

Inhibition of angiogenesis by IL-32: Possible role in asthma

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Background: IL-32 is a proinflammatory cytokine involved in various chronic inflammatory diseases. Chronic airway inflammation in asthmatic patients results in structural airway changes, including angiogenesis. Vascular endothelial growth factor (VEGF) is a key inducer of angiogenesis in the airways of asthmatic patients.

Objective: The aim of the study was to investigate the expression and function of IL-32 in patients with angiogenesis and asthma.

Methods: The expression and regulation of IL-32 in normal human bronchial epithelial (NHBE) cells was analyzed by using RT-PCR, ELISA, Western blotting, immunofluorescent staining, and flow cytometry. After knockdown of IL-32 in NHBE cells by small interfering RNA (siRNA) transfections, VEGF secretion was quantified by means of ELISA. New blood vessel formation was determined with human umbilical vein endothelial cells by culturing with supernatants from IL-32 siRNA-transfected NHBE cells. IL-32 was determined in serum and induced sputum samples of asthmatic patients and healthy control subjects by means of ELISA.

Results: IL-32 is expressed in NHBE cells on stimulation with IFN- γ , TNF- α , T_H1 cells, and rhinovirus. Inhibition of IL-32

expression resulted in significantly increased secretion of the proangiogenic factors VEGF and platelet-derived growth factor by NHBE cells. Human umbilical vein endothelial cells cultured in supernatants from IL-32 siRNA-transfected NHBE cells showed enhanced *in vitro* angiogenesis. IL-32 is detectable in induced sputum from asthmatic patients. IL-32 serum levels were significantly higher in asthmatic patients compared with those seen in healthy control subjects and correlated with response to asthma treatment.

Conclusion: IL-32 is induced by IFN- γ , TNF- α , T_H1 cells, and rhinovirus in bronchial epithelial cells. It inhibits angiogenesis, and its serum levels are associated with a good treatment response in asthmatic patients. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Bronchial epithelial cells, asthma, IL-32, angiogenesis, vascular endothelial growth factor, endotype

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The authors' laboratory is supported by the European Allergy and Asthma Center Davos (EACD), Swiss National Science Foundation grant nos. 32-132899 and 32-112306, the Christine Kühne Center for Allergy Research and Education (CK-CARE), and the Predicta FP7 7th frame European Network. H.M. received a European Academy of Allergy and Clinical Immunology fellowship.

Disclosure of potential conflict of interest: C. Rhyner has received research support from and is an employee of the Swiss Institute of Allergy and Asthma Research. M. Soyka has received research support from the Ulrich Miller Gierok Foundation and the University Hospital of Zurich. M. Akdis has received research support from the Swiss National Foundation and the European Union. C. Akdis has received research support from Novartis, Stallergenes, the Swiss National Science Foundation, the Global Allergy and Asthma European Network, and the Christine Kühne Center for Allergy Research; has served as an expert witness on the topics of T_H2-specific receptors (for Accellion) T-cell and B-cell regulation (for Aventis), and allergen-specific immunotherapy (for Allergopharma); is a Fellow and interest group member for the American Academy of Allergy, Asthma & Immunology; is Vice President of the European Academy of Allergy and Clinical Immunology; and is an ex-committee member and WP leader for GA²LEN. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication April 11, 2011; revised December 8, 2011; accepted for publication December 21, 2011.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2011.12.1002

Asthma is a chronic inflammatory disease of the airways characterized by structural airway changes, which are known as airway remodeling, including smooth muscle hypertrophy,¹ goblet cell hyperplasia,² subepithelial fibrosis,³ and angiogenesis.⁴ These structural changes could influence the reversibility of airway obstruction in certain subgroups of asthmatic patients⁵ and correlate with asthma severity.⁶ In this context it was demonstrated that in asthmatic patients with chronic persistent obstruction, FEV₁ values correlate inversely with the thickness of bronchial smooth muscle cell layers in bronchial biopsy specimens.⁷ There is a strong increase in the number of microvessels in lung biopsy specimens from asthmatic patients as a result of increased endothelial proliferation and blood vessel formation mediated by a number of angiogenic growth factors. The major angiogenic growth factors that induce vascular remodeling belong to the vascular endothelial growth factor (VEGF) family,⁸ which includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D, or the platelet-derived growth factor (PDGF) family.⁹ VEGF-A is a key regulator of blood vessel growth in the airways of asthmatic patients through promotion of proliferation and differentiation of endothelial cells and induction of vascular leakage and increased permeability.^{10,11} VEGF production by bronchial epithelial cells is upregulated by T_H17 cells,¹² which is in contrast to T_H1 cells, which suppress VEGF.¹³ Moreover, VEGF is increased during rhinovirus-associated asthma exacerbations, and its secretion is induced by rhinovirus infections of bronchial epithelial cells.¹⁴ In addition to rhinovirus infections, many other trigger factors are important for the pathogenesis of asthma, indicating that asthma is a heterogeneous disorder with various

Abbreviations used

ECP: Eosinophilic cationic protein
 HUVEC: Human umbilical vein endothelial cell
 NHBE: Normal human bronchial epithelial
 PDGF: Platelet-derived growth factor
 poly[I:C]: Polyinosinic:polycytidylic acid
 siRNA: Small interfering RNA
 TLR: Toll-like receptor
 Treg: Regulatory T
 VEGF: Vascular endothelial growth factor

phenotypes.¹⁵ The characterization of inflammatory pathways and new molecular mechanisms in asthmatic patients, which are associated with clinical outcome parameters, is important for the identification of new biomarkers or targets for therapy.¹⁶

IL-32 does not belong to a currently known cytokine family, and a receptor for IL-32 has not yet been described.¹⁷ The *IL32* gene is expressed by many cells, including T cells,¹⁸ dendritic cells,¹⁹ endothelial cells,²⁰ and keratinocytes.²¹ However, not all these cell types secrete IL-32. COS-7 cells secrete IL-32,²² whereas it is not secreted from endothelial cells,²⁰ T cells,¹⁸ or keratinocytes.²¹ Recombinant IL-32 induces the proinflammatory cytokines TNF- α , IL-8, and macrophage inflammatory protein 2 in monocytes, macrophages, and PBMCs.²² In addition, endogenous IL-32 increases the secretion of proinflammatory cytokines from PBMCs²³ and THP-1 cells.²⁴ Endogenous IL-32 also exhibits anti-inflammatory properties by promoting the production of IL-10 and by decreasing the production of IL-12 in dendritic cells.²⁵ Moreover, depletion of IL-32 in keratinocytes through small interfering RNA (siRNA) transfection decreases keratinocyte apoptosis.²¹ IL-32 has been implicated in many inflammatory disorders, and its expression was correlated with disease severity in patients with Crohn disease and rheumatoid arthritis.^{26,27} IL-32 expression was detected in skin biopsy specimens from patients with chronic atopic dermatitis,²¹ in which keratinocyte apoptosis induced by T_H1 plays an important role in eczema formation.²⁸⁻³⁰ IL-32 also appears to play an important role in host defense against *Mycobacterium tuberculosis* or HIV infection.^{31,23}

In the present study we investigated the expression and role of IL-32 in asthmatic patients. Silencing of IL-32 in normal human bronchial epithelial (NHBE) cells increases VEGF secretion. Subsequently, new blood vessel formation by human umbilical vein endothelial cells (HUVECs) cultured with the supernatants of IL-32 siRNA-transfected NHBE cells was upregulated by increased VEGF concentration. Moreover, IL-32 in sera of asthmatic patients was analyzed. The hypothesis of the clinical study was that asthmatic patients with high or low serum IL-32 levels differ in terms of clinical parameters and response to therapy. We demonstrate that asthmatic patients with high serum IL-32 levels responded better to intensive asthma treatment with significant lung function improvement.

METHODS**Cell culture**

NHBE cells (Lonza, Visp, Switzerland) were maintained in BEBM medium (Lonza), which was changed every second day, and the cells between passages 2 and 5 were used in all experiments. Cells were plated in 6-well or 24-well plates to be 90% confluent on the day of the experiment and were incubated

with recombinant cytokines: 10 ng/mL IFN- γ (R&D Systems, Abingdon, United Kingdom), 20 ng/mL TNF- α (R&D Systems), 10 ng/mL IL-1 β (PeproTech EC, London, United Kingdom), 10 ng/mL IL-4 (R&D Systems), 10 ng/mL IL-13 (R&D Systems), 10 ng/mL IL-17A (R&D Systems), 1 μ g/mL Pam3-Cys-Ser-(Lys)4 (Toll-like receptor ligand 2 [TLRL2]; Calbiochem c/o Merck, Darmstadt, Germany), 5 μ g/mL polyinosinic:polycytidylic acid (poly[I:C]; Toll-like receptor ligand 3 [TLRL3]; Amersham, Otelfingen, Switzerland), 100 ng/mL LPS (Toll-like receptor ligand 4 [TLRL4]; Sigma-Aldrich, Buchs, Switzerland), 1 ng/mL flagellin (Toll-like receptor ligand 5 [TLRL5], Calbiochem), 10 μ M/mL ssRNA40 (Toll-like receptor ligand 8 [TLRL8]; InvivoGen, Toulouse, France), and 1 μ M/mL CpG (Toll-like receptor ligand 9 [TLRL9]; Microsynth, Balgach, Switzerland).

