

Anti-inflammatory and corticosteroid-enhancing actions of vitamin D in monocytes of patients with steroid-resistant and those with steroid-sensitive asthma

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Background: Vitamin D is known for its anti-inflammatory effects.

Objective: Vitamin D regulation of responses in patients with steroid-resistant (SR) versus steroid-sensitive (SS) asthma has not been studied.

Methods: Peripheral blood cells from 11 patients with SR asthma and 8 patients with SS asthma were preincubated with 1,25-dihydroxyvitamin D (1,25[OH]₂D [VitD]), followed by dexamethasone (DEX) treatment and LPS stimulation. LPS-induced phosphorylated p38 mitogen-activated protein kinase (p-p38) in monocytes was examined by means of flow cytometry. Mitogen-activated protein kinase phosphatase-1 (MKP-1) mRNA expression, which inhibits p-p38, was analyzed by means of real-time PCR. Glucocorticoid receptor (GR) binding and histone H4 acetylation in the glucocorticoid response element of the MKP-1 promoter in monocytes were analyzed by means of chromatin immunoprecipitation.

Results: DEX significantly inhibited LPS-induced p-p38 in monocytes from patients with SS asthma but not those from patients with SR asthma ($P < .01$). VitD inhibited LPS-induced p-p38 in monocytes from both patient groups ($P < .01$) but enhanced DEX suppression of LPS-induced p-p38 only in monocytes from patients with SS asthma ($P < .01$). VitD induced MKP-1 expression and enhanced DEX induction of MKP-1 in both patients with SS asthma and patients with SR asthma. VitD/DEX-induced MKP-1 mRNA levels remained significantly lower in monocytes from patients with SR asthma ($P < .05$). DEX-stimulated recruitment of GR and histone H4 acetylation at the glucocorticoid response element 4.6 kbp upstream of the MKP-1 gene were significantly lower in monocytes from patients with SR asthma compared with those from patients with SS asthma. VitD pretreatment enhanced DEX-induced GR binding and histone acetylation in monocytes from both patient groups. However, GR binding and histone H4 acetylation remained significantly lower in monocytes from patients with SR asthma.

Conclusion: VitD demonstrated anti-inflammatory and corticosteroid-enhancing effects in monocytes of patients with

SR asthma and patients with SS asthma. However, the responses to corticosteroids in patients with SR asthma remained significantly lower than those in patients with SS asthma. (J Allergy Clin Immunol 2014;■■■■:■■■■-■■■■.)

Key words: Asthma, steroid resistance, corticosteroids, vitamin D

Current scientific literature supports the importance of the anti-inflammatory function of vitamin D in health and disease beyond its known role in calcium metabolism and bone health.^{1,2} It has been reported that vitamin D deficiency, which is defined as a serum concentration of the vitamin D prohormone 25-hydroxyvitamin D (25[OH]D) of less than 20 ng/mL, is very common in the general population.^{1,3} Epidemiologic studies have suggested that adequate levels of 25(OH)D are critical because studies have linked vitamin D insufficiency to a variety of conditions, including cancers, autoimmune diseases, infections, and diabetes. Vitamin D deficiency has been reported to be associated with a higher rate of asthma risk.^{4,5} Recent publications by our research group and others have found that asthmatic patients with low serum vitamin D levels have increased corticosteroid requirements.⁶⁻¹⁰ We recently reported^{6,7} that in adult and pediatric patients with mild-to-moderate asthma, reduced vitamin D levels are associated with impaired lung function, increased airway hyperreactivity, and reduced corticosteroid response.

Corticosteroids are currently the gold standard for controlling inflammation in asthmatic patients.¹¹ The anti-inflammatory effects of corticosteroids are mediated through cognate binding to glucocorticoid receptor (GR), which translocates to the cell nucleus from cytoplasm and binds to glucocorticoid response elements (GREs) to regulate the transcription of specific genes, including mitogen-activated kinase phosphatase-1 (MKP-1).^{12,13} MKP-1 is a phosphatase that selectively inactivates p38 mitogen-activated protein kinase (MAPK),¹⁴ thus inhibiting the production of proinflammatory cytokines regulated by p38 MAPK, which is critical for the anti-inflammatory functions of corticosteroids.¹⁵ In a recent study of monocytes from healthy subjects, we demonstrated that 1,25-dihydroxyvitamin D (1,25[OH]₂D [VitD]) mediated inhibition of LPS-induced p38 MAPK activation and proinflammatory cytokine production. This was mediated by VitD-induced MKP-1.¹⁶ We also found *in vitro* evidence for steroid-sparing effects of VitD⁷ and reported that VitD enhancement of cellular responses to dexamethasone (DEX) in human monocytes was mediated by MKP-1.¹⁷

It is estimated that up to 25% of asthmatic patients do not respond to corticosteroids.¹⁸⁻²¹ GR-mediated signaling has been a focus of research to understand the mechanisms that modulate steroid sensitivity and resistance.²² Whether vitamin D can exert anti-inflammatory and steroid-enhancing effects in patients with steroid-resistant (SR) asthma and those with steroid-sensitive (SS) asthma is of great interest because oral supplementation

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Abbreviations used

ChIP: Chromatin immunoprecipitation
 DEX: Dexamethasone
 GR: Glucocorticoid receptor
 GRE: Glucocorticoid response element
 MAPK: Mitogen-activated protein kinase
 MED14: Mediator complex subunit 14
 MKP-1: Mitogen-activated kinase phosphatase 1
 p-p38: Phosphorylated p38 mitogen-activated protein kinase
 VitD: 1,25-Dihydroxyvitamin D (1,25[OH]₂D)

with vitamin D might represent an inexpensive approach to enhance corticosteroid responsiveness, improve asthma control, and reduce asthma burden. In this study the anti-inflammatory effects of vitamin D and the effects of vitamin D on cellular responses to corticosteroids *in vitro* were examined in monocytes of patients with SR asthma and those of patients with SS asthma.

