

Staphylococcal exotoxins are strong inducers of IL-22: A potential role in atopic dermatitis

Margarete Niebuhr, MD, Helena Scharonow, MS, Merle Gathmann, MS, Diana Mamerow, MS, and Thomas Werfel, MD *Hannover, Germany*

Background: Patients with atopic dermatitis (AD) and psoriasis are frequently colonized with *Staphylococcus aureus* that produces staphylococcal enterotoxin B (SEB) and α -toxin. In patients with AD, *S aureus* colonization is positively correlated with the severity of their eczema. Moreover, IL-22-producing cells have been shown to accumulate in AD skin and to correlate with disease severity.

Objective: To assess IL-22 production in response to SEB and sublytic α -toxin stimulation in patients with AD and psoriasis compared with healthy controls.

Methods: IL-22 induction was investigated in PBMCs, T cells, and autologous cocultures of keratinocytes and T cells on SEB and α -toxin stimulation in a time-dependent and dose-dependent manner at the mRNA and protein (ELISA and flow cytometry) level. Anti-IL-1 receptor or anti-IL-6 antibodies were used in blocking experiments.

Results: Staphylococcal enterotoxin B and sublytic α -toxin concentrations induced IL-22 production in PBMCs and isolated CD4⁺ T cells. IL-22 secretion was enhanced by α -toxin stimulation in autologous cocultures of keratinocytes and T cells. In T cells and PBMCs from patients with AD, IL-22 secretion was significantly enhanced on α -toxin stimulation compared with patients with psoriasis and healthy controls.

Conclusion: Increased IL-22 secretion induced by staphylococcal exotoxins in the skin partially explains how skin colonization and infection with *S aureus* can contribute to chronic skin inflammation in AD. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: SEB, α -toxin, IL-22, T_H22, human, atopic dermatitis, psoriasis

IL-22 is a member of the IL-10 family that was recently discovered to be preferentially produced by T_H17 cells and therefore has been considered an effector cytokine of T_H17 cells.^{1,2} More recently, 2 independent groups have identified a distinct subset of

Abbreviations used

AD: Atopic dermatitis
IL-1RA: IL-1 receptor antagonist
SEB: Staphylococcal enterotoxin B

IL-22-producing CD4⁺ T cells, namely T_H22 cells that produce negligible or only low amounts of IL-17. These cells concomitantly express the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10 and produce high levels of IL-22, but do not express IL-17 or IFN- γ . In addition, T_H22 cells were found to produce high levels of IL-13 and TNF- α .^{3,4}

IL-22 is thought to play a central role in inflammatory diseases with marked epidermal acanthosis, such as psoriasis.⁵⁻⁷ In patients with plaque psoriasis, Wolk et al⁸ showed an increased IL-22 expression in skin biopsies and greatly elevated IL-22 levels in the blood compared with healthy controls.⁸ In keratinocytes, IL-22 increases the expression of the antimicrobial peptides human β -defensin 2 and 3, indicating an involvement in early host defense against microbial pathogens.⁹ It was demonstrated recently that unique IL-22-producing CD4⁺ and CD8⁺ T cells accumulate in lesional skin of patients with atopic dermatitis (AD). Moreover, the frequency of IL-22-producing CD8⁺ T cells in AD skin positively correlates with disease severity.¹⁰ Eyerich et al¹¹ showed that the frequency of IL-22⁺ T cells was increased in inflamed skin from patients with psoriasis, AD, and allergic contact dermatitis.

A hallmark of AD is the striking susceptibility to colonization by *Staphylococcus aureus*. Between 80% and 100% of patients with AD are colonized with *S aureus*.^{12,13} In contrast, *S aureus* can be isolated from the skin of only 5% to 30% of healthy individuals, mainly from intertriginous areas.¹³ There is a positive correlation between disease severity and *S aureus* colonization of lesional and nonlesional skin.¹² This could be a result of IgE-mediated hypersensitivity^{14,15} or production of exotoxins with superantigenic properties such as staphylococcal enterotoxin B (SEB).^{16,17} A study detected *S aureus* in nonlesional skin of 27% of patients with psoriasis and in 46% of skin lesions.¹⁸ Tomi et al¹⁹ investigated 25 patients with psoriasis: the skin of 60% of patients was positive for *S aureus*, and isolated *S aureus* strains were toxigenic for staphylococcal enterotoxin A-D in 36%. Furthermore, the Psoriasis Area and Severity Index score significantly correlated with enterotoxin-producing *S aureus* strains.

On the other hand, the severity of AD decreased in patients colonized with nontoxigenic *S aureus* strains on antimicrobial treatment, which suggests the involvement of pathogenic factors other than superantigens derived from *S aureus*.²⁰ A subset of *S aureus* strains can produce α -toxin, a potent 33-kD cytolyisin that is not an enterotoxin (superantigen).²¹ In 2 previous studies,

From the Department of Dermatology and Allergy, Division of Immunodermatology and Allergy Research, Hannover Medical School.

Supported by SFB 566 and GRK 1441.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication March 16, 2010; revised June 24, 2010; accepted for publication July 23, 2010.

Reprint requests: Margarete Niebuhr, MD, Department of Dermatology and Allergy, Division of Immunodermatology and Allergy Research, Hannover Medical School, Ricklinger Str 5, D-30449 Hannover, Germany. E-mail: niebuhr.margarete@mh-hannover.de.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2010.07.041

we found that in patients with AD, (1) 30% of *S aureus* strains isolated from the skin of untreated patients²² produced α -toxin whereas (2) 63% of *S aureus* strains produced α -toxin in patients on standard anti-inflammatory and antiseptic treatment.²³

At higher concentrations, α -toxin may lead to necrosis in a wide range of human cells by forming small pores (1–2 nm diameter) in cell membranes.²¹ We previously showed that sublytic concentrations of α -toxin were able to activate T lymphocytes effectively, which led to cell proliferation and induction of IFN- γ at both the mRNA and protein levels.²² This indicates that besides pore-forming activity, sublytic concentrations of α -toxin have immunologic properties and therefore may act as a pathogenetic factor in chronic inflammatory skin diseases.

It remains unclear whether staphylococcal exotoxins effect IL-22 production of T cells from patients with chronic inflammatory skin diseases. Our data indicate that α -toxin induces IL-22 in patients with AD compared with healthy controls, whereas it failed to upregulate IL-22 secretion in patients with psoriasis. This could partially explain why skin colonization and infection with *S aureus* can contribute to chronic skin inflammation in AD.

METHODS

Patients

Peripheral blood samples were taken from informed consented healthy donors and patients with exacerbated psoriasis (n = 8) and AD (n = 10) who presented to our outpatient and inpatient departments as previously described.²⁴ AD was determined by the diagnostic criteria of Hanifin and Rajka.²⁵ Further information can be found in this article's Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org).

Hair keratinocyte isolation and culture

Isolation of outer root sheath cells from plucked human hair follicles was performed as described previously.²⁶

Isolation of monocytes and T cells

Monocyte²⁴ and T-cell isolation from buffy coats as well as T-cell polarization and expansion were performed as previously described.²⁷ Further information can be found in this article's Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org).

