219	RV +	16,4
220	-	22,3
221	RV ++	25,5
222	-	34,1
226	-	20,3
227	-	16,5
232	RV +++	13,7
233	RV +, Other	25,9
234	RV ++, Other	12,6
235	RV ++	14,2
236	-	/
237	Other	10,7
240	RV+, Other	18,3
241	RV ++	25,9
244	/	/
245	-	28,4
246	RV+	24,4
Mean		20,15
SEM		1,46

Figure

Figure 7 not available with this version.

Figures

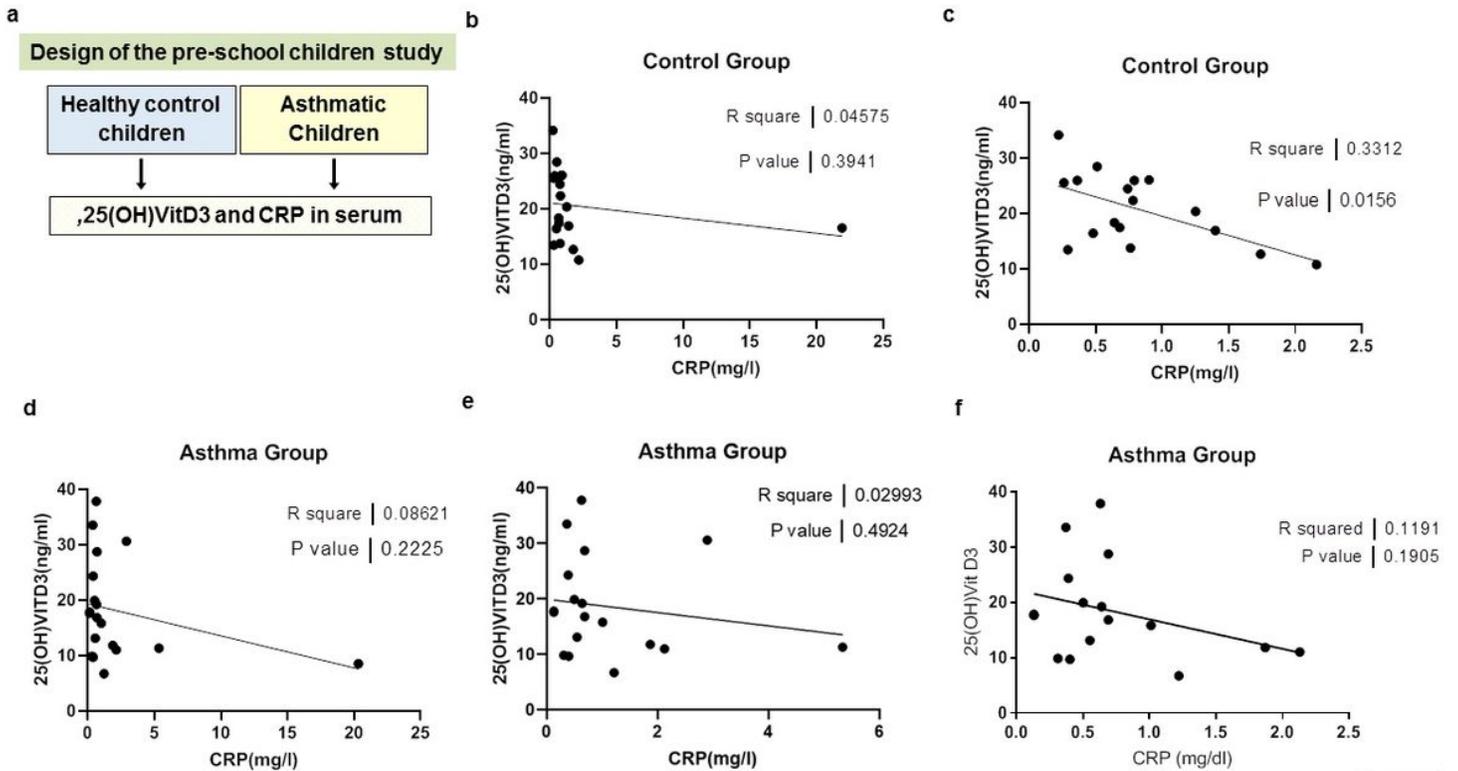


Figure 1

Figure 1
Higher serum levels of 1,25(OH) VitaminD3(=VitD3) are associated with lower CRP levels in the blood of control pre-school children. a: Experimental design of the study. b,c Linear regression analysis of serum levels of CRP and 25(OH)VitD3 in control and asthmatic children (d-f).