METHODS**Materials**

LPS, DEX, VitD (an active form of vitamin D), and anti- β -actin mAb were purchased from Sigma (St Louis, Mo). HyQTase was purchased from Hyclone Laboratories (Logan, Utah). Rabbit polyclonal antibody to GR and rabbit IgG were purchased from Abcam (Cambridge, Mass). Rabbit polyclonal antibody to GR phosphorylated at S211 was purchased from Cell Signaling (Danvers, Mass). Rabbit polyclonal antibodies to histone H4 and acetylated histone H4 were purchased from Millipore (Temecula, Calif). Anti-mouse and anti-rabbit horseradish peroxidase-labeled IgG were purchased from Amersham Biosciences (Piscataway, NJ). Chemiluminescent reagent was purchased from Perkin Elmer Life Sciences (Waltham, Mass). ChIP-IT Express kit was purchased from Active Motif (Carlsbad, Calif). Mouse mAb to GR and all the antibodies and reagents used for flow cytometry were purchased from BD Biosciences (San Diego, Calif).

Study subjects

Nineteen adult asthmatic patients with a baseline FEV₁ percent predicted of less than 85% were recruited. None of the subjects had received systemic corticosteroids for at least 6 months before the study. Smokers were excluded from participation in the study. All study subjects were symptomatic, as determined by using the Juniper Asthma Control Questionnaire.²³ Mean 7-item Asthma Control Questionnaire scores are provided in Table I, which details the characteristics of the study subjects. Some of the study participants were not taking inhaled corticosteroids at the time of enrollment but had used inhaled corticosteroids in the past. Patients' clinical responses to corticosteroids were determined based on change in prebronchodilator morning FEV₁ percent predicted after a 1-week oral prednisone burst (40 mg/d). Asthmatic patients were defined as having SR asthma if they had less than 10% improvement in FEV₁ and as having SS asthma if they had more than 12% improvement in FEV₁. Eight patients were classified as having SS asthma, and 11 patients were classified as having SR asthma (Table I). Blood samples were collected from all patients. The study was approved by the Institutional Review Board at National Jewish Health (Denver, Colo).

Cell culture and treatment

PBMCs were isolated from heparinized venous blood of asthmatic donors by means of Ficoll-Hypaque density gradient centrifugation. Cells were cultured in hormone-free medium (phenol red-free RPMI containing 5% charcoal-stripped FCS, 50 μ g/mL streptomycin, and 50 U/mL penicillin) during hormone and LPS treatments. PBMCs from patients with SS asthma and patients with SR asthma were preincubated with 10 nmol/L VitD for 21

hours, followed by 10 nmol/L DEX treatment for 3 hours. The adherent PBMC fraction was collected in RNA preservation solution (Qiagen, Valencia, Calif) or lysed for total protein extraction. Cells were stimulated with 10 ng/mL LPS for 10 minutes for MAPK activation to examine cellular responses to LPS after VitD with or without DEX treatment. Phosphorylated p38 MAPK (p-p38) expression in CD14⁺ cells was examined by using flow cytometry, as described by us earlier.²⁴ PBMCs were cultured in the presence of 10 nmol/L VitD for 24 hours, followed by cell stimulation with 10 ng/mL LPS with and without 10 nmol/L DEX for 24 hours, to examine the effects of VitD pretreatment on LPS-induced cytokine production. Cell-culture supernatants were collected and stored at -80°C for cytokine analysis by using ELISA.

Real-time PCR

Total RNA from cells was prepared with the RNeasy Mini kit (Qiagen). After reverse transcription, cDNA from each sample was analyzed by means of real-time PCR with the dual-labeled fluorogenic probe method on an ABI Prism 7300 real Time PCR system (Applied Biosystems, Foster City, Calif), as previously described.²⁵ Specific gene expressions were determined with primers for MKP-1, IL-6, and β -actin purchased from Applied Biosystems.

Western blotting

Protein lysates were prepared from adherent PBMC fractions cultured with 10 nmol/L VitD for 21 hours, followed by media or 10 nmol/L DEX culture for 3 hours. Cells were rinsed with ice-cold PBMCs and lysed in complete RIPA buffer supplemented with protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, Calif) for 10 minutes on a plate, transferred to Eppendorf tubes, and lysed for an additional 30 minutes on ice. Cell lysates were spun at 10,000 rpm for 10 minutes at 4°C , and supernatants were stored at -80°C . Protein samples were resolved on 4-12% Bis-tris gel (Invitrogen, Carlsbad, Calif) and transferred to polyvinylidene difluoride membranes. The membranes were incubated in PBS containing specific antibodies, 5% dry milk, and 0.1% Tween 20 at 4°C overnight. Subsequently, membranes were washed in PBS/0.1% Tween 20, incubated for 1 hour at room temperature with horseradish peroxidase-labeled secondary antibodies, washed, incubated with chemiluminescent reagent, and processed for autoradiography.

Developed x-ray films were scanned, and the densitometry of the bands was quantified with ImageJ (version 1.47) software (available at <http://rsbweb.nih.gov/ij/>). The densitometry of the bands was adjusted by subtracting the background densitometry readings. In this study blots detected by antibody against Ser211-phosphorylated GR and total GR were developed together for cell lysates from each study subject.

ELISA analysis

IL-6 levels in cell-culture supernatants were tested with the Human IL-6 ELISA ready-set-go! Kit from eBioscience (San Diego, Calif).

Chromatin immunoprecipitation assay

GR binding to the GRE in the MKP-1 promoter in monocytes of patients with SR asthma and patients with SS asthma was assessed by using the chromatin immunoprecipitation (ChIP) assay with the ChIP-IT Express kit, according to the manufacturer's instructions. PBMCs were preincubated with 10 nmol/L VitD for 21 hours, followed by DEX treatment at 10 nmol/L for 3 hours. GR binding to the GRE in the human MKP-1 gene promoter and histone H4 acetylation in this region were examined. Rabbit polyclonal antibodies were used in chromatin precipitations. Precipitated DNA was quantified by means of quantitative real-time PCR with SYBR green (Applied Biosystems). Primers used to detect MKP-1 promoter GREs were synthesized according to the method of Tchen et al.²⁶ The quantity of anti-GR antibody-precipitated DNA was normalized to Input DNA; anti-acetylated histone H4 (Anti-acH4) antibody-precipitated DNA was normalized to anti-histone H4 (Anti-H4) antibody-precipitated DNA.