Stimulation and blocking of monocytes, T cells, and keratinocytes

Monocytes or T cells were stimulated in a time-dependent and dose-dependent manner with α -toxin (Sigma Aldrich, Deisenhofen, Germany) at the sublytic concentrations indicated. LPS was not detected in any reagent, as determined by the limulus amebocyte assay (Haemochrom Diagnostika, Essen, Germany). For blocking experiments, anti-IL-1-receptor antagonist (Kineret; Biovitrum, Stockholm, Sweden) and/or neutralizing anti-hIL-6 (R&D Systems) were added.

Flow-cytometric analysis

Flow-cytometric analysis was performed as described previously.^{24,27} Further information can be found in this article's Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org).

Cytokine assessment by ELISA

Cell free culture supernatants were harvested and analyzed for IL-4, IL-17, IFN- γ , IL-22, and TNF- α (Duo Set; R&D Systems, Minneapolis, Minn) by using a commercially available ELISA system, following the manufacturer's

instructions. The α -toxin ELISA was performed by using a polyclonal *S aureus* α -hemolysin catching antibody and a polyclonal biotinylated detection antibody (both Abcam, Cambridge, United Kingdom).

RNA isolation, reverse transcription, and real-time PCR

RNA isolation, reverse transcription, and quantitative real-time PCR were performed as previously described.^{24,27} Further information can be found in this article's Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org).

Statistical analysis

For statistical analyses, the software Sigma stat for Windows (Systat Software, San Jose, Calif) was used. Statistical analyses were performed by using the Student *t* test.

RESULTS

Induction of IL-22 by SEB and α -toxin in human PBMCs and T cells

IL-22 expression and secretion on SEB and α -toxin stimulation were investigated at the mRNA level by quantitative RT-PCR and at the protein level by ELISA. The relative intracellular concentration of IL-22 in single cells was assayed by flow cytometry.

PBMCs and CD4⁺ T cells were left unstimulated or were stimulated with SEB or α -toxin for 4, 8, and 24 hours. Stimulation with α -toxin (n = 6) and SEB (n = 4), respectively, yielded a significant upregulation of IL-22 mRNA in PBMCs (Fig 1, A) as well as in CD4⁺ T cells (Fig 1, B). Because SEB belongs to the group of superantigens, binding to the T-cell receptor on T cells as well as to MHC II on antigen-presenting cells leads to full T-cell activation. Alternatively, MHC II can be expressed in purified CD4⁺ T cells after preactivation. Therefore, purified CD4⁺ T cells were preactivated with anti-CD3 (450 ng/mL) and anti-CD28 (200 ng/mL) for 72 hours before SEB stimulation, but not before α -toxin stimulation (Fig 1, B). SEB without preactivation induced only low levels of IL-22 in CD4⁺ T cells (see this article's Fig E1, A, in the [Online Repository at www.jacionline.org](http://www.jacionline.org)). Moreover, CD4⁺ T cells secreted IL-17, IFN- γ , and IL-4 on SEB stimulation (Fig E1, B–D).

α -Toxin induced an approximately 2.5-fold increase in IL-22 protein secretion of CD4⁺ T cells compared with PBMCs as assessed by ELISA in a time-dependent manner (24 hours, 48 hours, 72 hours, and 120 hours; Fig 1, C). PBMCs and CD4⁺ T cells secreted significantly more IL-22 on stimulation with α -toxin than the appropriate medium control (Fig 1, C). In contrast, no IL-22 secretion was detectable on stimulation with lytic concentrations of α -toxin (≥ 1 μ g/mL; see this article's Fig E2, C, in the [Online Repository at www.jacionline.org](http://www.jacionline.org)).

CD4⁺ T cells were preactivated with anti-CD3 and anti-CD28 for 72 hours before SEB stimulation. CD4⁺ T cells secreted significantly more IL-22 on 72-hour and 120-hour SEB stimulation than the appropriate medium control (Fig 1, D). To determine the optimal SEB and α -toxin concentrations for further experiments, we stimulated PBMCs with a range of SEB and α -toxin concentrations (0.1 ng/mL to 1 μ g/mL) for 24 hours. Stimulation with SEB yielded in an enhanced IL-22 secretion for all SEB concentrations tested with an optimum of 100 ng/mL (Fig E2, A). α -Toxin stimulation induced IL-22 continuously with a steep increase at 50 ng/mL; concentrations ≥ 1 μ g/mL led to cell death (Fig E2, C). To explore the best period for SEB and α -toxin stimulation, we performed a time kinetic study (24–120 hours) with

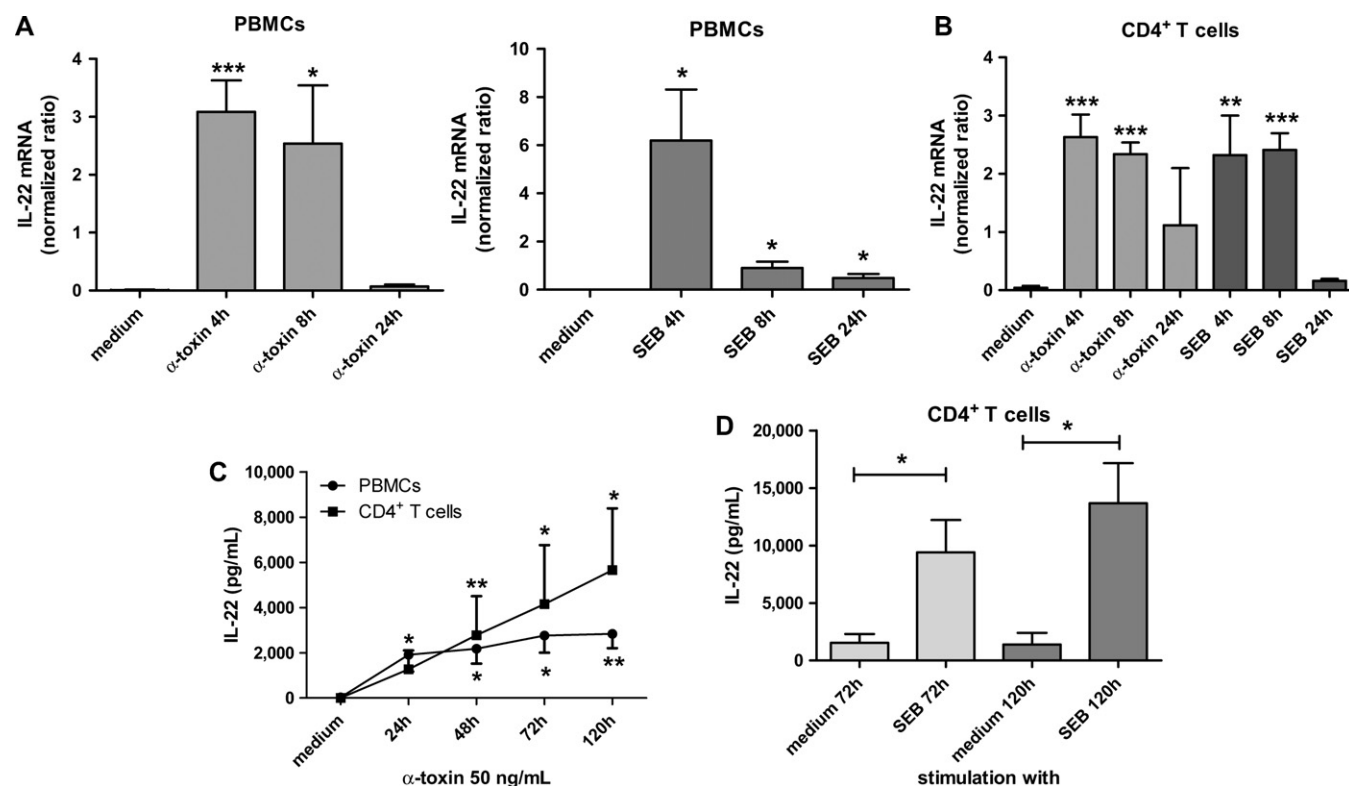


FIG 1. IL-22 is induced on SEB (100 ng/mL) and α -toxin (50 ng/mL) stimulation in PBMCs and CD4⁺ T cells at the mRNA (A and B) and protein (C and D) levels. Data are shown as mean IL-22/glyceraldehyde-3-phosphat-dehydrogenase ratio + SEM (A and B) or as the mean IL-22 value (pg/mL) $\pm$ SEM (C and D). * $P < .05$; ** $P < .01$; *** $P < .001$.