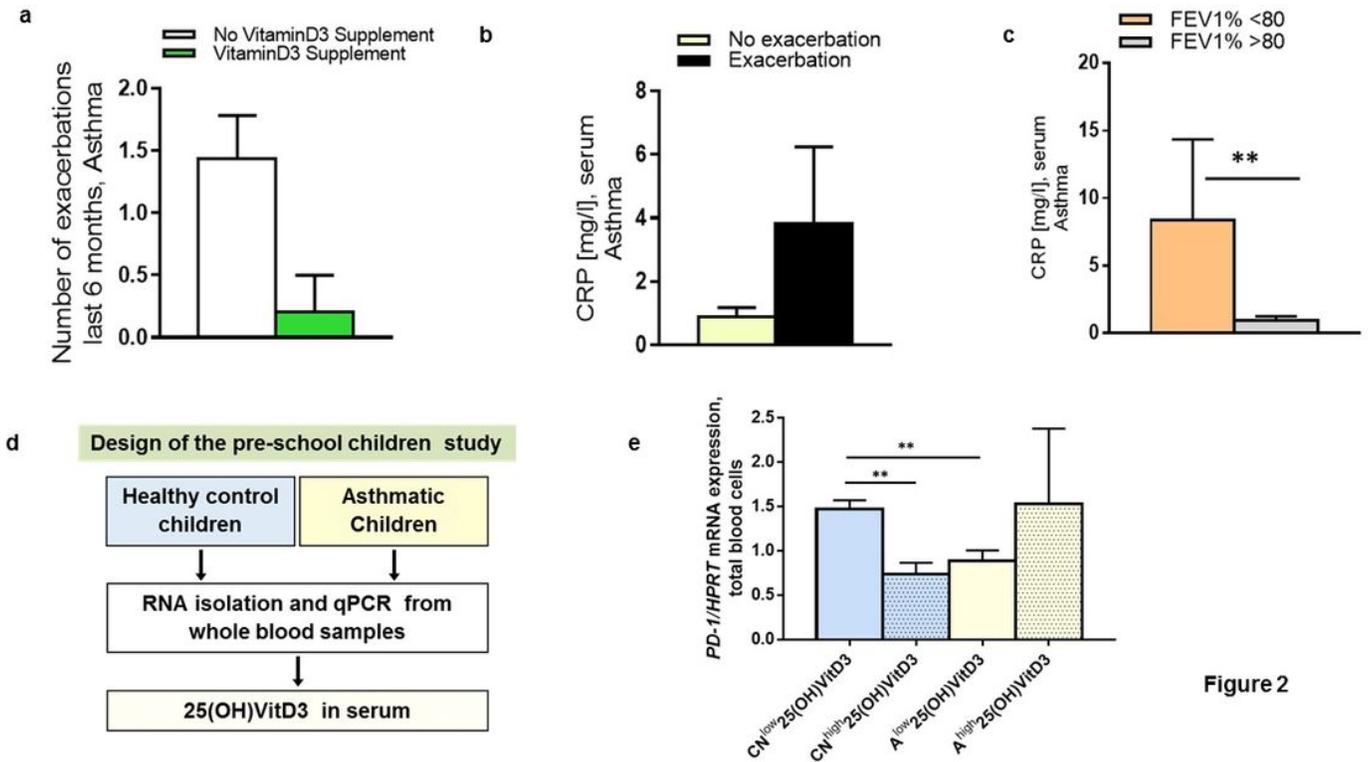


Figure 2

Figure 2

VitD3 supplementation is correlated with less exacerbations in preschool asthma children and PD1 is decreased in control children with higher serum levels of 1,25 (OH) Vitamin D3. (a) Exacerbations in asthmatic children during last 6 months (B0) depending on Vitamin D3 supplement intake (0=no Vitamin D3 supplement, 1= VitaminD3 supplement) as infant (No VitaminD3 supplement n=16,Vitamin D3 supplement n=5). (b) C-Reactive protein (CRP) measured in serum of asthmatic children at B0, in relation to number of exacerbations during the last 6 months retrospective from B0 (n= 8 in both groups). (c) CRP in same children related to their FEV1%, investigated at B0 (n=3-16 per group). p=0,0059. Two-tailed Student's T Test. d. Experimental design. b. Total blood cells (tempus tube) RNA isolation for qPCR from healthy and asthmatic children. e.PD1/HPRT mRNA expression in control children (CN) and asthmatic children (A), considering the serum level of 25 (OH) VitD3 lower than 20 ng/ml (low) and higher than 20 ng/ml (high). (N=6-6-7-5; CN low 25(OH)VitD3 versus CN high 25(OH)VitD3: p=0.001; CN low 25(OH)VitD3 versus A low 25(OH)VitD3: p= 0.0033. Two-tailed Students T-test).