TABLE I. Patients' characteristics

	Patients with SR asthma (n = 11)	Patients with SS asthma (n = 8)
Age (y), mean $\pm$ SD	42.5 $\pm$ 12.3	41.4 $\pm$ 13.8
Sex (male/female)	5/6	4/4
Race (white/African American/other)	7/1/3	7/0/1
BMI (kg/m ²), mean $\pm$ SD	27.9 $\pm$ 5.9	31.3 $\pm$ 9.8
IgE (U/mL), mean $\pm$ SD	178 $\pm$ 181	127 $\pm$ 87
ACQ-7 score	2.5 $\pm$ 1.2	2.7 $\pm$ 0.9
Baseline FEV ₁ (% predicted), mean $\pm$ SD	66.2 $\pm$ 16.2	65.0 $\pm$ 11.3
FEV ₁ (% reversal with albuterol), mean $\pm$ SD	27.8 $\pm$ 22.4	38.0 $\pm$ 24.6
FEV ₁ (% change after prednisone burst), mean $\pm$ SD	0.1 $\pm$ 5.3*	33.1 $\pm$ 17.5
Corticosteroid medications†		
ICS/LABA	4	3
ICS	3	0
None	4	5

ACQ-7, Seven-item Asthma Control Questionnaire; ICS, inhaled corticosteroid; LABA, long-acting β -agonist.

* $P < .0001$ compared with patients with SS asthma.

†For patients with SR asthma and patients with SS asthma who received ICSs/LABAs or ICSs, the mean $\pm$ SE of the ICS dose in budesonide equivalents was 1000 $\pm$ 344 and 940 $\pm$ 681 μ g, respectively.

Statistical analyses

Results were expressed as means $\pm$ SEMs or means $\pm$ SDs. Statistical analysis was conducted with GraphPad Prism, version 5 (GraphPad Software, La Jolla, Calif). Data were analyzed by using the paired Student *t* test (pairing by experimental conditions within the same patient group) and the unpaired Student *t* test (comparing the same treatment condition between 2 patient groups [ie, patients with SR asthma and patients with SS asthma]) if the data followed normal distribution; nonparametric tests were applied for the data that were not normally distributed. Differences were considered significant at a *P* value of less than .05. A minimum of 3 independent experiments was conducted to allow for statistical comparisons. In all figures values represent means $\pm$ SEMs.

RESULTS

Study subjects

Clinical characteristics of the study participants are summarized in Table I. In this study patients were defined as having SR or SS asthma based on changes in lung function after 1 week of oral prednisone treatment (40 mg/d). Asthmatic patients were defined as having SR asthma if they had less than 10% improvement in prebronchodilator FEV₁ percent predicted after a 7-day prednisone burst and as having SS asthma if they had greater than 12% improvement in FEV₁. These definitions of SR and SS asthma have been used in all of our research studies; our previous publications indicate that most patients with SR asthma have less than 5% improvement in FEV₁ after a 1-week oral prednisone burst.²⁷⁻³² As documented based on prebronchodilator FEV₁ percent predicted values, all study participants had bronchoconstriction with an FEV₁ percent predicted (mean $\pm$ SD) of 66.9% $\pm$ 16.2% and 65.0% $\pm$ 11.3% for the SR and SS asthma groups, respectively. After a 1-week prednisone burst, patients defined as having SS asthma had significant improvement in prebronchodilator FEV₁ percent predicted, with an FEV₁ percent predicted (mean $\pm$ SD) of 85.4% $\pm$ 11.7% ($P = .0002$), whereas no significant change in lung function was observed in the SR asthma group (ie, FEV₁ percent predicted [mean $\pm$ SD] of 66.1% $\pm$ 16.4%, $P = .96$).

Fig E1 in this article's Online Repository at www.jacionline.org summarizes changes in individual prebronchodilator FEV₁ percent predicted values in patients with SR asthma and patients

with SS asthma before and after a 1-week oral prednisone burst. After 7 days of oral prednisone treatment, patients with SS asthma had a substantial improvement in lung function, with a Δ FEV₁ percent predicted value (mean $\pm$ SD) of 33.1% $\pm$ 17.5%. No significant change in FEV₁ percent predicted values was observed in the SR asthma group, with a Δ FEV₁ percent predicted value (mean $\pm$ SD) of 0.1% $\pm$ 5.3% ($P < .0001$ compared with patients with SS asthma). Both patients with SR asthma and patients with SS asthma demonstrated greater than 12% improvement in FEV₁ percent predicted in response to bronchodilator administration (albuterol), which is consistent with the American Thoracic Society's definition of asthma (Table I).³³

VitD inhibits LPS-induced p38 phosphorylation and enhances corticosteroid responses in patients with SS asthma

In this study we examined the effects of VitD and DEX on suppression of responses to LPS in cells from patients with SS asthma versus patients with SR asthma. PBMCs from patients with SS asthma and patients with SR asthma were preincubated with 1,25(OH)₂D₃ for 21 hours, followed by DEX treatment for 3 hours. Then the cells were stimulated with 10 ng/mL LPS for 10 minutes. As shown by using flow cytometry (Fig 1), 4.6% $\pm$ 1.1% and 2.4% $\pm$ 0.6% CD14⁺ cells expressed p-p38 before LPS treatment in patients with SS asthma and patients with SR asthma, respectively. LPS treatment significantly increased the percentage of CD14⁺ cells that express p-p38 to 46.1% $\pm$ 6.3% ($n = 8$, $P < .01$) and 50.3% $\pm$ 6.7% ($n = 9$, $P < .01$) in patients with SS asthma and patients with SR asthma, respectively. DEX significantly inhibited LPS-induced p-p38 by 29% ($n = 8$, $P < .01$) in patients with SS asthma, but no significant inhibition of LPS-induced p-p38 by DEX was observed in patients with SR asthma. At the same time, 1,25(OH)₂D₃ pretreatment significantly inhibited LPS-induced p-p38 by 56% ($n = 8$, $P < .01$) and 50% ($n = 9$, $P < .01$) in patients with SS asthma and patients with SR asthma, respectively. Combination of 1,25(OH)₂D₃ and DEX inhibited LPS-induced p-p38 by 74% ($n = 8$, $P < .01$) in patients with SS asthma; this was significantly higher than the inhibition by 1,25(OH)₂D₃ or DEX alone ($n = 8$, $P < .01$).