100 ng/mL SEB and 50 ng/mL α -toxin in PBMCs. SEB stimulation induced a continuous increase of IL-22 secretion within 72 hours, with a steep increase after 120 hours (Fig E2, B). α -Toxin led to a continuous increase of IL-22 (Fig E2, D). In a previous study, we described an induction of T-cell proliferation on α -toxin stimulation.²² To discount the possibility that the higher IL-22 concentrations in the cell culture supernatants were a result of T-cell proliferation in α -toxin-stimulated cultures compared with the medium control, we performed an intracellular IL-22 staining in single cells assayed by flow cytometry. There was a significant induction of IL-22 secretion on stimulation with α -toxin (50 ng/mL) and SEB (100 ng/mL) in single CD4⁺ T cells (Fig 2, A). Fig 2, B, shows a representative experiment.

IL-22 secretion is upregulated in purified CD4⁺ T cells after stimulation with supernatants from α -toxin-stimulated purified monocytes

We next investigated whether secreted mediators from antigen-presenting cells are involved in IL-22 secretion of T cells. Purified CD14⁺ monocytes were stimulated for 24 hours with 50 ng/mL α -toxin. Afterward, α -toxin was washed out very carefully, and cells were incubated in fresh culture medium for 72 hours to produce cytokines. Supernatants were harvested and used for stimulation of allogenic CD4⁺ T cells. No carried over α -toxin was detected in the supernatants that were used for further T-cell stimulation as determined by ELISA (data not shown). Stimulation of CD4⁺ T cells with supernatants from α -toxin-stimulated but not from unstimulated monocytes yielded a significant induction of

IL-22 mRNA after 4, 8, and 24 hours of stimulation (Fig 3, A) as well as protein (Fig 3, B) production after 72 hours of stimulation in CD4⁺ T cells as determined by quantitative reverse transcription polymerase chain reaction and ELISA, respectively. However, the effect was lower when compared with direct stimulation of CD4⁺ T cells with α -toxin (Fig 1, C).

α -Toxin-induced IL-22 secretion in CD4⁺ T cells can be partially blocked with an IL-1 receptor antagonist but not with anti-IL-6

To explore further the influence of α -toxin on cytokine induction in monocytes, which in turn can influence IL-22 secretion in T cells, we investigated proinflammatory cytokines involved in T_H22 polarization and IL-22 induction (IL-1, IL-6, and TNF- α).

We stimulated highly purified CD14⁺ monocytes for 4 hours and 24 hours with α -toxin. Afterward, α -toxin was washed out very carefully, and cells were incubated in fresh culture medium for 72 hours to produce cytokines. Supernatants from these monocytes were harvested and used for stimulation of allogenic CD4⁺ T cells for 72 hours.

One hour before stimulation with these supernatants, T cells were incubated with IL-1 receptor antagonist (IL-1RA) to block IL-1 receptors and with neutralizing anti-IL-6 antibodies. α -Toxin-induced IL-22 secretion by purified CD4⁺ T cells could be partially blocked with IL-1-RA (Fig 4) but not anti-IL-6 antibodies (50-200 ng/mL; data not shown). α -Toxin stimulation of monocytes had no effect on TNF- α secretion (data not shown).