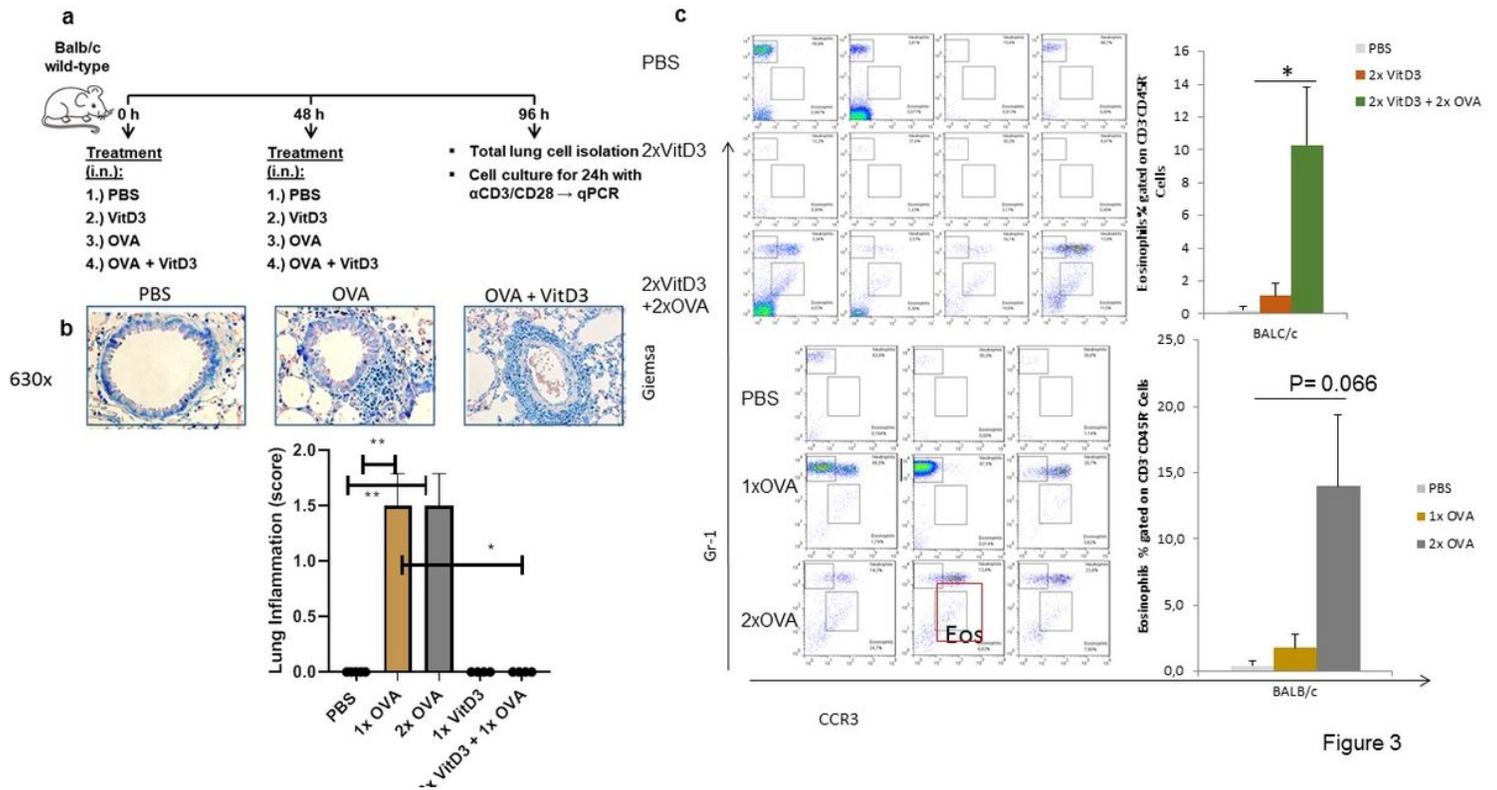


Figure 3

VitD3 given intranasally limits airways inflammation. a. Experimental design. b. Representative histological sections of the lung after Giemsa staining (n=4 per group) and lung inflammation score (PBS versus 1x and 2xOVA p=0.079;1xOVA versus VitD3+1xOVA p= 0.028 after Mann Whithney).c. BALF was collected and Eosinophils (into the red square) were stained by FACS staining (n=4 or 3 per group as indicated) and analysed by using Two tailed T test analysis (PBS versus 2xVitD3 p= 0.029 ; PBS versus 2xOVA p=0.066).