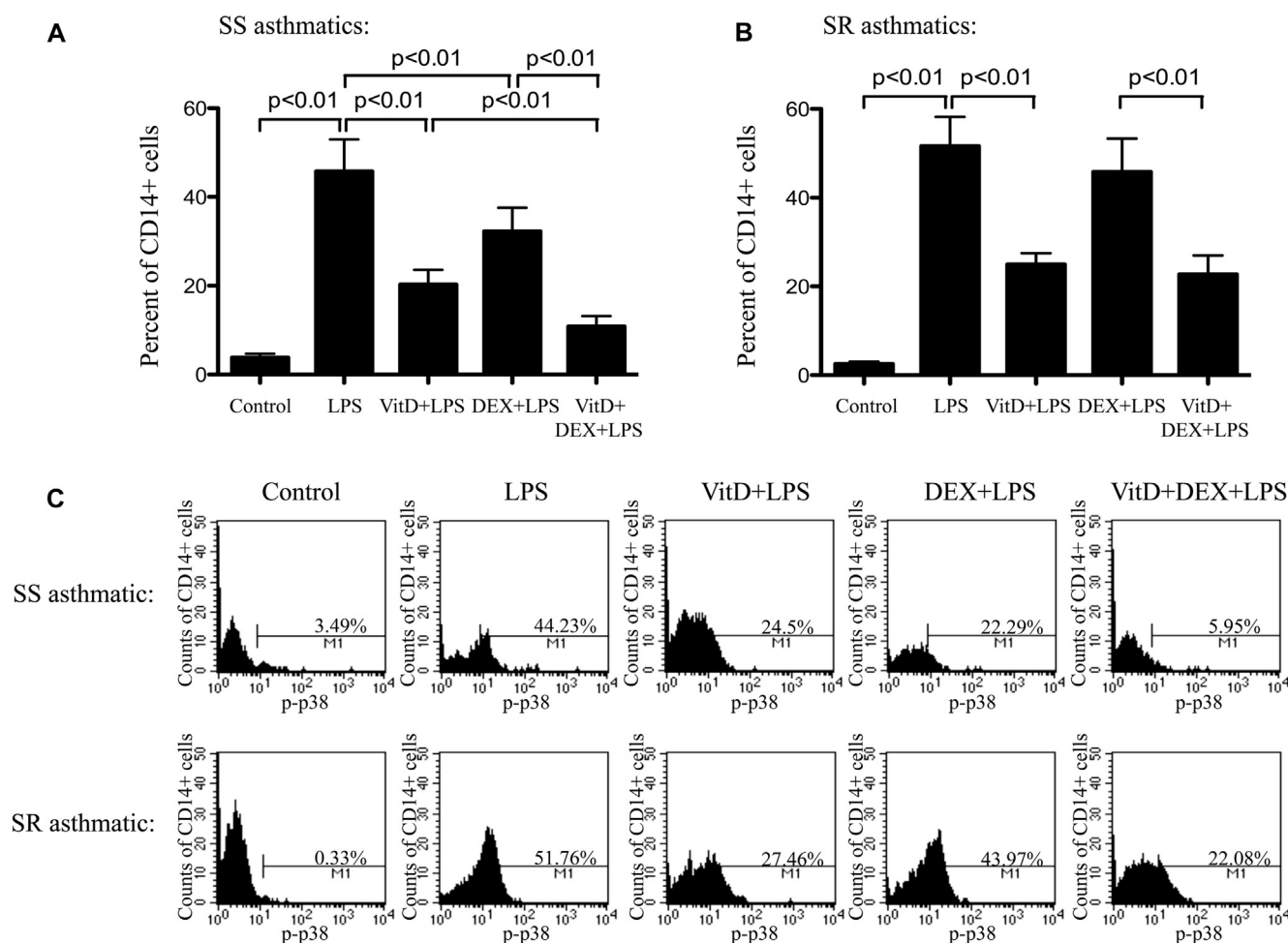


FIG 1. A and B, VitD enhances DEX inhibition of LPS-induced p38 phosphorylation in monocytes from patients with SS asthma ($n = 8$) (Fig 1, A) but not those from patients with SR asthma ($n = 9$) (Fig 1, B), as shown by means of flow cytometry. C, Representative flow cytometric data on the effects VitD, DEX, and VitD plus DEX on LPS-induced p-p38 in CD14⁺ cells from patients with SS asthma and patients with SR asthma are shown.

However, the combination of $1,25(\text{OH})_2\text{D}_3$ and DEX did not further enhance the inhibitory effect caused by $1,25(\text{OH})_2\text{D}_3$ alone ($n = 9$) in the cells from patients with SR asthma.

VitD enhances DEX inhibition of LPS-induced IL-6 production in monocytes from patients with SS asthma and patients with SR asthma

PBMCs from patients with SS asthma and patients with SR asthma were preincubated with $1,25(\text{OH})_2\text{D}_3$ for 24 hours, followed by LPS stimulation with or without DEX for an additional 24 hours, to examine whether the differential suppression of LPS-induced p38 MAPK activation by VitD and DEX influenced cytokine production. IL-6 levels in cell-culture supernatants were examined by using ELISA. On stimulation with LPS, the amount of IL-6 in culture supernatants increased from a basal level of 31.1 ± 8.5 and 66.6 ± 10.0 pg/mL to 749.5 ± 31.8 ($P < .01$) and 1443.2 ± 44.2 pg/mL ($P < .01$) in patients with SS asthma and patients with SR asthma, respectively. Treatments of $1,25(\text{OH})_2\text{D}_3$ or DEX significantly inhibited LPS-induced IL-6 production in cells from patients with SS asthma. This was further inhibited by the combination of $1,25(\text{OH})_2\text{D}_3$ and DEX in cells from these patients

(Fig 2). Similarly, $1,25(\text{OH})_2\text{D}_3$ or DEX significantly inhibited LPS-induced IL-6 production in cells from patients with SR asthma. The combination of $1,25(\text{OH})_2\text{D}_3$ and DEX further decreased IL-6 levels (Fig 2). However, significantly greater inhibition of LPS-induced IL-6 production was observed in the cells of patients with SS asthma in the presence of DEX or the VitD/DEX combination compared with that seen in cells of patients with SR asthma ($P < .05$ and $P < .01$ for DEX and VitD/DEX treatment conditions, respectively; Fig 2).