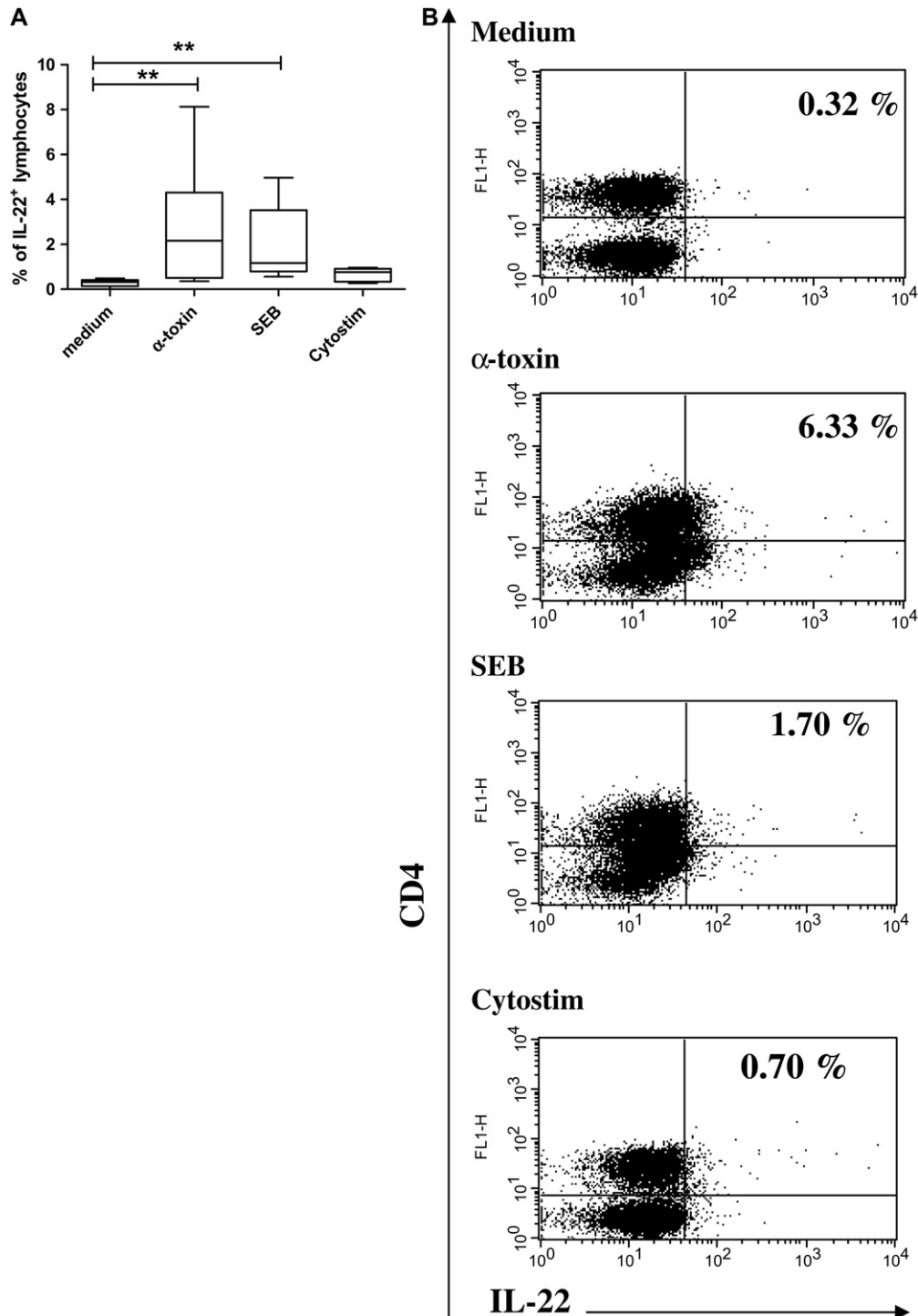


FIG 2. Increased percentage of IL-22⁺ CD4⁺ T cells in PBMCs induced by staphylococcal exotoxins (SEB and α-toxin). PBMCs were either left unstimulated (medium control) or were stimulated for 24 hours with α-toxin or SEB. Cytostim was used as a positive control. IL-22 was assessed by intracellular fluorescent activated cell sorter staining. Data are shown as a summary of *n* = 5 to 7 experiments (**A**) with percentages of IL-22⁺ CD4⁺ T cells or as 1 representative experiment (**B**). ***P* < .01.

Enhanced IL-22 secretion in autologous cocultures of T cells and keratinocytes on α-toxin stimulation

Autologous keratinocytes, CD4⁺ T cells, and PBMCs from healthy donors were cultured either separately or in combinations as indicated and left unstimulated or stimulated with α-toxin or

SEB. CD4⁺ T cells plus keratinocytes secreted significantly more IL-22 on 48-hour α-toxin stimulation compared with CD4⁺ T cells alone. A similar trend (*P* = .3) was observed on SEB stimulation. No IL-22 secretion from CD4⁺ T cells, PBMCs, or keratinocytes and CD4⁺ T-cell cocultures was observed

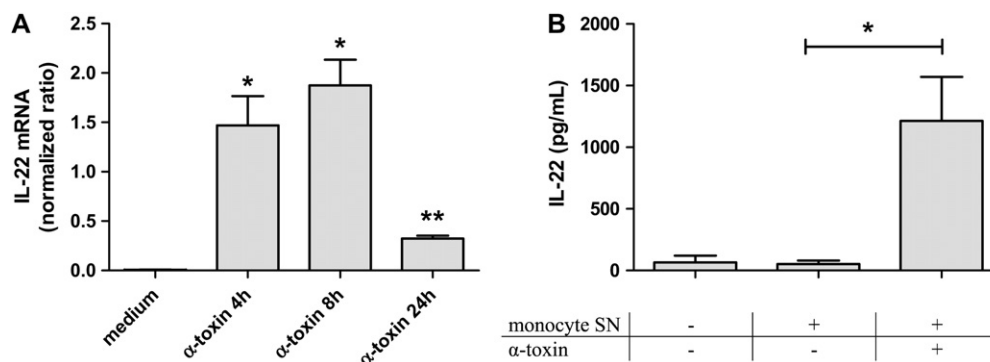


FIG 3. IL-22 mRNA (**A**) and protein (**B**) are upregulated in purified CD4⁺ T cells after stimulation with supernatants (SN) from α-toxin-stimulated purified monocytes compared with stimulation with SN from medium-treated monocytes as determined by qRT-PCR or ELISA. Data are shown as mean IL-22/glyceraldehyde-3-phosphat-dehydrogenase ratio + SEM of n = 3 (**A**) or as mean values + SEM of n = 6 experiments (**B**). **P* < .05; ***P* < .01.

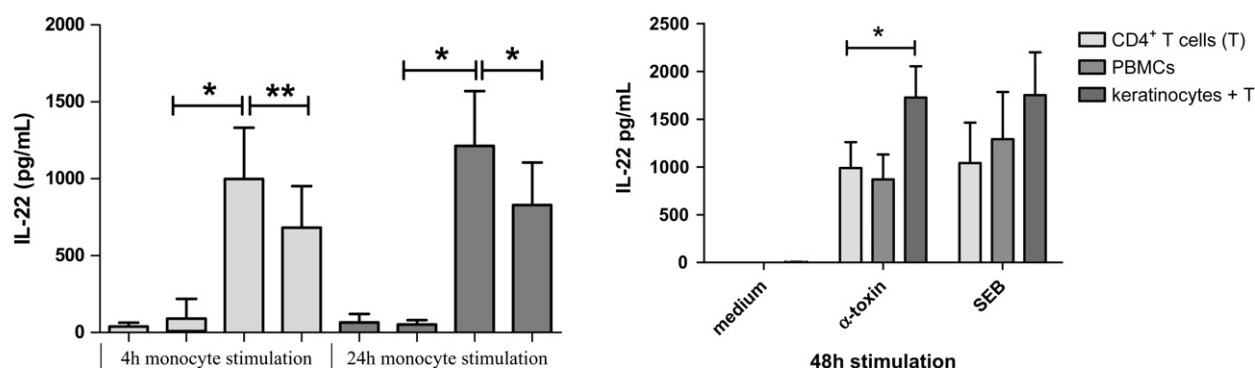


FIG 4. α-Toxin-induced IL-22 secretion in CD4⁺ T cells can be partially blocked with the IL-1 receptor antagonist Kineret. CD4⁺ T cells were stimulated as indicated for 72 hours. One hour before stimulation, T cells were preincubated with 0.5 μg/mL IL-1RA to block IL-1 receptors. The mean value + SEM of n = 6 independent experiments is shown. **P* < .05; ***P* < .01. IL-1R, IL-1 receptor; SN, supernatant.

without staphylococcal exotoxin stimulation (medium) (Fig 5). Unstimulated, α-toxin-stimulated, or SEB-stimulated monocultured keratinocytes did not secrete IL-22 (data not shown).

Enhanced IL-22 secretion in PBMCs and CD4⁺ T cells from patients with atopic dermatitis on α-toxin stimulation

PBMCs and CD4⁺ T cells from patients with AD or psoriasis and healthy controls were either left unstimulated or were stimulated with 50 ng/mL or 100 ng/mL α-toxin for up to 5 days. IL-22 secretion in cell free culture supernatants was quantified by ELISA. Both α-toxin concentrations increased IL-22 secretion in PBMCs (Fig 6, A and B) and T cells (Fig 6, C and D) from patients with AD, patients with psoriasis, and healthy controls compared with the appropriate medium control. CD4⁺ T cells from patients with AD stimulated with 50 ng/mL α-toxin for 48 hours and 72 hours yielded significantly increased IL-22 secretion compared with T cells from patients with psoriasis (Fig 6, C). Stimulation of CD4⁺ T cells with 100 ng/mL α-toxin for 48 hours and 120 hours led to significantly enhanced secretion of IL-22 in

FIG 5. Enhanced IL-22 secretion in autologous cocultures of T cells and keratinocytes on α-toxin stimulation. Autologous keratinocytes, CD4⁺ T cells (T), and PBMCs from healthy donors were cultured either separately or in combinations as indicated and were stimulated for 48 hours with α-toxin (n = 8; 50 ng/mL) or SEB (n = 5; 100 ng/mL). Supernatants were quantified for IL-22 by ELISA. Data are shown as mean values + SEM. **P* < .05.

patients with AD compared with patients with psoriasis as well as in patients with AD compared with healthy controls on α-toxin stimulation for 48 hours (Fig 6, D). PBMCs and T cells from patients with AD secreted more IL-22 compared with patients with psoriasis and healthy controls on α-toxin stimulation at all time points tested (Fig 6, A-D). To compare IL-22 induction on α-toxin stimulation with T_H17, T_H1, and T_H2 and cytokine induction, we assessed IL-17, IFN-γ, and IL-4. α-Toxin induced IFN-γ, IL-4, and IL-17 in PBMCs and T cells from patients with AD and psoriasis as well as healthy controls. By trend, patients with AD secreted more IL-17 on α-toxin stimulation compared with patients with psoriasis and healthy controls. As expected, PBMCs from patients with AD secreted less IFN-γ compared with patients with psoriasis and healthy controls on α-toxin stimulation. We observed a low IL-4 secretion in PBMCs and CD4⁺ T cells on α-toxin stimulation without significant differences among patients with AD, patients with psoriasis, and healthy controls (see this article's Fig E3, A-C, in the Online Repository at www.jacionline.org).