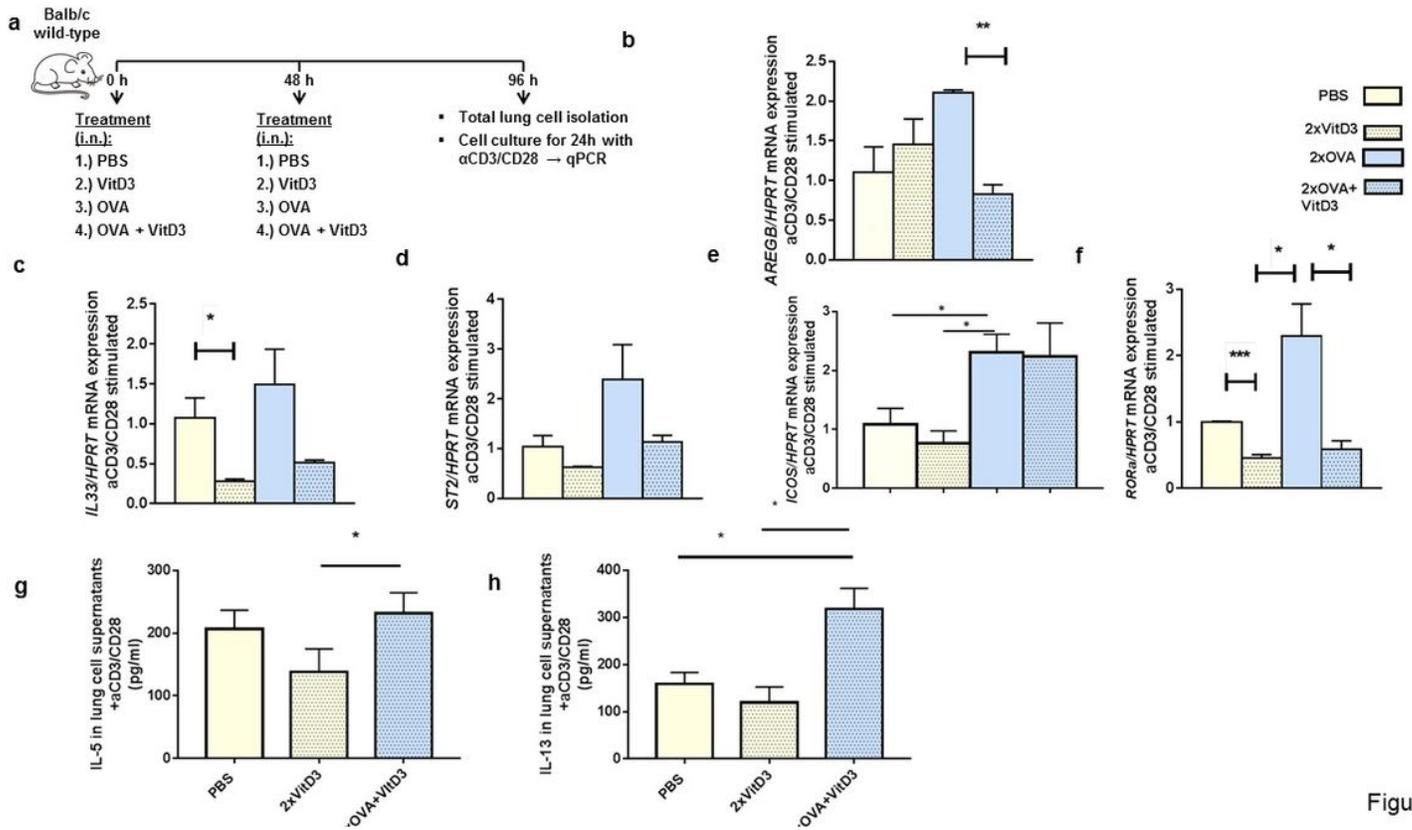


Figure 4

Figure 4
 Airway challenge with 1,25 (OH) Vitamin D3 inhibited IL-33, Ror-alpha in the airways. a. Experimental design of airway tolerance. b. AREGB/HPRT mRNA expression in αCD3/CD28 stimulated lung cells. $p=0,0038$. Two-tailed T-test. c. IL-33/HPRT mRNA expression in αCD3/CD28 stimulated lung cells. $P=0,035$. Two-tailed T-test. d. ST2/HPRT mRNA expression in αCD3/CD28 stimulated lung