VitD enhances DEX induction of MKP-1 in monocytes from patients with SS asthma and patients with SR asthma

We previously reported that VitD suppression of LPS-induced p38 MAPK activation in human monocytes from healthy subjects is mediated by MKP-1¹⁶ and that VitD/DEX enhanced the expression of MKP-1 in human monocytes.¹⁷ In this study we examined the expression of MKP-1 mRNA in response to VitD, DEX, or VitD/DEX in monocytes of patients with SR asthma and patients with SS asthma. PBMCs from patients with SS asthma and patients with SR asthma were preincubated with $1,25(\text{OH})_2\text{D}_3$

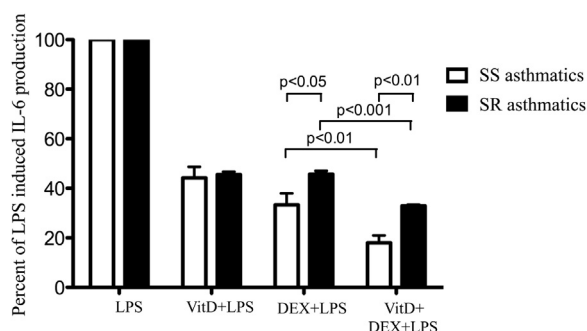


FIG 2. VitD enhances DEX inhibition of LPS-induced IL-6 production in monocytes from patients with SS asthma and patients with SR asthma, as detected by means of ELISA. Percentages of LPS-induced IL-6 levels for each treatment condition compared with the amount of IL-6 produced in response to LPS alone (100%) are shown ($n = 5$ patients with SR asthma and $n = 5$ patients with SS asthma).

or vehicle control for 21 hours, followed by DEX treatment for 3 hours. The adherent fraction of PBMCs, which contains mainly monocytes,¹⁶ was collected to examine MKP-1 mRNA expression. $1,25(\text{OH})_2\text{D}_3$ or DEX individually significantly induced MKP-1 expression in both patients with SS asthma and patients with SR asthma ($P < .05$ and $P < .01$ for VitD or DEX treatment conditions individually compared with untreated cells, respectively). Preincubation of cells with $1,25(\text{OH})_2\text{D}_3$ significantly enhanced DEX induction of MKP-1 in both patients with SS asthma and patients with SR asthma ($P < .05$; Fig 3, A and B). The level of MKP-1 induced by DEX or the VitD/DEX combination was significantly greater in patients with SS asthma than in patients with SR asthma ($P < .05$ and $P < .05$ for the corresponding treatment conditions in patients with SS asthma; Fig 3, C).

VitD enhanced GR binding and histone H4 acetylation to the GRE of the MKP-1 promoter in monocytes from patients with SS asthma and patients with SR asthma

The above data indicated that DEX differentially upregulated MKP-1 in patients with SS asthma and patients with SR asthma. To further understand whether this effect was influenced by differential GR binding to the GRE in the MKP-1 promoter, we performed ChIP assays to assess GR binding to the GRE in the MKP-1 promoter. GRE, which is located 4.6 kb upstream of the MKP-1 gene transcriptional start site, is a predominant GR binding site in the human MKP-1 gene promoter.²⁶ A schematic drawing of the human MKP-1 gene promoter, the location of the GRE, and potential histone acetylation sites are shown in Fig 4, A.

The results of ChIP assays showed that DEX treatment significantly induced GR binding to the GRE by 3.5 ± 0.1 -fold ($P < .05$) and 2.9 ± 0.6 -fold ($P < .05$), respectively, comparing media control in patients with SS asthma and patients with SR asthma (Fig 4, B). In response to DEX treatment, GR binding to this GRE in the MKP-1 promoter was significantly greater in patients with SS asthma compared with that seen in patients with SR asthma ($P < .05$; Fig 4, B). Preincubation with $1,25(\text{OH})_2\text{D}_3$ significantly enhanced DEX-induced GR binding to 6.0 ± 0.4 -fold ($P < .05$) and 7.6 ± 1.8 -fold ($P < .05$), respectively, in patients with SS asthma and patients with SR asthma (Fig 4, B). The

amount of GR bound to the MKP-1 promoter in response to VitD/DEX treatment was significantly greater in the cells of patients with SS asthma compared with those of patients with SR asthma ($P < .05$).

Because histone acetylation is an indication of active gene transcription,^{16,34} we tested histone H4 acetylation levels around the region of the GRE in the MKP-1 promoter using a ChIP assay. The results showed that DEX treatment significantly increased histone H4 acetylation by 17.5 ± 4.1 -fold ($P < .05$) and 9.1 ± 1.1 -fold ($P < .05$), respectively, compared with media control in both patients with SS asthma and patients with SR asthma (Fig 4, C). Significantly higher histone H4 acetylation was observed in patients with SS asthma than in patients with SR asthma after DEX treatment ($P < .05$; Fig 4, C). Preincubation with $1,25(\text{OH})_2\text{D}_3$ significantly enhanced DEX-mediated histone H4 acetylation to 36.3 ± 2.8 -fold ($P < .05$) and 18.3 ± 0.9 -fold ($P < .05$), respectively, in patients with SS asthma and patients with SR asthma (Fig 4, C). In response to VitD/DEX treatment, significantly greater histone H4 acetylation was observed at the MKP-1 promoter of patients with SS asthma compared with that seen in patients with SR asthma ($P < .01$).

DEX differentially upregulated GR phosphorylation at S211 in monocytes from patients with SS asthma and patients with SR asthma

To determine whether the differential induction of MKP-1 by DEX in the monocytes of patients with SS asthma and patients with SR asthma is related to differences in GR phosphorylation status after treatment with DEX, we tested GR phosphorylation at S211 site. This site is associated with a transcriptionally active GR.^{24,35} PBMCs from patients with SS asthma and patients with SR asthma were preincubated with 10 nmol/L VitD for 21 hours or vehicle control, followed by DEX treatment at 10 nmol/L for 3 hours. An adherent PBMC fraction was collected. Western blot results demonstrated that DEX induced GR phosphorylation at S211 by 2.6 ± 0.1 -fold ($P < .01$) and 2.3 ± 0.1 -fold ($P < .01$), respectively, compared with media control in patients with SS asthma and patients with SR asthma (Fig 5, B and D). However, the S211 phosphorylation induced by DEX was significantly greater in patients with SS asthma than in patients with SR asthma (Fig 4, D). Pretreatment with $1,25(\text{OH})_2\text{D}_3$ had no effect on GR phosphorylation at S211. VitD pretreatment did not enhance GR phosphorylation at S211 in response to DEX, and GR S211 phosphorylation levels remained significantly greater in the cells of patients with SS asthma compared with those of patients with SR asthma ($P < .05$; Fig 5, D).