DISCUSSION

Atopic dermatitis and psoriasis are very common chronic inflammatory skin diseases. AD is considered a polar T_H2 disease in the acute phase, with a (partial) shift to T_H1 during the chronic

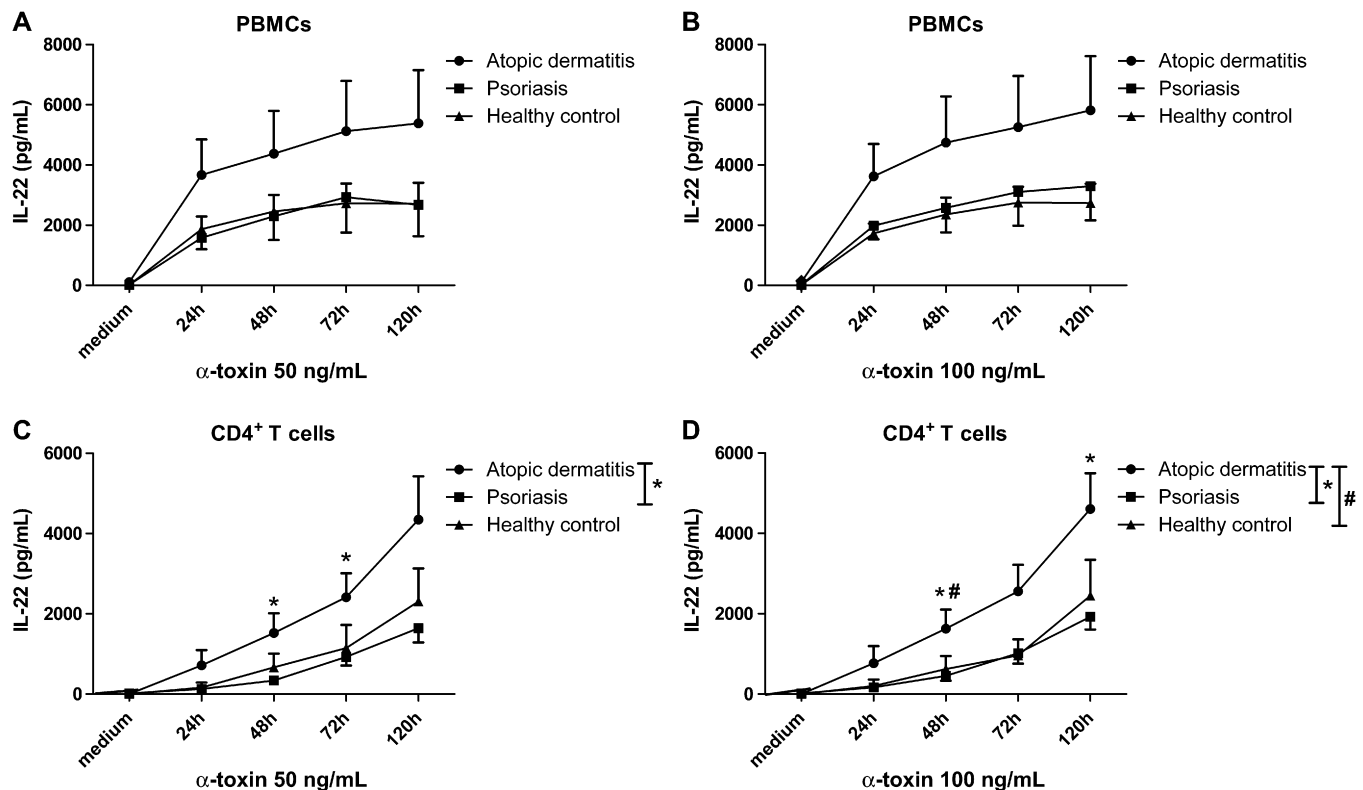


FIG 6. Enhanced IL-22 production in PBMCs and CD4⁺ T cells from AD compared with psoriasis and healthy controls on α -toxin stimulation. PBMCs (**A and B**) and CD4⁺ T cells (**C and D**) from patients with AD ($n = 10$) and psoriasis ($n = 8$) as well as healthy controls ($n = 8$) were either left unstimulated or stimulated as indicated. Supernatants were quantified for IL-22 by ELISA. Data are shown as mean values $\pm$ SEM. *Differences between AD and psoriasis, $P < .05$. #Differences between AD and healthy control, $P < .05$.

phase.²⁸ Psoriasis has been considered a model T_H1 disease.^{29,30} Recently, T_H17 cells have challenged this classic paradigm, and they are implicated in a growing number of autoimmune and inflammatory diseases.³¹ Although IL-22 production was initially linked to IL-17 expression by T_H17 cells,^{1,2} IL-22 production can also occur in an apparently unique subset of cells that do not express IL-17 or IFN- γ but coexpress CCR6 and the skin homing receptors CCR4 and CCR10.^{3,4} Because evidence has increased that IL-22 secretion is critical in chronic inflammatory skin disorders, namely AD and psoriasis, we investigated whether microbial products such as SEB and α -toxin were sufficient to stimulate IL-22 secretion.

We demonstrated for the first time that SEB and sublytic concentrations of α -toxin are strong inducers of IL-22 in PBMCs, T cells, and autologous cocultures of keratinocytes and CD4⁺ T cells. This effect was both direct, on stimulation with α -toxin, and IL-1-dependent, as shown by blocking experiments with IL-1RA and stimulation of CD4⁺ T cells with supernatants from α -toxin-stimulated monocytes. However, the indirect effect on IL-22 induction was lower compared with direct stimulation of CD4⁺ T cells with α -toxin (Fig 1, C). In this regard, regulation of IL-22 seems to be analogous to IL-17. Rao et al³² demonstrated by using a model of late graft failure that IL-1 was able to increase the frequency of alloreactive memory CD4⁺ T cells that produced IL-17 but not those that produced IFN- γ . Conversely, IL-1RA was able to block IL-17 secretion in cocultures of epithelial cells with allogeneic CD4⁺ T cells. IL-1 induction in monocytes by α -toxin was first demonstrated by Bhakdi et al³³ in 1989.