cells. e. ICOS/HPRT mRNA expression in αCD3/CD28 stimulated lung cells. $p=0,042$. $p=0,015$. f. RORα/HPRT mRNA expression in αCD3/CD28 stimulated lung cells. PBS vs. 2xVitD3: $p=0,0005$. 2xVitD3 vs 2xOVA: $p=0,011$. 2xOVA vs 2xVitD3: $p=0,026$. g. IL-5 ELISA; $p=0,047$. Two-Tailed T-test. h. IL-13 ELISA. PBS vs. VitD3: $p=0,0188$; PBS vs. 2xOVA+VitD3: $p=0,0109$. Both two-tailed T-test.

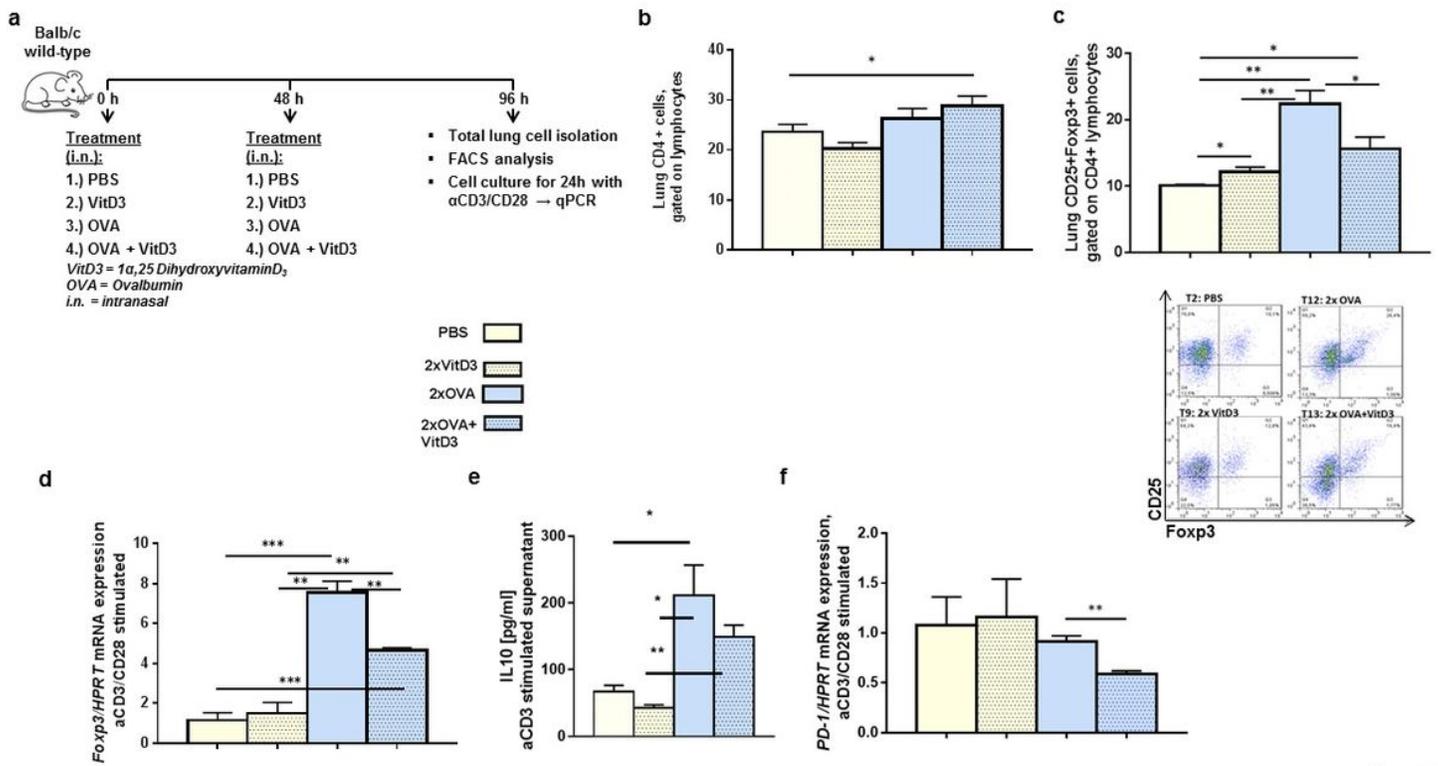


Figure 5