Coculture with T cells

T-cell subsets were generated as previously described.^{32,33} For detailed information, see the **Methods** section in this article's Online Repository at www.jacionline.org.

NHBE cells were plated in a 6-well plates to be 90% confluent on the day of the experiment, and T cells were stimulated for 4 hours with anti-CD2, anti-CD3, and anti-CD28 mAbs. Then T cells were washed to remove the cytokines used for differentiation and added to NHBE cells at 5×10^5 cells per well (ratio, 5:1). After 24 hours of coculture, T cells were washed away with PBS, and NHBE cells were harvested for flow cytometric analysis.

HUVEC angiogenesis

Fifteen hours after siRNA transfection of NHBE cells that were cultured in 6-well plates, the NHBE cell medium was replaced by 2 mL of HUVEC medium (Angiokit; TCS CellWorks, Buckingham, United Kingdom), and the NHBE cells were stimulated with IFN- γ (10 ng/mL) and TNF- α (20 ng/mL). After 24 hours, the supernatants were harvested, and 1 μ g/mL anti-IFN- γ (R&D Systems) and 5 μ g/mL anti-TNF- α (R&D Systems) were added. In some conditions 0.4 μ g/mL anti-VEGF (R&D Systems) was added to the supernatants. The supernatants were mixed 1:1 with fresh medium for HUVECs and then added to the HUVECs cultured in fibroblasts (Angiokit, TCS CellWorks). This procedure was repeated on days 4, 7, and 9. After 11 days, the HUVECs cultured in fibroblasts were stained for platelet endothelial cell adhesion molecule 1, according to the manufacturer's protocol. New blood vessel formation was analyzed by measuring tubular length with light microscopy (Axiovert 405M; Carl Zeiss, Feldbach, Switzerland).

Rhinovirus infection

NHBE cells were seeded in 6-well dishes and infected with rhinovirus serotype 1b at a concentration of 1 multiplicity of infection. The proportion of the virus in infection medium was 1:5.7 (75 μ L of virus in 425 μ L of medium). Infection medium consisted of BEBM medium enriched with 4% FCS, 4% tryptase, and 0.01% MgCl₂. The plates were shaken at room temperature for 1 hour and then washed with PBS and replaced with new BEBM medium. T cells were added, and infected NHBE cells cocultured with T cells were incubated at 33°C in a 5% CO₂ atmosphere for 24 hours.

Clinical study report

Serum samples were obtained from 99 patients with asthma diagnosed according to the Global Initiative for Asthma (www.ginasthma.org) guidelines at the Hochgebirgsklinik in Davos, Switzerland, and from 16 healthy subjects. All subjects were at least 18 years old. The medication of asthmatic patients is shown in **Table I**. Power analysis calculated a total sample size of 58 patients (effect size = 1.0, α = .05, and 1- β = 0.95). Seventy-five percent of all asthmatic patients had allergic asthma, and 39.4% were classified as having severe asthma, according to the American Thoracic Society criteria for severe asthma. The study started on April 1, 2010, and the collection of patients was finished on October 30, 2010. The treatment duration was exactly 20 days. In addition, induced sputum from 22 asthmatic patients and 9 healthy subjects was collected. The study was approved by the responsible ethical

TABLE I. Characterization of asthmatic patients with or without serum IL-32

	Sex	Age (y)	Age of onset (y)	Patients with aspirin-induced asthma	Patients with allergic asthma	Patients with exercise-induced asthma	Body mass index (kg/m ²)	Patients fulfilling ATS criteria for severe asthma	IgE level (kU/L)
IL-32–negative patients	47.5% male	51.0 ± 14.3 (18-79)	24.1 ± 16.0 (0-53)	20.3%	74.1%	25.4%	25.1 ± 3.4 (19-33)	37.2%	458.6
IL-32–positive patients	47.5% male	49.5 ± 11.4 (21-72)	22.4 ± 16.2 (0-54)	17.5%	77.5%	35.0%	26.0 ± 4.2 (19-36)	42.5%	355.0
<i>P</i> value	.997	.57	.61	.725	.739	.304	.28	.603	.58
No.	99	99	99	99	99	99	99	99	90

	Patients with systemic steroids (entry)	Systemic steroid doses (entry) if patients take systemic steroids (mg)	Patients with systemic steroids (discharge)	Systemic steroid doses (discharge) if patients take systemic steroids	Patients with inhaled steroids (entry)	Patients with inhaled steroids (discharge)	Patients with long-acting β ₂ -agonists	Patients with theophylline
IL-32–negative patients	35.6%	50.3 ± 52.9 (4-250)	31.0%	42.3 ± 40.1 (4-140)	93.2%	93.2%	89.8%	22.0%
IL-32–positive patients	35.0%	57.0 ± 96.8 (20-400)	32.5%	53.6 ± 75.7 (20-300)	100%	100%	97.7%	35.0%
<i>P</i> value	.952	.72	.834	1.0	.093	.093	.144	.155
No.	99	35	99	31	99	99	99	99

	Exacerbations during last year	Juniper Asthma Symptom Score	Current smoker	Smoke exposure (pack years)	Short β ₂ -agonists (puffs/d) entry	Short β ₂ -agonists (puffs/d) discharge	FEV ₁ /FVC ratio	Family history of asthma	Asthma control
IL-32–negative patients	4.0 ± 3.3 (0-12)	2.3 ± 1.3 (0-8)	6.8%	5.2 ± 9.0 (0-35)	1.8 ± 2.2 (0-10)	0.8 ± 1.2 (0-7)	0.91 ± 0.20 (0.30-1.32)	53.0%	FC: 22.0% PC: 33.9% NC: 44.1%
IL-32–positive patients	5.1 ± 3.2 (0-12)	2.3 ± 1.2 (0-5)	7.5%	2.7 ± 5.6 (0-20)	2.2 ± 3.6 (0-17)	1.1 ± 2.0 (0-10)	0.96 ± 0.17 (0.55-1.47)	73.0%	FC: 22.5% PC: 35.0% NC: 42.5%
<i>P</i> value	.072	.903	.891	.326	.926	.987	.146	.046	
No.	99	99	99	99	96	96	97	99	99

Demographic and clinical characterization of patients with (IL-32-positive) or without (IL-32-negative) detectable serum IL-32 is shown. Percentage indicates the percentage of asthmatic patients fulfilling the indicated criteria. Systemic steroid doses are shown in the equivalent milligram dosage of cortisone (means ± SDs). For calculation of *P* values, Pearson χ^2 tests for binary variables and Mann-Whitney *U* tests for continuous variables were used.

ATS, American Thoracic Society; FC, fully controlled; FVC, forced vital capacity; NC, not controlled; PC, partly controlled.

commissions of cantons of Graubünden and Zürich. For more information on sputum induction, see the [Methods](#) section in this article's Online Repository.

Statistical interpretations

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, Inc, San Diego, Calif). Results are shown as means ± SEMs. Nonparametric statistical comparison between groups was performed by using the Mann-Whitney *U* test. For comparison of binary variables between groups, the Pearson χ^2 test was used. For comparison of pair-linked cell-culture experiments, the paired *t* test was used. For correlation analyses, the linear regression test was used. Differences in *P* values of .05 or less were considered significant.

IL-32 immunofluorescent staining, IL-32 ELISA, IL-32 flow cytometry, IL-32 Western blotting, siRNA transfection, and real-time PCR mRNA expression analysis³⁴

For more information, see the [Methods](#) section in this article's Online Repository. The primers used for real-time PCR analysis are shown in [Table E1](#) (in this article's Online Repository at www.jacionline.org).

RESULTS

Induction and regulation of IL-32 in NHBE cells

NHBE cells were incubated with various cytokines that are suggested to be involved in the pathogenesis of asthma to investigate IL-32 expression. After 24 hours of stimulation with TNF- α or IFN- γ , *IL32* mRNA expression increased 7.8 ± 3.7 -fold or 38.5 ± 3.3 -fold, respectively. The combination of both cytokines induced a 176.2 ± 121.3 -fold increase after 24 hours, demonstrating that IFN- γ and TNF- α synergize for the induction of *IL32*. Increasing the incubation time to 72 hours did not further increase *IL32* mRNA expression in NHBE cells by TNF- α and IFN- γ ([Fig 1, A](#)). Stimulation with IL-1 β , which is known to induce IL-32 in endothelial cells,²⁰ marginally increased, whereas IL-4, IL-13, and IL-17 did not alter *IL32* expression in NHBE cells ([Fig 1, A](#)).