DISCUSSION

In the current study we examined the anti-inflammatory effects of VitD, DEX, and the VitD/DEX combination in monocytes from patients with SS asthma and patients with SR asthma. We demonstrated that DEX had a significantly reduced ability to suppress LPS-induced cell activation in the cells of patients with SR asthma compared with those of patients with SS asthma. At the same time, we demonstrated significant suppression of LPS-induced p38 MAPK activation and cytokine production in these cells by VitD, irrespective of whether the cells were from patients with SR asthma or patients with SS asthma. The data demonstrate

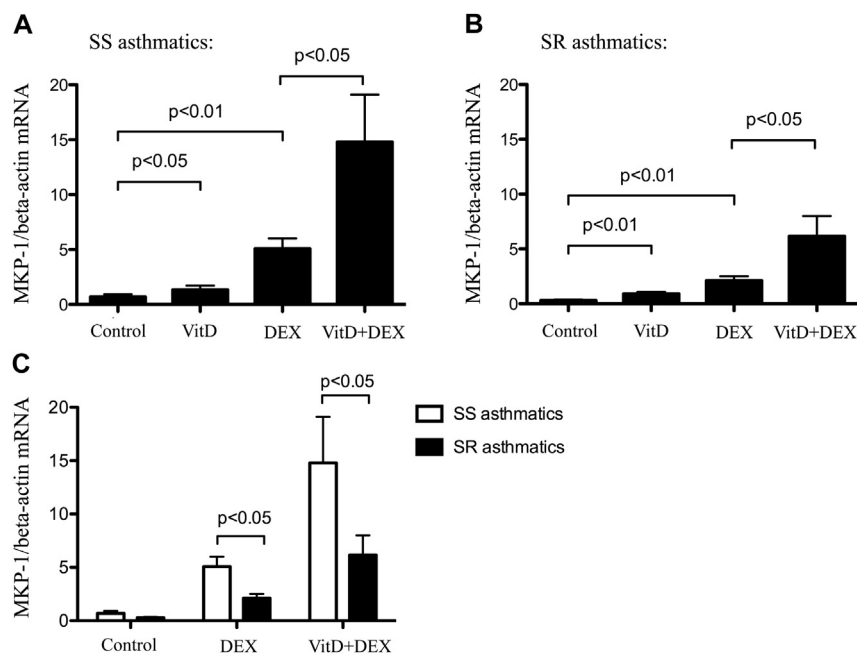


FIG 3. A and B, VitD enhances DEX induction of MKP-1 in monocytes from patients with SS asthma and patients with SR asthma. PBMCs from patients with SS asthma (Fig 3, A) and patients with SR asthma (Fig 3, B) were preincubated with 10 nmol/L VitD for 21 hours, followed by DEX treatment at 10 nmol/L for 3 hours. An adherent PBMC fraction was collected. MKP-1 mRNA levels were detected by using real-time PCR and normalized to β -actin mRNA ($n = 8$ patients with SR asthma and $n = 8$ patients with SS asthma). C, MKP-1 induction by DEX and VitD+DEX was significantly greater in cells from SS asthma patients.

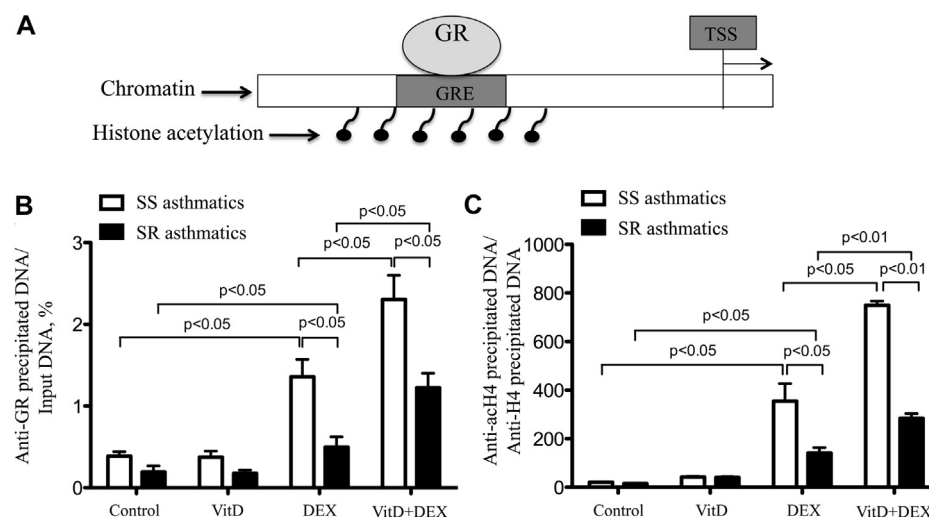


FIG 4. Differential recruitment of GR to the GRE in the MKP-1 gene promoter in monocytes from patients with SS asthma and patients with SR asthma. A, Schematic representation of GR binding to GRE 4.6 and histone acetylation in the human MKP-1 promoter. The number represents kilobase pairs upstream of the transcriptional start site (TSS). B and C, Recruitment of GR (Fig 4, B) and histone H4 acetylation (Fig 4, C) at the GRE 4.6 site ($n = 3$).

that VitD compared with DEX has superior anti-inflammatory function in the cells of patients with SR asthma. In addition, we showed an enhancement of cellular responses to corticosteroids in cells from patients with SR asthma and patients with SS asthma if the cells were pretreated with VitD. However, regardless of enhancement of DEX responses by VitD, the cells of patients with SR asthma remained less responsive to DEX treatment compared with the cells from patients with SS asthma.