In keratinocytes, IL-22 increases the expression of the antimicrobial peptides human β -defensin 2 and 3, indicating an involvement in early host defense against microbial pathogens.⁹ Eyerich et al¹¹ showed that the frequency of IL-22⁺ T cells is increased in inflamed skin from patients with psoriasis, AD, and allergic contact dermatitis. In contrast, Guttman-Yassky et al²⁹ found an impaired IL-22, IL-17, and IL-23 expression in chronic eczematous skin from patients with AD compared with psoriasis skin and concluded that the absence of the IL-23/T_H17 axis in AD may account for recurrent skin infections. These conflicting results may be explained by the different methods used. Guttman-Yassky et al²⁹ used immunohistochemistry, whereas Eyerich et al¹¹ investigated T-cell clones from lesional skin. By performing coculture experiments with autologous keratinocytes and T cells, we demonstrated for the first time that α -toxin stimulation of keratinocytes cocultured with T cells significantly enhanced IL-22 secretion compared with CD4⁺ T cells alone. Moreover, α -toxin induced more IL-22 in cocultures of T cells and keratinocytes compared with T cells and PBMCs (data not shown). The IL-22 receptor is known to consist of 2 subunits, called IL-22 receptor 1 and IL-10 receptor 2. Whereas IL-10 receptor 2 is widely expressed on immune cells, IL-22 receptor 1 expression is restricted to nonhematopoietic cells such as keratinocytes of the skin and epithelial cells of the gastrointestinal tract and the lung.^{9,34} Therefore, IL-22 is known to act exclusively on epithelial cells and not on immune cells. The ability of α -toxin to induce inflammasome-mediated caspase-1 activation, which is required for IL-1 β cleavage and secretion, has also been

reported.^{35,36} Therefore, α -toxin induced activation of the NOD-like receptor family, pyrin domain containing 3 inflammasome with consecutive IL-1 secretion could be the underlying mechanism for keratinocyte activation in our coculture system. Our data indicate that crosstalk between keratinocytes and T cells on stimulation with staphylococcal α -toxin may in turn enhance IL-22 production in T cells, pointing to a positive feedback loop.

Because IL-22-producing T cells might play an important role in AD, we wanted to elucidate the effects of α -toxin stimulation on IL-22 production in patients with AD and compare these effects with psoriasis and healthy controls. Surprisingly, we demonstrated an enhanced IL-22 secretion by PBMCs and CD4⁺ T cells obtained from patients with AD compared with patients with psoriasis and healthy controls on stimulation with α -toxin. This observation was significant in T cells from patients with AD compared with patients with psoriasis and healthy controls. Furthermore, IL-22 levels in PBMCs and CD4⁺ T cells from patients with psoriasis did not differ significantly from healthy controls on stimulation with staphylococcal α -toxin, pointing to a distinct role for IL-22 in AD.

Nogralles et al¹⁰ demonstrated that unique IL-22-producing CD4⁺ and CD8⁺ T cells are increased in chronic lesional skin of patients with AD, whereas IL-17-producing CD4⁺ T cells accumulated in psoriasis compared with AD skin. The authors used qRT-PCR and flow cytometry for their investigations, which could explain the conflicting results of the previous study published by the same group.²⁹ The authors argue that upregulation of IL-22, independent of IL-17, might drive different features of epidermal pathology in inflammatory skin diseases, including the induction of antimicrobial peptides by T_H17 T cells and epidermal hyperplasia for T_H22 cells. However, they did not demonstrate differences in IL-17, IL-22, IL-4, and IFN- γ secretion by CD4⁺ and CD8⁺ T cells obtained from the peripheral blood of patients with psoriasis compared with patients with AD.¹⁰ This is partly in line with our findings because we failed to detect any differences in medium controls of PBMCs and CD4⁺ T cells from patients with AD and psoriasis compared with each other and with healthy controls. However, α -toxin stimulation increased IL-22 secretion of PBMCs and CD4⁺ T cells obtained from patients with AD and psoriasis and healthy controls. Under these conditions, PBMCs and CD4⁺ T cells from patients with AD secreted more IL-22 compared with patients with psoriasis and healthy controls. Future studies—for example, with tissue-derived T cells—are necessary to explore the role of α -toxin in chronic inflammatory skin diseases.

T_H17 and T_H1 cells and to a lesser extent T_H22 cells were found to be elevated in the blood of patients with psoriasis before infliximab treatment. T_H17 and T_H1 cells, but not T_H22 cells, decrease during therapy.³⁷ Harper et al³⁸ investigated psoriasis lesions immunohistochemically and found abundant IL-17A-positive cells and fewer IL-22-positive cells.³⁸ This is in contrast with Wolk et al,⁸ who showed in patients with plaque psoriasis an increased IL-22 expression in skin biopsies and greatly elevated IL-22 levels in the blood, compared with healthy controls.⁸ However, we failed to detect increased IL-22 secretion in PBMCs and CD4⁺ T cells from patients with psoriasis compared with healthy controls.

Because α -toxin is present on the skin of 30% and 63% of patients with AD untreated²² and treated with antimicrobials,²³ respectively, IL-22 may have a high clinical impact in the pathogenesis, flare-ups, and maintenance of eczema.

Our data could, at least in part, explain why skin colonization with *S aureus* can contribute to chronic skin inflammation. Further investigations are necessary to explore the role of IL-22 and IL-1RA in chronic inflammatory skin diseases because they could represent interesting targets for drug development.

We thank Mrs Gabriele Begemann and Mrs Kathrin Baumert for their excellent technical assistance.

Key messages

- Staphylococcal exotoxins are strong inducers of IL-22 in human PBMCs and T cells.
- Staphylococcal α -toxin can induce IL-22 directly and via IL-1 from antigen-presenting cells.
- Patients with AD secrete significantly more IL-22 on stimulation with α -toxin compared with patients with psoriasis and healthy controls.

REFERENCES

- Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 2008;28:454-67.
- Spolski R, Leonard WJ. Cytokine mediators of Th17 function. *Eur J Immunol* 2009;39:658-61.
- Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from Th-17, Th1 and Th2 cells. *Nat Immunol* 2009;10:864-71.
- Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol* 2009;10:857-63.
- Nogralles KE, Zaba LC, Guttman-Yassky E, Fuentes-Duculan J, Suarez-Farinas M, Cardinale I, et al. Th17 cytokines interleukin (IL) -17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br J Dermatol* 2008;159:1092-102.
- Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 2007;445:648-51.
- Boniface K, Guignouard E, Pedretti N, Garcia M, Delwail A, Bernard FX, et al. A role for T cell-derived interleukin-22 in psoriatic skin inflammation. *Clin Exp Immunol* 2007;150:407-15.
- Wolk K, Witte E, Wallace E, Docke WD, Kunz S, Asadullah S, et al. IL-22 regulates the expression of genes responsible for antimicrobial defence, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *Eur J Immunol* 2006;36:1309-23.
- Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity* 2004;21:241-54.
- Nogralles CE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing T22 T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing Th17 T cells. *J Allergy Clin Immunol* 2009;123:1244-52.
- Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodelling. *J Clin Invest* 2009;119:3573-85.
- Breuer K, Kapp A, Werfel T. Bacterial infections and atopic dermatitis. *Allergy* 2001;56:1034-41.
- Matsui K, Nishikawa A, Suto H, Tsuboi R, Ogawa H. Comparative study of *Staphylococcus aureus* isolated from lesional and non-lesional skin of atopic dermatitis. *Microbiol Immunol* 2000;44:945-7.
- Breuer K, Wittmann M, Bosche B, Kapp A, Werfel T. Severe atopic dermatitis is associated with sensitization to staphylococcal enterotoxin B (SEB). *Allergy* 2000;55:551-5.
- Leung DYM, Harbeck R, Bina P, Reiser RF, Yang E, Norris DA, et al. Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. *J Clin Invest* 1993;92:1374-80.
- Skov L, Olsen JV, Gjorno L, Schlievert PM, Baadsgaard O, Leung DY. Application of staphylococcal enterotoxin B on normal and atopic skin induces up-regulation of T-cells by a superantigen-mediated mechanism. *J Allergy Clin Immunol* 2000;105:820-6.