IL-32 protein was detected in NHBE cell lysates but not in supernatants after stimulation with TNF- α and IFN- γ for 24 hours, indicating that IL-32 is not secreted under these culture conditions ([Fig 1, B](#)). IL-1 β did not further induce IL-32 secretion in the presence of TNF- α and IFN- γ ([Fig 1, B](#)). Immunofluorescent staining also demonstrated that IL-32 was located

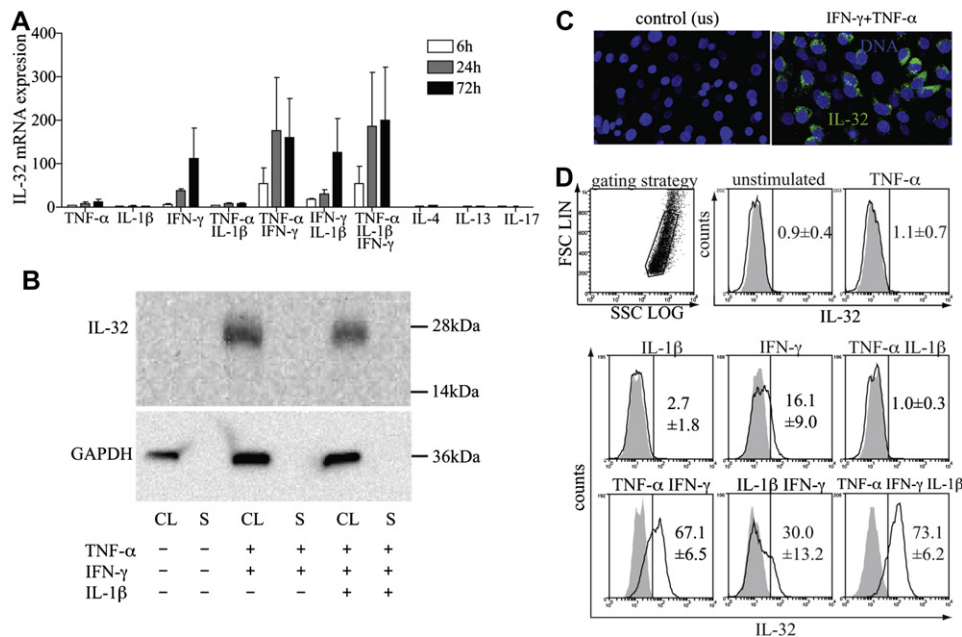


FIG 1. IL-32 is regulated by TNF- α and IFN- γ in NHBE cells. **A**, *IL32* mRNA expression was analyzed in NHBE cells incubated with TNF- α , IFN- γ , IL-1 β , IL-4, IL-13, and IL-17 for 6, 24, or 72 hours. **B**, IL-32 in cell lysates and supernatants was detected by using Western blotting after incubation with TNF- α and IFN- γ or TNF- α , IFN- γ , and IL-1 β for 24 hours. **C**, IL-32 was stained in NHBE cells cultured on air-liquid interfaces after incubation with TNF- α and IFN- γ or medium control for 24 hours. IL-32 was stained with Alexa Fluor 488 (green), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). **D**, IL-32 production in NHBE cells after stimulation with TNF- α , IFN- γ , and IL-1 β or combinations was quantified by means of flow cytometry. Fig 1, A: Means $\pm$ SEMs, $n = 3$. Fig 1, B: $n = 1$. Fig 1, C: One representative staining, $n = 3$. Fig 1, D: Mean percentages $\pm$ SEMs, $n = 5$. CL, Cell lysate; FSC, forward scatter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S, supernatant; SSC, side scatter.

in the cytoplasm of NHBE cells after stimulation with TNF- α and IFN- γ (Fig 1, C; for isotype control see, Fig E1 in this article's Online Repository at www.jacionline.org). Resting NHBE cells did not express IL-32 protein (Fig 1, B-D). In addition, flow cytometric analyses demonstrated that 16.1% $\pm$ 9.0% of the NHBE cells produced IL-32 after stimulation with IFN- γ for 24 hours (Fig 1, D). Although TNF- α or IL-1 β alone did not induce IL-32, both IL-1 β and TNF- α synergize for the induction of IL-32 with IFN- γ and increased the number of IL-32-expressing NHBE cells up to 30.0% $\pm$ 13.2% or 67.1% $\pm$ 6.5%, respectively (Fig 1, D).

IL-32 is induced during rhinovirus infection and Toll-like receptor triggering

Because TNF- α and IFN- γ are the main inducers of IL-32 in NHBE cells and play a role during host defense, the effect of Toll-like receptor (TLR) stimulation on IL-32 expression was investigated. TLR3 activated by poly(I:C) increased *IL32* mRNA expression by 7.6 $\pm$ 3.6-fold and 58.0 $\pm$ 7.9-fold in the presence of IFN- γ , whereas there was no effect on IL-32 mRNA expression after TLR2, TLR4, TLR5, TLR8, or TLR9 stimulation (Fig 2, A). In addition, the percentage of IL-32-expressing NHBE cells increased from 24.0% $\pm$ 11.2% to 62.5% $\pm$ 13.9% in the presence of IFN- γ and poly(I:C), indicating that poly(I:C) and IFN- γ synergize in the promotion of IL-32 induction. However, poly(I:C) alone did not induce IL-32 protein expression in NHBE cells (Fig 2, B).

Similarly, NHBE cells infected with rhinovirus for 24 hours did not express IL-32. However, rhinovirus infection of NHBE cells cocultured with T_H1 cells increased IL-32-expressing cells from 7.1% $\pm$ 5.2% to 23.4% $\pm$ 16.5%. T_H2 or regulatory T (Treg) cells showed no effect on IL-32 expression in NHBE cells (Fig 2, C).

Silencing of endogenous IL-32 in NHBE cells upregulates VEGF and PDGF

IL-32 was depleted by IL-32 siRNA transfection followed by analysis of cytokines and chemokines secreted in the supernatants by NHBE cells to determine the function of intracellular IL-32 in NHBE cells. IL-32-expressing NHBE cells stimulated for 24 hours with IFN- γ and TNF- α were reduced from 87.4% $\pm$ 4.6% to 6.7% $\pm$ 1.8% after transfection with IL-32 siRNA (Fig 3, A). In the supernatants of the NHBE cells transfected with IL-32 siRNA, VEGF levels were 58.1% $\pm$ 20.7% and PDGF levels were 39.0% $\pm$ 12.2% higher compared with those seen in the negative control cells transfected with scrambled siRNA (Fig 3, B and C). The concentrations of IL-6, interferon-inducible protein 10, RANTES, monocyte chemoattractant protein 1, granulocyte colony-stimulating factor, IL-1 β , and IL-1 receptor antagonist in the supernatants of NHBE cells were not affected by silencing IL-32 (Fig 3, C). Because the amount of secreted VEGF in NHBE cell supernatants was relatively high (1.0-5.3 ng/mL) compared with the PDGF concentration (0.07-0.22 ng/mL), we focused on the role played by VEGF in subsequent experiments.