Despite the excellent efficacy of corticosteroids in controlling asthma symptoms, some asthmatic patients (patients with SR asthma) do not respond to corticosteroids.^{18,36} Patients with SR asthma have persistent airway inflammation despite treatment with corticosteroids and therefore could be predisposed to increased airway remodeling and irreversible lung disease.³⁰ This has become a challenging health problem that contributes to the high costs of asthma care.^{37,38} Therefore it is desirable to

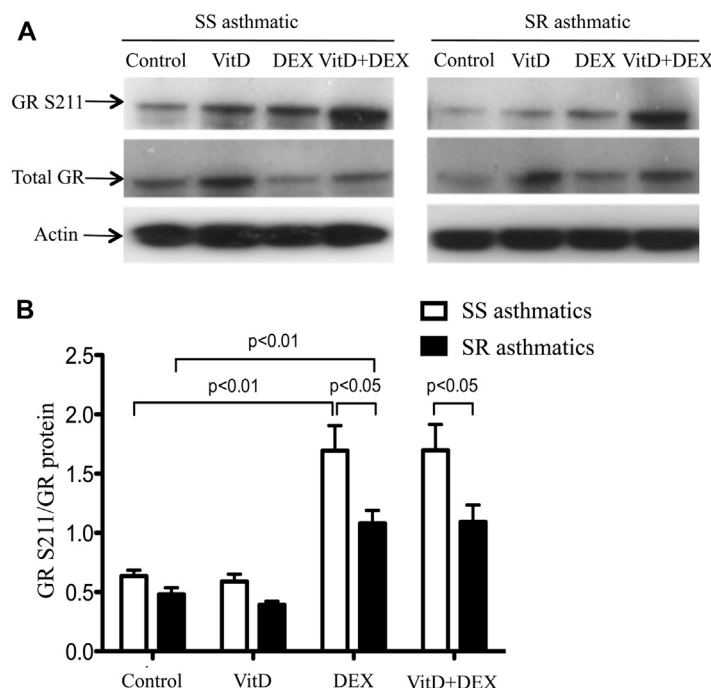


FIG 5. S211 GR phosphorylation in response to DEX with and without VitD pretreatment in cells of patients with SS asthma and patients with SR asthma. **A**, Results from a representative Western blot comparing monocytes from patients with SR asthma and patients with SS asthma are shown out of 5 patients with SR asthma and 5 patients with SS asthma studied. **B**, Blotting densitometric readings summary of GR S211 phosphorylation normalized to total GR levels.

know the mechanisms of differential actions of corticosteroids in patients with SS asthma and patients with SR asthma and also to develop approaches to enhance responses to corticosteroids in patients with SR asthma.

Recent studies demonstrated that administration of vitamin D to healthy subjects and patients with SR asthma enhanced subsequent responsiveness to DEX for induction of IL-10 *in vitro*.³⁹ Also, vitamin D had been shown to inhibit T_H17 cytokine production *in vitro* in patients with severe asthma, irrespective of their clinical responsiveness to corticosteroids.⁴⁰ In our previous study using monocytes from healthy control donors, we found that VitD inhibited LPS-induced cell activation by reducing p38 MAPK phosphorylation caused by increased MKP-1 production.¹⁶ In the current study VitD exerted similar effects in cells from asthmatic patients. However, DEX inhibited LPS-induced p38 phosphorylation only in patients with SS asthma. This observation prompted us to test MKP-1 expression in response to DEX and VitD treatment in monocytes of patients with SR asthma and patients with SS asthma. The results showed a significantly higher induction of MKP-1 by DEX in patients with SS asthma than in patients with SR asthma, which is consistent with our p38 MAPK phosphorylation data. We then tested GR binding to a GRE located 4.6 kbp upstream of the MKP-1 promoter; this GRE is reported to be the predominant GR binding site in the human MKP-1 promoter.²⁶ Increased GR binding to the GRE and a concomitant higher acetylation of histone H4 around the GRE in the MKP-1 promoter were observed in the cells of patients with SS asthma compared with those of patients with SR asthma, indicating a higher GR activity in patients with SS asthma for MKP-1 gene transcriptional activation.

In this study we had a limited number of cells available and were not able to evaluate MKP-1 protein and mRNA expression in response to LPS stimulation after treatment with DEX and VitD both individually and in combination. However, we have previously demonstrated changes in MKP-1 mRNA production after VitD treatment and LPS stimulation in monocytes from healthy control subjects. LPS treatment resulted in upregulation of MKP-1 mRNA expression ($P < .05$). Cellular MKP-1 mRNA levels were further induced (mean $\pm$ SD) 4.5 ± 1.4 -fold in response to LPS/DEX ($P < .01$ compared with LPS-treated cells). MKP-1 mRNA expression in response to LPS was significantly higher in VitD-pretreated cells ($P < .01$ compared with cells cultured in media alone for 24 hours, followed by 6 hours of LPS treatment). MKP-1 mRNA expression was highest in cells pretreated with VitD and stimulated with DEX and LPS compared with those undergoing all other treatment conditions.¹⁷ In addition, we previously reported changes in MKP-1 protein expression in response to VitD treatment in monocytes from healthy control subjects.¹⁶

We noted that in monocytes from patients with SR asthma, the effect of VitD on DEX induction of MKP-1 does not translate into an effect on p-p38 because some degree of MKP-1 mRNA enhancement by the VitD/DEX combination was observed in monocytes of patients with SR asthma, whereas this enhancement did not result in increased suppression of LPS-induced p38 MAPK activation by VitD/DEX combination compared with VitD alone. These differences are likely related to the different readouts used in these assays (ie, p38 MAPK phosphorylation vs MKP-1 mRNA induction, with MKP-1 mRNA changes being more sensitive). In addition, as assessed based on VitD and DEX effects on IL-6 production, some measure of cooperativity between VitD and DEX was observed in patients with SR asthma. However, in the

presence of VitD/DEX, approximately 40% of LPS-induced IL-6 was still in cultures from patients with SR asthma, whereas only 20% of the LPS-induced levels were present in cultures from patients with SS asthma, suggesting that VitD/DEX-enhancing effects were less efficient in the SR asthma cell cultures.