17. Mc Fadden JP, Noble WC, Camp RDR. Superantigenic exotoxin-secreting potential of staphylococci isolated from atopic eczematous skin. *Br J Dermatol* 1993; 128:631-2.
18. Marples RR, Heaton CL, Kligman AM. Staphylococcus aureus in psoriasis. *Arch Dermatol* 1973;107:568-70.
19. Tomi NS, Kränke B, Aberer E. Staphylococcal toxins in patients in patients with psoriasis, atopic dermatitis, and erythroderma, and in healthy control subjects. *J Am Acad Dermatol* 2005;53:67-72.
20. Breuer K, Haussler S, Kapp A, Werfel T. Staphylococcus aureus: colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. *Br J Dermatol* 2002;147:55-61.
21. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of Staphylococcus aureus. *Clin Microbiol Rev* 2000;13:16-34.
22. Breuer K, Wittmann M, Kempe K, Kapp A, Mai U, Dittrich-Breiholz O, et al. Alpha-toxin is produced by skin colonizing Staphylococcus aureus and induces a T helper type 1 response in atopic dermatitis. *Clin Exp Allergy* 2005;35:1088-95.
23. Wichmann K, Uter W, Weiss J, Breuer K, Heratizadeh A, Mai U, et al. Isolation of alpha-toxin-producing Staphylococcus aureus from the skin of highly sensitized adult patients with severe atopic dermatitis. *Br J Dermatol* 2009;161:300-5.
24. Niebuhr M, Langnickel J, Begemann G, Baumert K, Draing C, Werfel T. Dysregulation of TLR-2 induced effects in monocytes from patients with atopic dermatitis: Impact of the TLR-2 R753Q polymorphism. *Allergy* 2008;63:728-34.
25. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980;92(suppl):44-7.
26. Wang D, Drenker M, Eiz-Vesper B, Werfel T, Wittmann M. Evidence for a pathogenetic role of IL-18 in cutaneous lupus erythematosus. *Arthritis Rheum* 2008;58:3205-15.
27. Gutzmer R, Mommert S, Gschwandtner M, Zwingmann K, Stark H, Werfel T. The histamine H4 receptor is functionally expressed on Th2 cells. *J Allergy Clin Immunol* 2009;123:619-25.
28. Werfel T. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol* 2009;129:1878-91.
29. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Zaba LC, Cardinale I, Nogales KE, et al. Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol* 2008;181:7420-7.
30. van Beelen A, Teunissen MBM, Kapsenberg ML, de Jong EC. Interleukin-17 in inflammatory skin disorders. *Curr Opin Allergy Clin Immunol* 2007;7:374-81.
31. McKenzie BS, Kastelein RA, Cua DJ. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol* 2006;27:17-23.
32. Rao DA, Tracey KJ, Pober JS. IL-1 α and IL-1 β are endogenous mediators linking cell injury to the adaptive alloimmune response. *J Immunol* 2007;179: 6536-46.
33. Bhakdi S, Muhly M, Korom S, Hugo F. Release of interleukin-1 beta associated with potent cytotoxic action of staphylococcal alpha-toxin on human monocytes. *Infect Immunol* 1989;57:3512-9.
34. Wolk K, Sabat R. Interleukin-22: a novel T and NK cell-derived cytokine that regulates the biology of tissue cells. *Cytokine Growth Factor Rev* 2006;17: 367-80.
35. Munoz-Planillo R, Franchi L, Miller LS, Nunez G. A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the NLRP3 inflammasome. *J Immunol* 2009;183:3942-8.
36. Gurcel L, Abrami L, Girardin S, Tschopp J, Gisou van der Goot F. Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. *Cell* 2006;126:1135-45.
37. Kagami S, Rizzo HL, Lee JJ, Koguchi Y, Blauvelt A. Circulating Th17, Th22 and Th1 cells are increased in psoriasis. *J Invest Dermatol* 2010;130:1373-83.
38. Harper EG, Guo C, Rizzo H, Lillis J, Kurtz SE, Skorcheva I, et al. Th17 cytokines stimulate CCL20 expression in keratinocytes in vitro and in vivo: implications for psoriasis pathogenesis. *J Invest Dermatol* 2009;129:2175-83.

METHODS

Patients

Patients did not receive any systemic treatment for psoriasis or AD during the 2 weeks before blood drawing and presented with an exacerbation of their disease to our department. For healthy controls, individuals were chosen without a history of atopic diseases, with normal total IgE and negative IgE specific for the most common seasonal and perennial inhalant allergens, who were taking no immunosuppressive medications. The study was approved by the local ethics committee and was in accordance with the protocols of the Declaration of Helsinki.

Isolation of monocytes and T cells

Buffy coats were used that were disposed of by the blood bank after thrombocyte preparation from anonymous healthy blood donors. PBMCs were isolated by density gradient centrifugation in lymphoprep (Fresenius Kabi Norge AS, Halden, Norway). CD4⁺ T cells and monocytes were isolated by using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec Inc, Bergisch Gladbach, Germany). Monocytes and T cells were cultured in Iscove medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (Gibco, Eggenstein, Germany), 2 M glutamine (Seromed, Berlin, Germany), 1% nonessential salts (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Seromed), and 0.5% gentamicin (Biochrom) at 37°C in a humidified atmosphere at 5% CO₂. For activation of T cells, CD4⁺ T cells were stimulated with activating anti-CD3 (450 ng/mL), anti-CD28 (200 ng/mL) antibodies (Pellicluster, Amsterdam, The Netherlands) as well as IL-2 (20 ng/mL; TEBU Peprotech, Offenbach, Germany) for 72 hours.

Contaminating cells were evaluated by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany) and included B lymphocytes (CD20, <0.5%), and natural killer cells (CD56, <3.0%). Granulocytes (CD16) were absent. Isolated T cells had a purity of at least 94% and monocytes of at least 88%.

Hair keratinocyte isolation and culture

Isolation of outer root sheath cells from plucked human hair follicles was performed as described previously.^{E1} In brief, approximately 5 anagen hairs were plucked from the scalp of healthy volunteers. The plucked hairs were cut down to the follicle, and the hair follicles were incubated overnight in HBSS buffered with 20 mmol/L HEPES (Sigma-Aldrich, Deisenhofen, Germany) and supplemented with 50 µg/mL gentamicin and 2.5 µg/mL amphotericin B (PromoCell, Heidelberg, Germany). The hairs were then placed in a 6-well plate that had been covered with a feeder layer of 3T3 fibroblasts (DSMZ, Braunschweig, Germany). Within 1 to 2 weeks, keratinocytes grew out (mainly from the bulge region). The medium (high-glucose Dulbecco's modified Eagle medium with Ham's F-12 supplemented with 10% FCS, 1.8×10^{-4} mol/L adenine [Sigma-Aldrich], 10^{-10} cholera toxin [Sigma-Aldrich], 2×10^{-9} T3, 10 ng/mL epidermal growth factor, 4 µg/mL hydrocortisone, 5 µg/mL transferrin, 50 µg/mL gentamicin, and 2.5 µg/mL amphotericin B [all components except adenine and cholera toxin were from PromoCell]) was changed every 2 to 3 days.