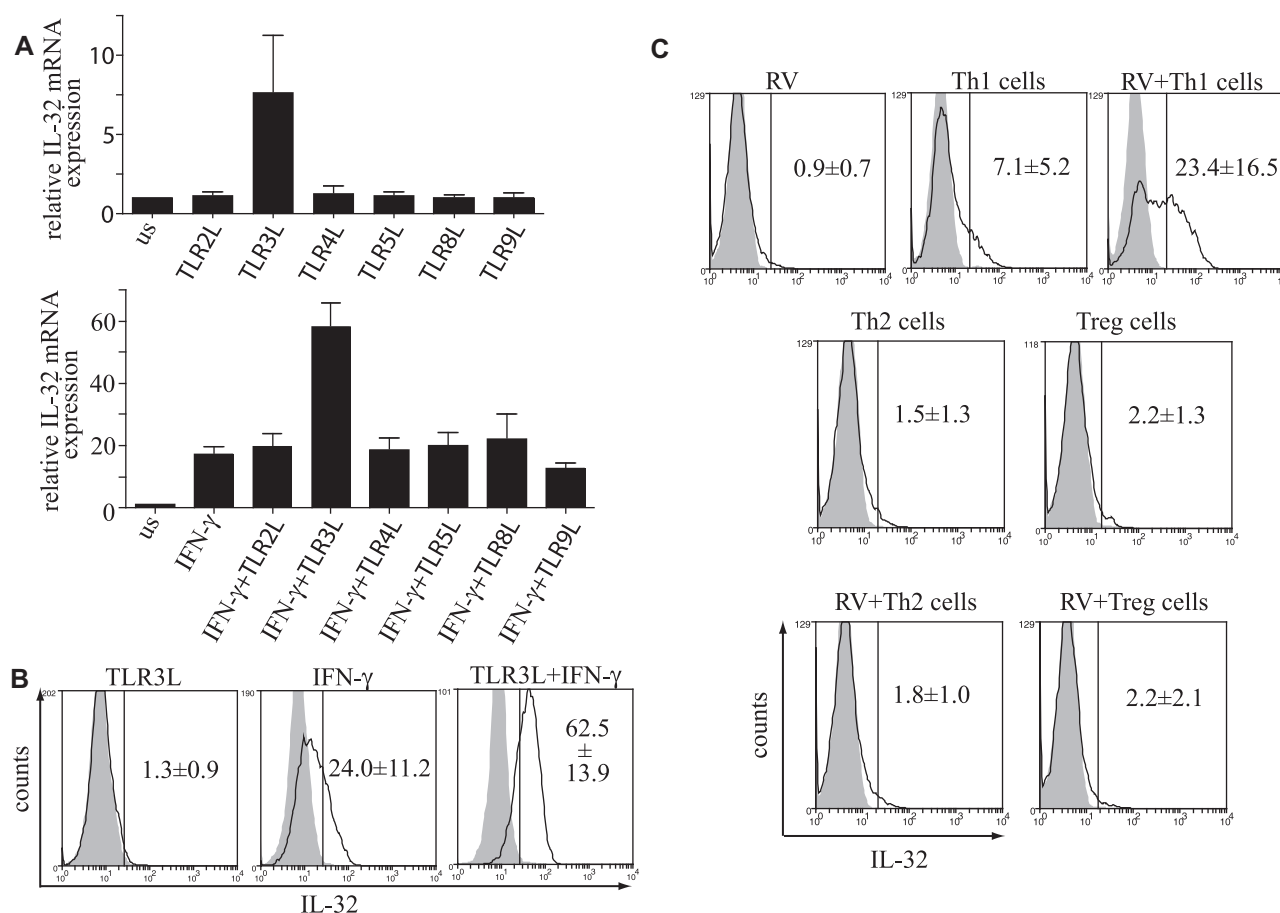


FIG 2. Rhinovirus infection and T_H1 cells synergize for IL-32 induction in NHBE cells. **A**, IL32 mRNA expression in NHBE cells incubated with TLR2, TLR3, TLR4, TLR5, TLR8, or TLR9 agonists and cultured with or without IFN-γ for 24 hours is shown. *us*, Unstimulated. **B**, IL-32 production was analyzed by means of flow cytometry after incubation with TLR3 agonist, IFN-γ, or a combination for 24 hours. **C**, NHBE cells were infected with rhinovirus (RV) and cultured alone or with T_H1, T_H2, or Treg cells for 24 hours, and IL-32 expression was evaluated by means of flow cytometry. Fig 2, A: Means ± SEMs, n = 3. Fig 2, B and C: Mean percentages ± SEMs, n = 3.

IL-32 depletion in NHBE cells increased angiogenesis through VEGF

To investigate the role of IL-32 and VEGF production by NHBE cells on angiogenesis, supernatants from IL-32 siRNA- or scrambled siRNA-transfected NHBE cells were added to HUVECs, and the tubular formation was measured. Again, IL-32 siRNA transfection decreased IL-32 protein expression in NHBE cells stimulated with TNF-α and IFN-γ for 24 hours (see Fig E2 in this article's Online Repository at www.jacionline.org). HUVECs cultured with supernatants from IL-32 siRNA-transfected NHBE cells showed significantly higher tubular formation compared with negative controls (Fig 4, A). After 11 days, the total tubular length of HUVECs cultured with supernatants from IL-32 siRNA-transfected NHBE cells was significantly higher (71.8 ± 21.2 mm) compared with the total tubular length of HUVECs cultured with supernatants from scrambled siRNA-transfected NHBE cells (40.1 ± 12.9 mm; Fig 4, B). After VEGF neutralization, the differences in the tubular formation disappeared, indicating that IL-32 inhibits angiogenesis by suppressing VEGF production (Fig 4).

Serum IL-32 levels in asthmatic patients correlate with serum TNF-α and IFN-γ levels and response to asthma therapy

IL-32 was detected in 40 (40.4%) serum samples from 99 asthmatic patients, whereas only 1 (6.3%) serum sample was positive in 16 healthy control subjects (Fig 5, A). The average IL-32 serum levels were significantly higher in asthmatic patients (24.2 ± 8.4 pg/mL) compared with those seen in healthy control subjects (0.7 ± 0.7 pg/mL; Fig 5, A). Serum cytokines and chemokines were analyzed in asthmatic patients with or without detectable serum IL-32 to investigate the proinflammatory molecules in relationship to IL-32 expression *in vivo*. Serum levels of TNF-α and IFN-γ were significantly higher in asthmatic patients with detectable serum IL-32 levels compared with those seen in asthmatic patients without detectable IL-32 levels in their serum. There was no difference in VEGF serum levels between these 2 groups (Fig 5, B-D). When IL-32 was detectable in serum, it correlated positively with serum TNF-α ($P = .0001$, $r^2 = 0.32$) and IFN-γ ($P = .0049$, $r^2 = 0.19$) levels (see Fig E3, A, in this article's Online Repository at www.jacionline.org). In addition, IL-17 and IL-13 levels were increased in sera from asthmatic patients