Using cells from healthy control donors, we recently reported that VitD enhancement of DEX-induced MKP-1 transcription occurred because of increased GR binding to the GRE 4.6k bp upstream of the MKP-1 gene, which was mediated by adjacent vitamin D receptor binding to the MKP-1 promoter and shared mediator complex subunit 14 (MED14) coactivator between GR and vitamin D receptor.¹⁷ GR transcriptional activity is closely related to the phosphorylation state at 3 serine residues (S203, S211, and S226) in the N-terminal transcriptional activation region of the GR.⁴¹ It has been demonstrated that S203-phosphorylated GR localizes to the cytoplasm, S226-phosphorylated GR inhibits gene transcription, and S211-phosphorylated GR is induced by ligand binding to the GR and directly correlates with the degree of GR nuclear translocation and transactivation.^{42,43} Therefore we tested DEX-induced GR phosphorylation at S211 in asthmatic monocytes. Significantly higher GR phosphorylation levels at S211 in response to DEX were observed in cells from patients with SS asthma compared with those from patients with SR asthma. VitD treatment did not affect GR phosphorylation at S211 and did not enhance GR phosphorylation at S211 in response to DEX.

It has been reported that S211 GR phosphorylation enhances GR interaction with the MED14 coactivator.^{35,43} Because we previously demonstrated that the enhancement of GR-induced MKP-1 transcription by VitD is mediated by the GR MED14 coactivator, we propose that reduced S211 GR phosphorylation in the cells from patients with SR asthma results in reduced efficacy of VitD-mediated enhancement of MKP-1 mRNA transcription in monocytes from patients with SR asthma.

Vitamin D levels are normally measured based on serum 25(OH)D₃ levels; because this form of vitamin D is more stable, the active form of vitamin D, 1,25(OH)₂D₃, has a short half-life.⁴⁴ Human monocytes have the ability to convert 25(OH)D₃ to an active form.^{45,46} Studies have concluded that serum concentrations of 25(OH)D₃ are important because the available circulating 25(OH)D₃ influences local tissue concentrations of the active vitamin D.^{45,46} Our previous data¹⁶ support the idea that to achieve optimal anti-inflammatory effects by vitamin D, it is important to maintain serum vitamin D levels of greater than 30 ng/mL in the physiologic range.^{1,47} We previously assessed the anti-inflammatory effects of 25(OH)D₃ doses that are related to vitamin D deficiency (15 ng/mL) and vitamin D sufficiency (30 ng/mL = lower normal range; 50–70 ng/mL = upper normal range for the serum vitamin D levels⁴⁸) and compared these effects to several doses of the active form of vitamin D 1,25(OH)₂D₃ by using cells from healthy control subjects.¹⁶ We demonstrated that 15 ng/mL of 25(OH)D₃ (a concentration considered vitamin D deficiency⁴⁸) did not suppress LPS-induced IL-6 and TNF- α production in human monocytes. We found that 25(OH)D₃ at 30 ng/mL and higher (ie, levels considered vitamin D sufficient in human subjects) significantly inhibited the IL-6 and TNF- α production induced by LPS. Furthermore, the degree of suppression of IL-6 and TNF- α production by 30 ng/mL 25(OH)D₃ was comparable with the effects achieved by 0.1 nmol/L of the active form of vitamin D.¹⁶ The effects achieved by 50 to 70 ng/mL 25(OH)D₃ were comparable with the effects of 10 nmol/L 1,25(OH)₂D₃.¹⁶ In this study we used 10 nmol/L 1,25(OH)₂D₃. On the basis of

the comparisons to effects of this dose of active form of vitamin D with the effects of 25(OH)D₃, the concentrations used in these experiments are realistically attainable and safe.

In summary, our study has several novel findings, which might have important clinical implications. First, despite reduced responses to DEX in the cells of patients with SR asthma, VitD alone can inhibit LPS-induced activation in cells from these patients, as shown by inhibition of LPS-induced p38 MAPK activation and IL-6 production. Second, VitD demonstrates corticosteroid-enhancing effects in monocytes from patients with SR asthma and patients with SS asthma, as shown by increased MKP-1 production in response to DEX after VitD pretreatment. Third, despite enhancement by VitD, the responses to corticosteroids in monocytes from patients with SR asthma remained significantly lower compared with those from patients with SS asthma. Lower MKP-1 mRNA induction in response to DEX or VitD/DEX treatment was observed in monocytes of patients with SR asthma because of reduced GR binding and histone H4 acetylation at MKP-1 promoter in these cells. Reduced GR binding to the MKP-1 promoter might be related to the reduced levels of DEX-induced GR phosphorylation at S211 in monocytes of patients with SR asthma. The conclusions of this work are limited by the small sample size studied, and the findings should be replicated. Nevertheless, this work sets the stage to address potential benefits and biomarkers for monitoring clinical VitD supplementation in asthmatic patients treated with corticosteroids.

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Key messages

- Monocytes of patients with SR asthma are less responsive to DEX *in vitro* compared with those of patients with SS asthma, as shown by DEX suppression of both LPS-induced p38 MAPK activation and IL-6 production and DEX induction of MKP-1.
- VitD alone demonstrates anti-inflammatory effects in the cells of patients with SR asthma and patients with SS asthma because it inhibits LPS-induced p38 MAPK activation and IL-6 production and stimulates monocyte MKP-1 expression.
- VitD pretreatment enhances DEX responses *in vitro* in monocytes of patients with SR asthma and patients with SS asthma through increased MKP-1 production as a result of increased GR binding to the MKP-1 promoter mediated by VitD receptor.
- Despite the enhancement of corticosteroid responses by VitD, VitD pretreatment does not overcome reduced responses to corticosteroids in the cells of patients with SR asthma. The levels of DEX-induced GR phosphorylation at S211 are significantly lower in monocytes from patients with SR asthma compared with those from patients with SS asthma. VitD pretreatment does not affect GR phosphorylation at this site.

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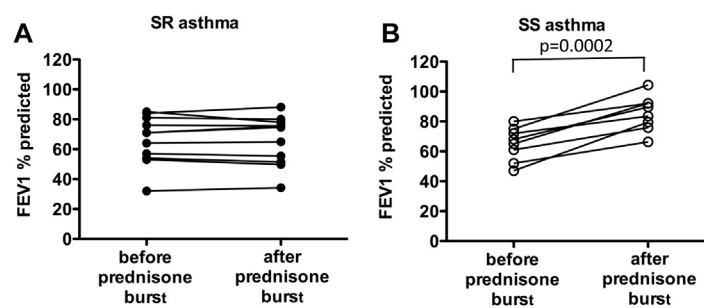


FIG E1. Prebronchodilator FEV₁ percent predicted before and after a 1-week oral prednisone burst in patients with SR asthma (**A**) and patients with SS asthma (**B**).