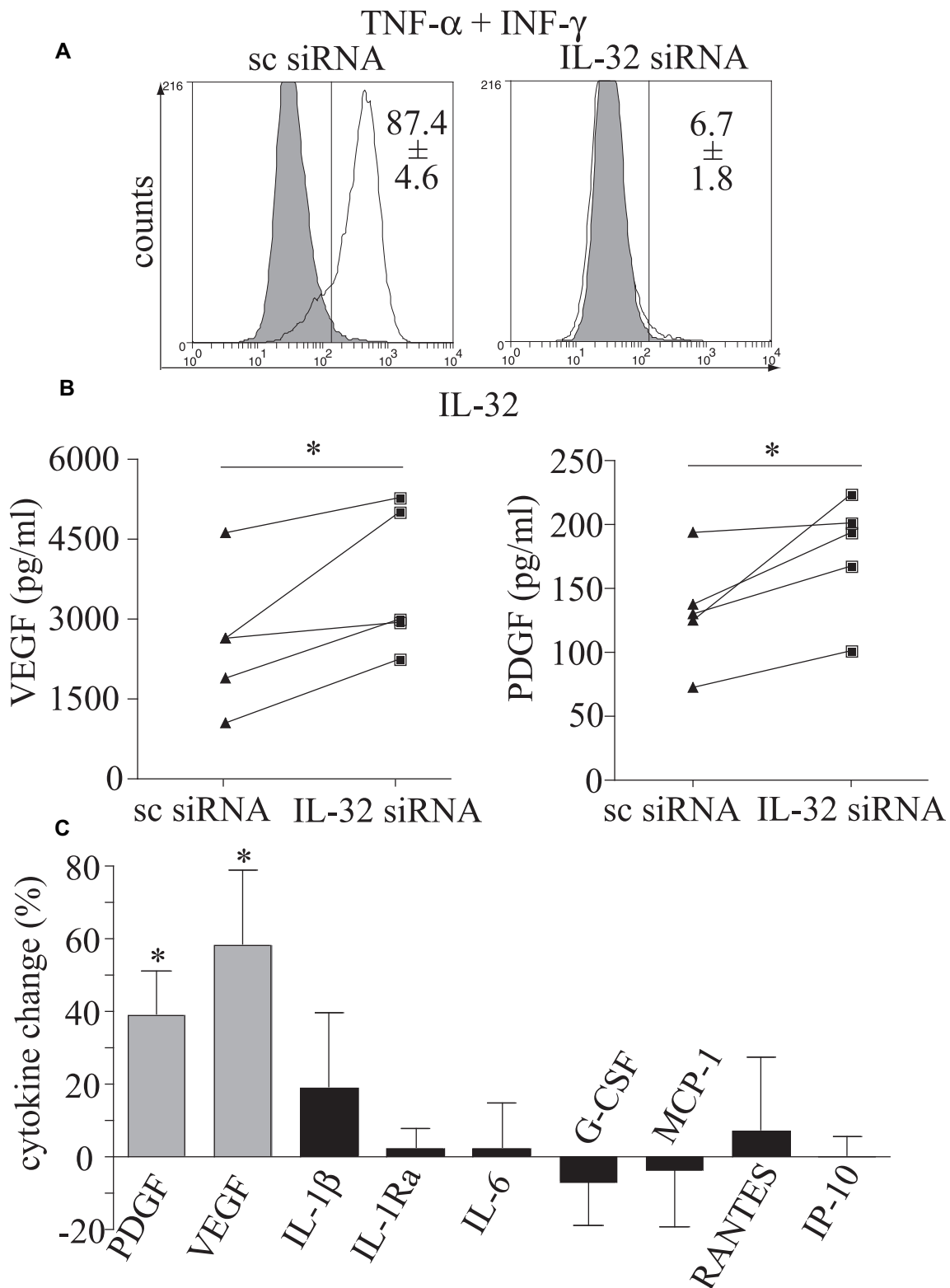


FIG 3. IL-32 depletion in NHBE cells increases secretion of proangiogenic factors. **A**, IL-32 expression after IL-32 siRNA or scrambled (sc) siRNA transfection of NHBE cells stimulated with TNF- α and INF- γ for 24 hours was analyzed by means of flow cytometry. **B** and **C**, Concentrations of VEGF and PDGF in supernatants of scrambled (sc) siRNA- and IL-32 siRNA-transfected NHBE cells were measured (Fig 3, **B**), and mean changes in cytokine and chemokine concentrations in supernatants between scrambled siRNA- and IL-32 siRNA-transfected NHBE cells were calculated (Fig 3, **C**). $n = 5$. * $P < .05$. G-CSF, Granulocyte colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1; IP-10, interferon-inducible protein 10.

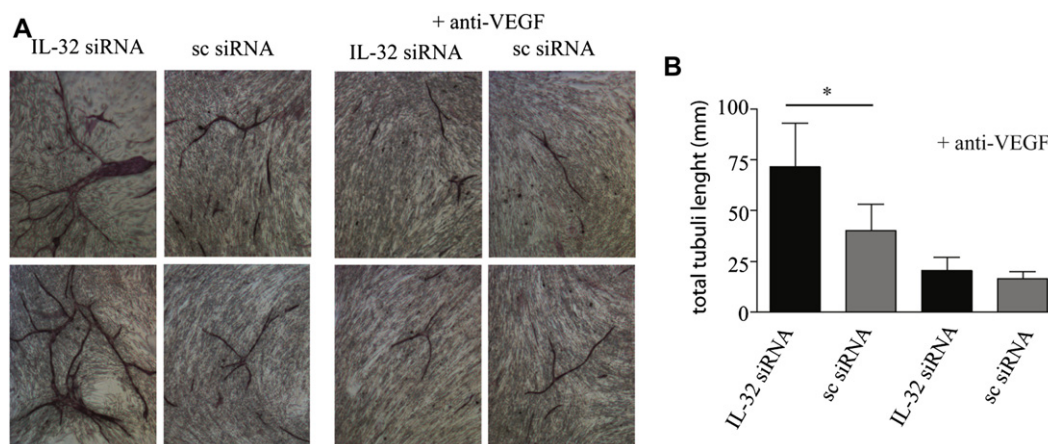


FIG 4. IL-32 knockdown in NHBE cells increases HUVEC tubular formation in a VEGF-dependent manner. HUVECs were cocultured with fibroblasts in the supernatants from IL-32 siRNA or scrambled (sc) siRNA-transfected NHBE cells for 11 days, and subsequently, platelet endothelial cell adhesion molecule 1 was stained. Tubular formation was analyzed by means of photography (**A**), and total tubular lengths were calculated (**B**). Fig 4, **A**: Two representative pictures from 4 independent experiments. Fig 4, **B**: Mean percent-ages $\pm$ SEMs, $n = 4$. * $P < .05$.

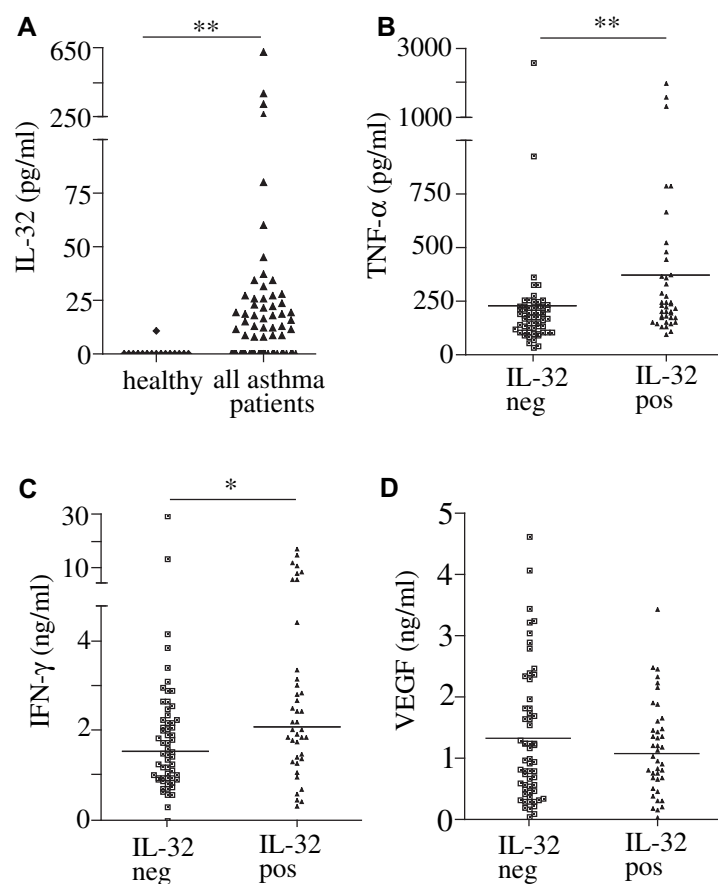


FIG 5. Serum IL-32 levels correlate with serum TNF- α and IFN- γ levels. **A**, IL-32 serum levels from 99 asthmatic patients and 16 healthy control subjects were measured by means of ELISA. **B-D**, Serum levels of TNF- α (Fig 5, **B**), IFN- γ (Fig 5, **C**), and VEGF (Fig 5, **D**) in asthmatic patients with detectable serum IL-32 (IL-32 pos) and without detectable serum IL-32 (IL-32 neg) were determined by means of ELISA. $n = 99$ for asthmatic patients and $n = 16$ for healthy control subjects. * $P < .05$ and ** $P < .01$.

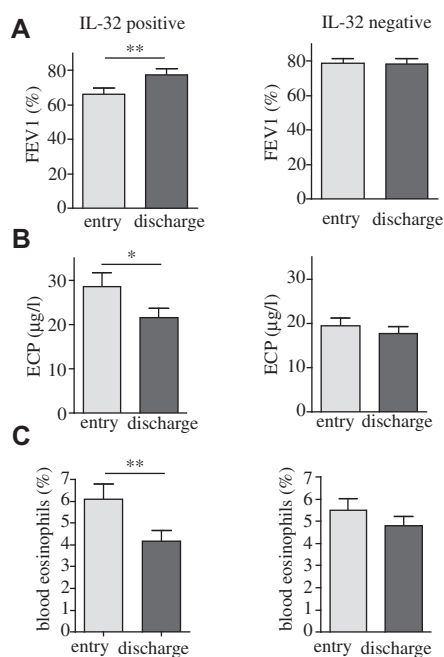


FIG 6. Serum IL-32 levels correlate with therapy response in asthmatic patients. **A-C**, FEV₁ (Fig 6, A), ECP levels (Fig 6, B), and blood eosinophil counts (Fig 6, C) from asthmatic patients with detectable serum IL-32 (IL-32 positive) and without detectable serum IL-32 (IL-32 negative) are shown at the beginning (entry) and after 3 weeks of intensive asthma therapy (discharge). All values are presented as means $\pm$ SEMs. Fig 6, A: n = 99. Fig 6, B: n = 95. Fig 6, C: n = 97. * $P < .05$ and ** $P < .01$.

with detectable serum IL-32 compared with levels in asthmatic patients without detectable serum IL-32, whereas there were no differences in serum concentrations of IL-8, eotaxin, granulocyte colony-stimulating factor, interferon-inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1 β , and IL-4 (see Fig E3, B). Moreover, IL-32 was detected in 8 (36.4%) of 22 sputum samples from asthmatic patients, whereas it was only detected in 1 (11.1%) of 9 induced sputum samples from healthy control subjects (see Fig E4 in this article's Online Repository at www.jacionline.org).

Next, the effect of intensive asthma therapy on lung function measured based on FEV₁ between asthmatic patients with and without detectable serum IL-32 was compared. At the first day of asthma therapy, the FEV₁ and lung function reversibility after inhalation of β_2 -agonists did not differ between asthmatic patients with or without detectable serum IL-32 (see Fig E5 in this article's Online Repository at www.jacionline.org). After 3 weeks of intensive asthma therapy in the high-altitude clinic in Davos, Switzerland, FEV₁ in asthmatic patients with detectable serum IL-32 increased from $77.3\% \pm 3.8\%$ to $84.0\% \pm 3.4\%$ in contrast to the FEV₁ in asthmatic patients without detectable serum IL-32, which did not change (FEV₁ entry, $78.6\% \pm 2.9\%$; FEV₁ discharge, $78.7\% \pm 2.9\%$; Fig 6, A). In addition, serum eosinophilic cationic protein (ECP) levels decreased from 28.5 ± 3.2 to 21.6 ± 2.1 $\mu\text{g/L}$, and blood eosinophil counts decreased from $5.5\% \pm 0.5\%$ to $4.8\% \pm 0.4\%$ in asthmatic patients with detectable serum IL-32 levels. In contrast, there was no significant change in blood eosinophil counts and ECP levels in asthmatic patients without serum IL-32 (Fig 6, B and C). Between asthmatic patients with or without serum IL-32, there was no

significant difference in sex, age, age of onset, smoking, family history of asthma, Juniper Asthma Symptoms Scores, number of exacerbations during the last year, asthma triggers, body mass index, serum IgE levels, and asthma medications, including systemic and topical steroids, inhaled β_2 -agonists, or theophylline (Table I).

DISCUSSION

In the present study we demonstrate that IL-32 is highly expressed in NHBE cells after stimulation with IFN- γ and TNF- α , rhinovirus infection, and T_H1 cells. IL-32 decreases VEGF production and angiogenesis. Detectable serum levels of IL-32 in asthmatic patients were a strong predictor for a positive response to asthma treatment.

IL-32 is expressed in many cells, including natural killer cells,³⁵ endothelial cells,³⁶ and keratinocytes.²¹ In keratinocytes IL-32 is mostly induced by TNF- α and IFN- γ ,²¹ whereas endothelial cells upregulate IL-32 after stimulation with IL-1 β ,²⁰ suggesting that the pathway for IL-32 induction is cell type specific. The expression of IL-32 in NHBE cells after stimulation with TNF- α , IFN- γ , or rhinovirus infection in the present study suggests that IL-32 plays an important role in the host defense against viral or bacterial pathogens. In this context it was shown that IL-32 is induced in PBMCs and monocytes by *M tuberculosis*²⁴ or in A549 cells after influenza A infection.³⁷ The importance of IL-32 for viral defense is also supported by a previous study demonstrating that IL-32 depletion in PBMCs from HIV-infected patients decreased T_H1 cytokine levels and impaired viral clearance, suggesting that IL-32 is a natural inhibitor of HIV infection.²³

Our data demonstrate that IL-32 was not secreted from NHBE cells and also acts as an intracellular protein. It is consistent with data from previous studies showing that IL-32 was detected in the cytoplasm and was not secreted by PBMCs,³¹ endothelial cells,²⁰ or T cells.¹⁸ However, the release of IL-32 by COS-7²² or intestinal epithelial³⁸ cells and detectable levels of IL-32 in the sera of asthmatic patients and patients with atopic dermatitis²¹ suggest that certain cell types secrete this cytokine. It is possible that secreted IL-32 activates different pathways than intracellular IL-32 and therefore exhibits other functions.

Angiogenesis leads to formation of new blood vessels, resulting in an increase of the vascular area of the airways, and is an important event in airway remodeling.⁴ Proinflammatory signals, such as viral and bacterial infections or allergens, in sensitized subjects can stimulate airway structural and inflammatory cells to produce VEGF, thereby promoting angiogenesis.^{14,39,40} Primary bronchial epithelial cells produce VEGF levels capable of inducing proliferation of HUVECs, demonstrating that basal VEGF secretion by bronchial epithelial cells is essential for angiogenesis and tissue remodeling in the lungs.⁴¹ The present study demonstrates that after IL-32 knock-down, VEGF secretion was upregulated, and tubular formation of HUVECs cultured with supernatants from IL-32 siRNA-transfected NHBE cells significantly increased, indicating that IL-32 is an important inhibitor of airway remodeling in asthmatic patients.

Although IL-32 induces proinflammatory cytokines in monocytes,⁴² macrophages,²² or endothelial cells,²⁰ endogenous IL-32 in dendritic cells promotes IL-10 secretion sufficient for suppression of inflammatory cells.²⁵ In addition to immune cell suppression, apoptosis of immune cells after antigen elimination and

apoptosis of tissue cells play indispensable roles in the development and modulation of immune reactivity and are important for maintaining immune system homeostasis.⁴³ Apoptosis induction of T cells,¹⁸ keratinocytes,²¹ and monocytes²⁴ by IL-32 suggests that IL-32 might limit immune and inflammatory reactions in patients with chronic inflammatory diseases. The IL-32-mediated decrease in VEGF secretion by NHBE cells during airway inflammation supports the anti-inflammatory and immune reaction-limiting function of IL-32.

The presence of IL-32 in sera from asthmatic patients was not only accompanied by increased TNF- α and IFN- γ serum levels but also by increased levels of IL-13 and IL-17, which upregulate VEGF production^{13,44} and thus generate an amplification circuit between angiogenesis and inflammation. The upregulation of IL-32 by the proinflammatory cytokines TNF- α and IFN- γ and its ability to suppress VEGF and PDGF suggest that IL-32 can restore the balance between angiogenesis stimulation and inhibition, which could interrupt the amplification loop between inflammation and angiogenesis. The upregulation of IL-32 during rhinovirus infection suggests that IL-32 could be an important factor counterregulating angiogenesis in rhinovirus-triggered asthma.

Although asthma-specific serum cytokine patterns might not exist, because asthma is regarded as a complex heterogeneous disease,^{15,45} serum cytokine levels are associated with different asthma subtypes and might be useful for identification of asthma subtype-specific inflammatory pathways.⁴⁶ Recently, the term “endotype” for asthma classification was introduced, which defines asthma subgroups functionally and pathologically by a molecular mechanism or by treatment response.^{16,45} Accordingly, we compared serum IL-32 levels in asthmatic patients with clinical characteristics, physical examination results, and serum levels of proinflammatory cytokines. IL-32 was present in 40% of the asthmatic patients, suggesting that 1 asthma endotype might be defined by the presence of serum IL-32. When IL-32 was present in serum, the levels of TNF- α , IFN- γ , IL-17A, and IL-13 were increased, indicating that these patients had a different activation of immune cells together with bronchial epithelial cells. However, serum VEGF levels were not increased when IL-32 was present, suggesting that IL-32 suppresses VEGF *in vivo*. Interestingly, asthmatic patients with detectable serum IL-32 levels showed a significant lung function improvement, as well as a significant decrease in ECP levels and blood eosinophil counts compared with those seen in asthmatic patients without detectable serum IL-32 after 3 weeks of intensive asthma treatment. Therefore serum IL-32 levels might distinguish between asthma subtypes with or without fixed airflow limitations. The IL-32-mediated decrease in angiogenesis in our cell-culture experiments suggests that asthmatic patients with detectable serum IL-32 levels might have less structural changes in the airways, which could explain the better therapy response. Importantly, there was no difference in Juniper Asthma Symptom Scores and the frequency of short-acting β_2 -agonist use between IL-32-positive and IL-32-negative patients. The upregulation of TNF- α , IFN- γ , IL-13, and IL-17 in IL-32-positive patients compared with IL-32-negative patients suggests that different and more broad molecular mechanisms have been activated. The involvement of different inflammatory pathways and the correlation between IL-32 levels and response to asthma therapy might be useful for the definition of asthma endotypes characterized by the presence of IL-32 in serum.

In conclusion, this study highlights IL-32 as a cytokine involved in the suppression of angiogenesis in asthmatic patients. The elucidation of IL-32 and its signaling mechanisms might represent interesting targets for the treatment of angiogenesis to prevent irreversible structural changes of the airways. In addition, the serum IL-32 level might be a useful predictor for response to asthma therapy.

Clinical implications: The current study demonstrates a novel role for IL-32, which suppresses proangiogenic signals from bronchial epithelial cells. The presence of serum IL-32 is a predictor for response to asthma therapy.

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METHODS

Real-time PCR mRNA expression analysis

Total RNA from NHBE cells was extracted with the RNeasy Mini Kit (Qiagen, Basel, Switzerland), according to the manufacturer's instructions. Reverse transcription was performed by using reverse transcriptions reagents with random hexamers (Fermentas GmbH, St Leon-Rot, Germany). Relative quantification was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif) and the $2^{-\Delta\Delta C_t}$ formula.^{E1} Glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene. The relative mRNA expression of the unstimulated control was set to 1. Primers were designed to flank or cross an exon-intron border, and primer sequences are listed in Table E1.

IL-32 immunofluorescent staining

NHBE cells grown on air-liquid interfaces (Costar, Cambridge, Mass) were fixed for 10 minutes with 1% formaldehyde (Fluka, Buchs, Switzerland), followed by permeabilization with detergent (0.1% Triton [Fluka] and 0.02% SDS [Roth, Karlsruhe, Germany] in PBS [Invitrogen, Basel, Switzerland]) for 5 minutes. Nonspecific staining was minimized by means of incubation in PBS with 2% BSA (Fluka) and 20% goat serum (Invitrogen) for 30 minutes. Subsequently, cells were incubated for 1 hour with a purified mouse anti-human IL-32 mAb (KU32-52; BioLegend, San Diego, Calif). After being washed in PBS with 2% BSA, cells were incubated with a secondary goat anti-mouse IgG polyclonal antibody linked to Alexa Fluor 488 (Invitrogen). Slides were mounted with VectaShield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, Calif) and analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems AG, Glattbrugg, Switzerland).

Detection of cytokines

A sandwich ELISA for the detection of IL-32 was developed previously.^{E2} IL-32 α and β isoforms were detected, but not γ and δ isoforms, and the detection limit was 7.8 pg/mL. The anti-IL-32 antibodies used (capture antibody, KU32-56; detection antibody, KU32-52; all from BioLegend) had no cross-reaction with other cytokines, such as IL-1 α , IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-18, and TNF- α .^{E3} VEGF levels and levels of other cytokines were measured in culture supernatants by using Bio-Plex (Bio-Rad Laboratories, Hercules, Calif). VEGF ELISA detected VEGF-A (VEGF165 and VEGF121).

Western blot analysis

Protein extraction was performed according to the following protocol. NHBE cells were centrifuged for 5 minutes at 500 *g*, and the pellet was resuspended in 30 μ L of buffer C containing protease inhibitor (420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA, and 1 μ mol/L NP-40) for 20 minutes at 4°C. After spinning at 14,000 *g* for 1 minute, supernatants were collected, and 60 μ L of buffer D (1.5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA, and 1 μ mol/L NP-40) was added.

Samples were loaded next to a prestained protein-mass ladder (Invitrogen) on a NuPAGE 12% bis-Tris gel (Invitrogen). The proteins were electroblotted onto a polyvinylidene difluoride membrane (GE Healthcare AG, Glattbrugg, Switzerland). Unspecific binding was blocked with TBS-Tween 5% milk powder. After blocking, the membrane was incubated with the anti-IL-32 mAb KU32-52 (final concentration of 0.1 μ g/mL, BioLegend) in blocking buffer overnight at 4°C. Next, the membrane was washed and incubated with an anti-mouse horseradish peroxidase-labeled antibody (1:10,000, GE Healthcare AG) at room temperature for 1 hour. The blot was developed with an ECL plus Western Blot Detection System (GE Healthcare AG) and visualized with an LAS 1000 camera (Fuji, Düsseldorf, Germany). Membranes were stripped, reblocked, and reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:4000, Applied Biosystems) to confirm equal sample loading and transfer.

Flow cytometric analysis

The anti-IL-32 mAb (KU32-52) and mouse IgG₁ κ isotype control, both labeled with Alexa Fluor 488, were from BioLegend. For fixation and permeabilization of NHBE cells, the BD Cytofix/Cytoperm kit was used according to the manufacturer's instructions (BD Biosciences, San Diego, Calif), and subsequently, NHBE cells were incubated in blocking solution (PBS, 0.5% BSA, and 10% rat serum) for 15 minutes at room temperature. The anti-IL-32 mAb or IgG₁ κ isotype control was added for 30 minutes at room temperature, and after washing twice with PBS containing 0.5% BSA, cells were analyzed by means of flow cytometry, which was performed on an EPICS XL-MCL with Expo32 software (Beckman Coulter, Fullerton, Calif).

siRNA transfection

Transfection reagents and siRNA were purchased from Applied Biosystems. For transfection, a proprietary blend of polyamines was used (siPORT Amine Transfection Agent, Applied Biosystems). The antisense sequence of IL-32 siRNA was 5'-UCUUUUUCAAGUAGAGGAGtg-3', and scrambled siRNA was used as negative control (Applied Biosystems). NHBE cells were counted, centrifuged at 270 *g*, and resuspended in prewarmed medium to 1×10^5 cells/mL. The siRNA/transfection agent complexes were prepared according to the manufacturer's instructions and subsequently distributed into the culture wells. NHBE cells were transferred to the culture plate, and after a recovery period of 15 hours, medium was changed, and NHBE cells were either stimulated or left untreated for 24 hours.

Differentiation of T_H1, T_H2, and Treg cells

CD45RA⁺ T cells were isolated from PBMCs by using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Treg cells were generated in the presence of anti-IL-4 mAbs (5 μ g/mL, R&D Systems), anti-IL-12 mAbs (5 ng/mL, R&D Systems), anti-IFN- γ mAbs (5 μ g/mL, R&D Systems), and TGF- β (5 ng/mL, R&D Systems). T_H1 cells were differentiated in the presence of IL-12 (25 ng/mL, R&D Systems) and anti-IL-4 mAbs (5 μ g/mL, R&D Systems). T_H2 cells were generated in the presence of IL-4 (25 ng/mL, R&D Systems), anti-IFN- γ mAbs (5 μ g/mL, R&D Systems), and anti-IL-12 mAbs (5 ng/mL, R&D Systems).

Induced sputum samples

Asthmatic patients or healthy control subjects inhaled 3% saline solution for 5 to 7 minutes, followed by a deep expectoration to collect at least 1 g of sputum. If an adequate sample amount could not be collected, the procedure was repeated up to 3 times under lung function control after every saline inhalation. If the FEV₁ decrease was more than 15%, the procedure for induced sputum production was stopped because of increased risk of an asthma attack. After successful sputum production, 0.1% dithiothreitol was added in a volume equal to the sputum volume, and subsequently, content was vortexed at room temperature for 10 minutes. Then PBS was added in a volume equal to the dithiothreitol volume, and content was filtered through a 40- μ m nylon mesh and subsequently centrifuged at 4°C and 270 *g* for 20 minutes. Supernatants were removed, and proteinase inhibitor was added (50 μ L/mL of sputum); supernatants were stored in batches at -80°C until ELISA was done.

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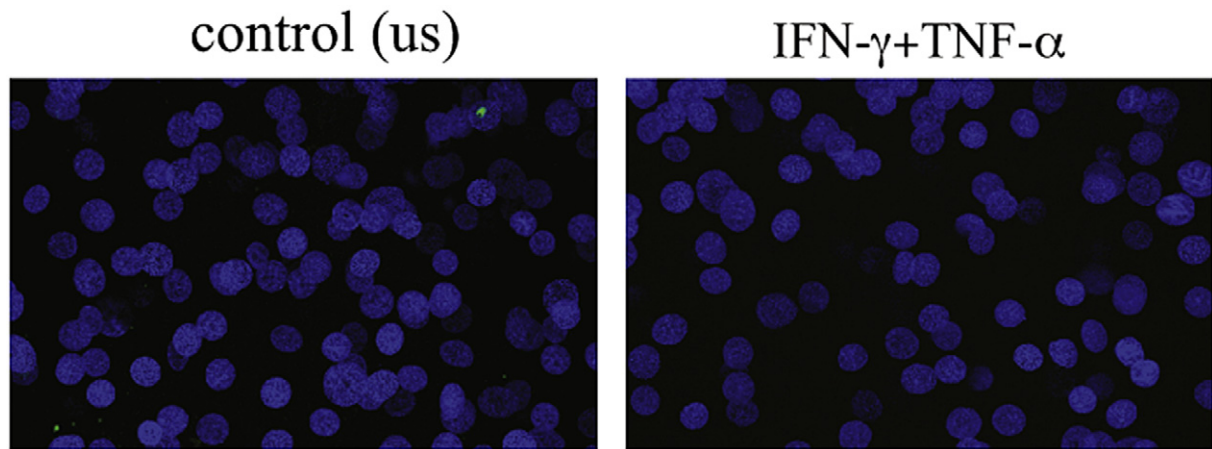


FIG E1. IL-32 isotype control for Fig 1, C. IgG₁ κ isotype control with Alexa Fluor 488 (*green*) isotype control staining for Fig 1, C (NHBE cells cultured on air-liquid interfaces), is shown. Nuclei were stained with 4',6-diamidino-2-phenylindole (*blue*). *us*, Unstimulated.

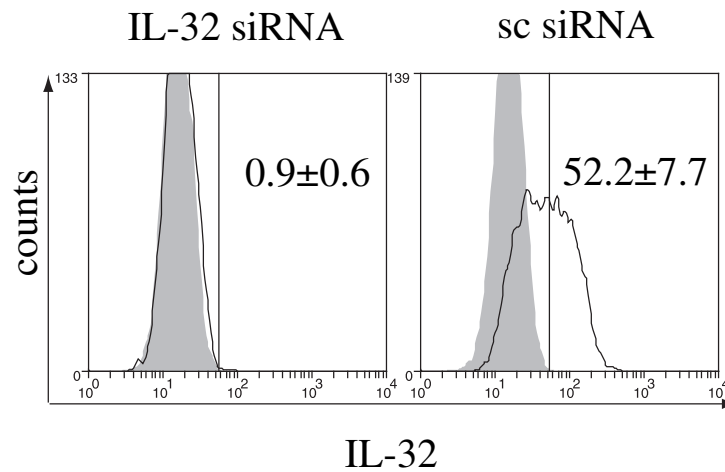


FIG E2. Analysis of IL-32 expression of NHBE cells from the experiment shown in Fig 4. IL-32 expression after IL-32 siRNA or scrambled (*sc*) siRNA transfection of NHBE cells stimulated with TNF- α and IFN- γ for 24 hours was analyzed by means of flow cytometry. All values are presented as mean percentages $\pm$ SEMs ($n = 4$).

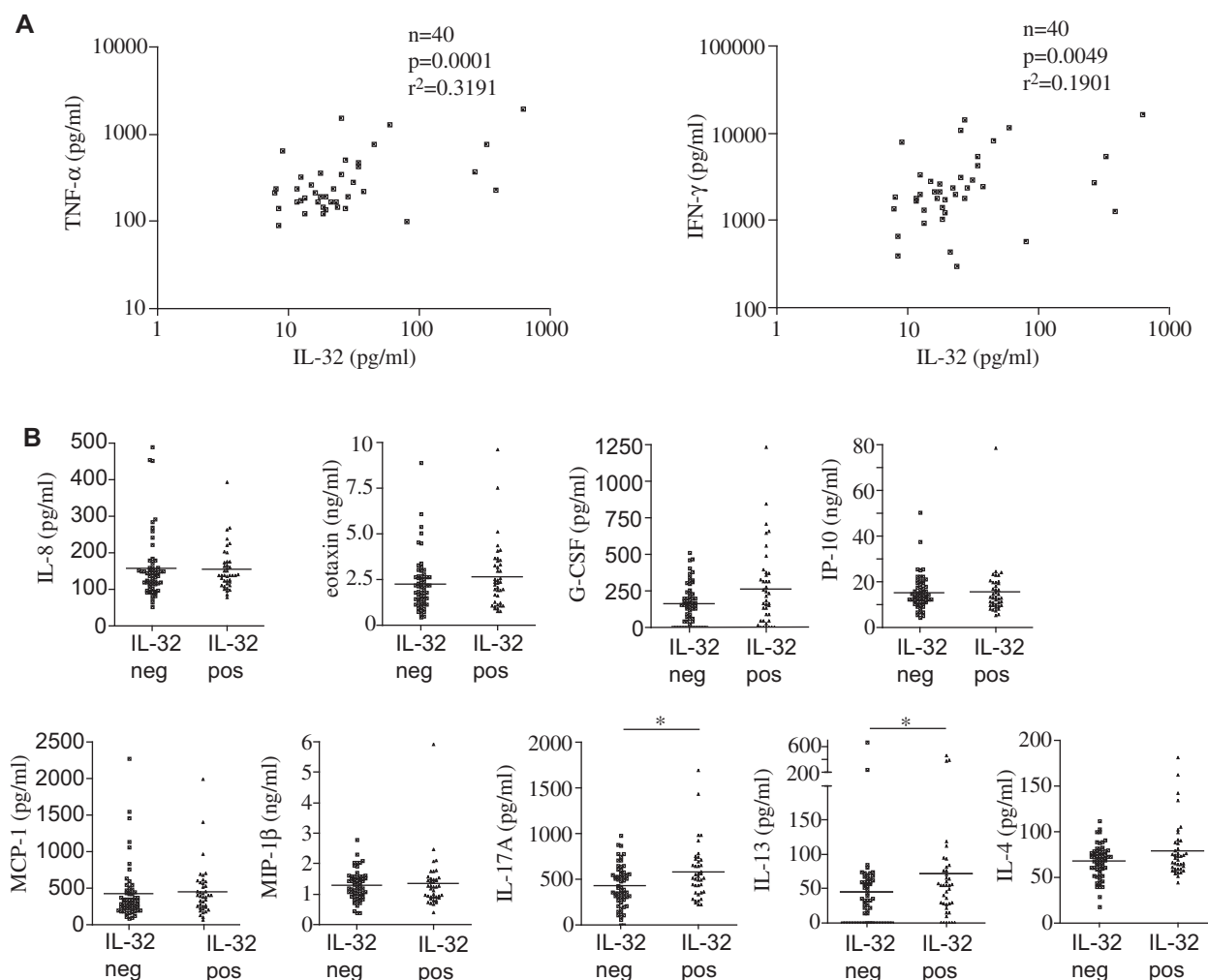


FIG E3. Characterization of serum cytokines in asthmatic patients with or without serum IL-32. **A**, In asthmatic patients with detectable serum IL-32, correlations between serum TNF- α and serum IL-32 levels or serum IFN- γ and serum IL-32 levels was analyzed by using linear regression. **B**, Serum levels of IL-8, eotaxin, granulocyte colony-stimulating factor (G-CSF), interferon-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 β (MIP-1 β), IL-17A, IL-13, and IL-4 in asthmatic patients with detectable serum IL-32 (IL-32 pos) and without detectable serum IL-32 (IL-32 neg) were determined by means of ELISA. Fig E3, A: $n = 40$. Fig E3, B: $n = 99$. * $P < .05$.

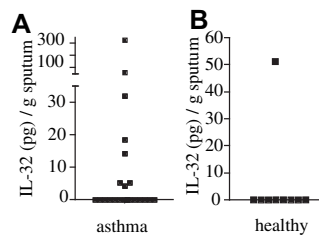


FIG E4. IL-32 is detected in induced sputum from asthmatic patients. IL-32 was detected in 8 of 22 sputum supernatants from induced sputum of asthmatic patients (**A**) by means of ELISA and in 1 of 9 sputum supernatants from induced sputum of healthy control subjects (**B**). n = 22 for asthmatic patients and n = 9 for healthy control subjects.

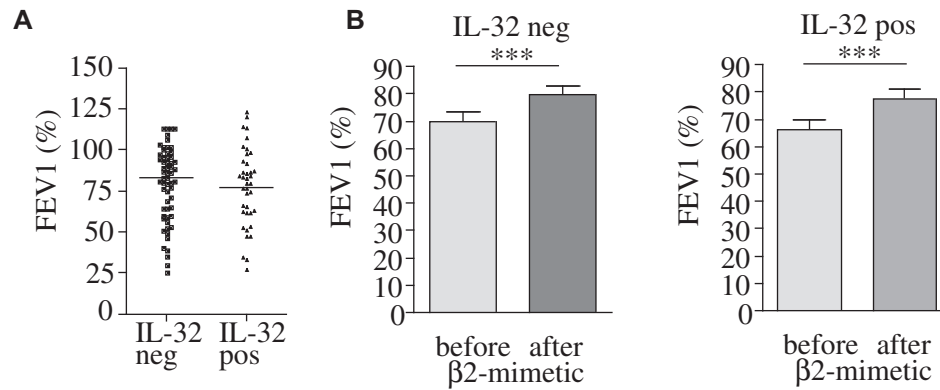


FIG E5. Lung function analysis of asthmatic patients with or without detectable serum IL-32 before therapy. **A**, FEV₁ was measured when patients arrived in the clinic (*entry*) and is shown for asthmatic patients with or without detectable serum IL-32. **B**, FEV₁ reversibility after β₂-agonist inhalation is shown for asthmatic patients with (*IL-32 pos*) or without (*IL-32 neg*) detectable serum IL-32. Fig E6, **A**: n = 99. Fig E6, **B**: n = 55 for IL-32-negative subjects and n = 37 for IL-32-positive subjects.

TABLE E1. Primer sequences used for real-time PCR

IL-32	
Forward	GAC AGT GGC GGC TTA TTA TGA G
Reverse	CCT CGG CAC CGT AAT CCA T
GAPDH	
Forward	TGC ACC ACC AAC TGC TTA
Reverse	GGC ATG GAC TGT GGT CAT

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.