When sufficient keratinocytes were outgrown, cells were detached by using first 0.02% EDTA and afterward 0.05% trypsin/0.02% EDTA (PAN Biotech, Aidenbach, Germany). Trypsin activity was stopped by the addition of an

equal volume of trypsin inhibitor (PAN Biotech). Keratinocytes were then cultured in serum-free keratinocytes growth medium (Keratinocyte Growth Medium 2 kit; PromoCell GmbH, Heidelberg, Germany), which was changed every 2 to 3 days. When cells reached 70% to 80% confluency, they were used for further experiments or were passaged. Cells were used between passages 3 and 7 for experiments in hydrocortisone-free and epidermal growth factor-free medium. The purity of hair keratinocytes was verified by the expression of the epithelial marker cytokeratin (antihuman cytokeratin antibody, clone: MNF-116; DakoCytomation, Hamburg, Germany) and the fibroblast-specific marker AS02 (CD90; Dianova, Hamburg, Germany). All cells (more than 95%) were found to be uniformly positive for cytokeratin.

RNA isolation, reverse transcription, and real-time PCR

RNA was isolated by using the RNA high purification kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instructions. The cDNA was synthesized with integrated removal of genomic DNA in the samples by using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Quantitative real-time RT-PCR (qRT-PCR) was performed on a LightCycler PCR machine (Roche Molecular Biochemicals) by using the Quantitect SYBR Green PCR kit (Qiagen) and Quantitect Primer for IL-1β, IL-17 and glyceraldehyd-3-phosphat-dehydrogenase (housekeeping gene). Purified PCR products were used as external calibrators. For quantitative analysis, standard curves for IL-1β and IL-17 were created. These standard curves described the PCR efficiencies of the target and the reference gene (glyceraldehyd-3-phosphat-dehydrogenase), which allowed an efficiency-corrected quantification by using the Relative Quantification software (Roche Molecular Biochemicals).

Flow-cytometric analysis

Cells were washed and resuspended in PBS containing 0.2% gelatin, 20 mmol/L sodium azide, and 10 µg/mL heat-aggravated human IgG (Sigma-Aldrich, Deisenhofen, Germany) to block FcR for 15 minutes. Subsequently, cells were incubated with the indicated mAbs, on ice for 30 minutes, or isotype-matched controls (Sigma). Stained cells were washed 3 times, fixed in PBS with 1% paraformaldehyde, and analyzed by flow cytometry (FACScalibur; Becton Dickinson, Heidelberg, Germany).

Intracellular cytokine staining was performed according to the protocol and with the reagents provided by BD Biosciences (Becton Dickinson, Heidelberg, Germany). Briefly, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 6 hours in the presence of monensin. After 2 hours, brefeldin A was added. After cell surface staining, cells were fixed, permeabilized with Cytofix/Cytoperm (BD Biosciences), and incubated with antibodies. Acquisition and analysis was performed with a FACS Calibur (Becton Dickinson).

REFERENCE

- E1. Wang D, Drenker M, Eiz-Vesper B, Werfel T, Wittmann M. Evidence for a pathogenetic role of IL-18 in cutaneous lupus erythematosus. *Arthritis Rheum* 2008; 58:3205-15.

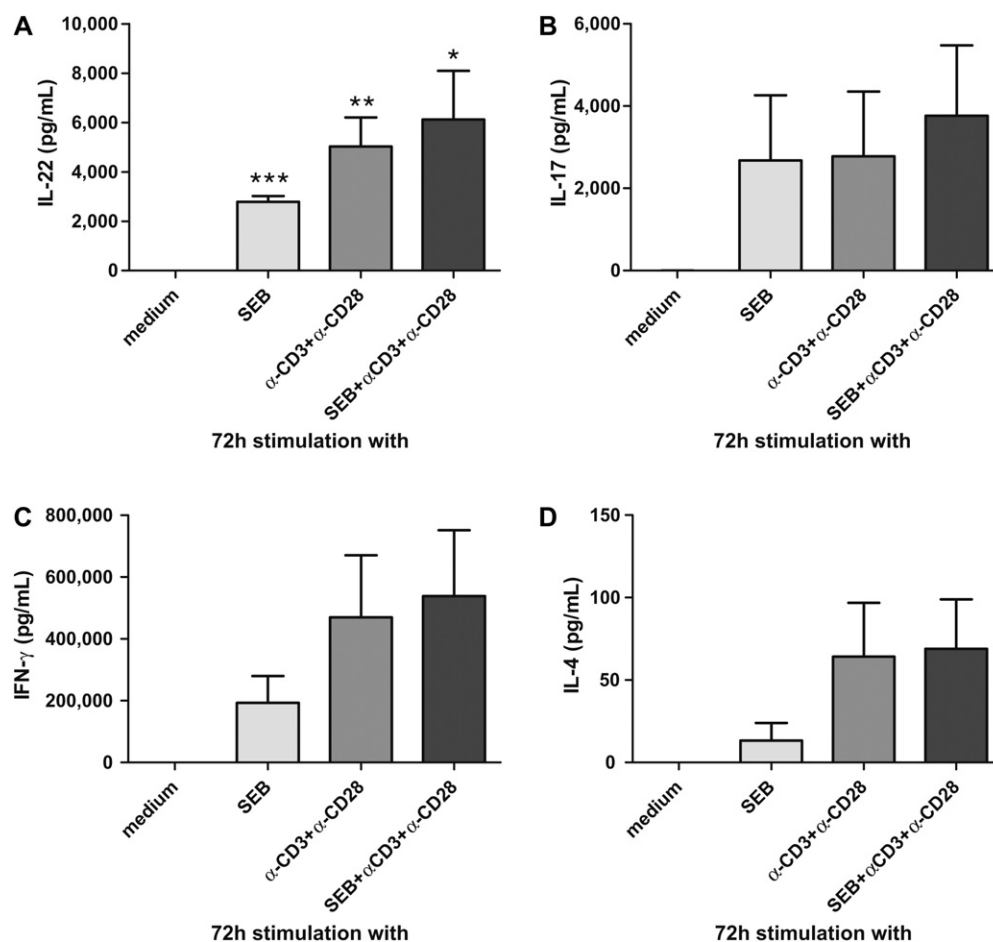


FIG E1. IL-22, IL-17, IFN- γ , and IL-4 production in CD4⁺ T cells on SEB stimulation. CD4⁺ T cells ($n = 4-6$) were either left unstimulated or stimulated with SEB, anti-CD3 plus anti-CD28, or SEB plus anti-CD3 plus anti-CD28 for 72 hours. Supernatants were quantified for IL-22 (**A**), IL-17 (**B**), IFN- γ (**C**), and IL-4 (**D**) by ELISA. Data are shown as mean values + SEM. * $P < .05$; ** $P < .01$; *** $P < .001$.

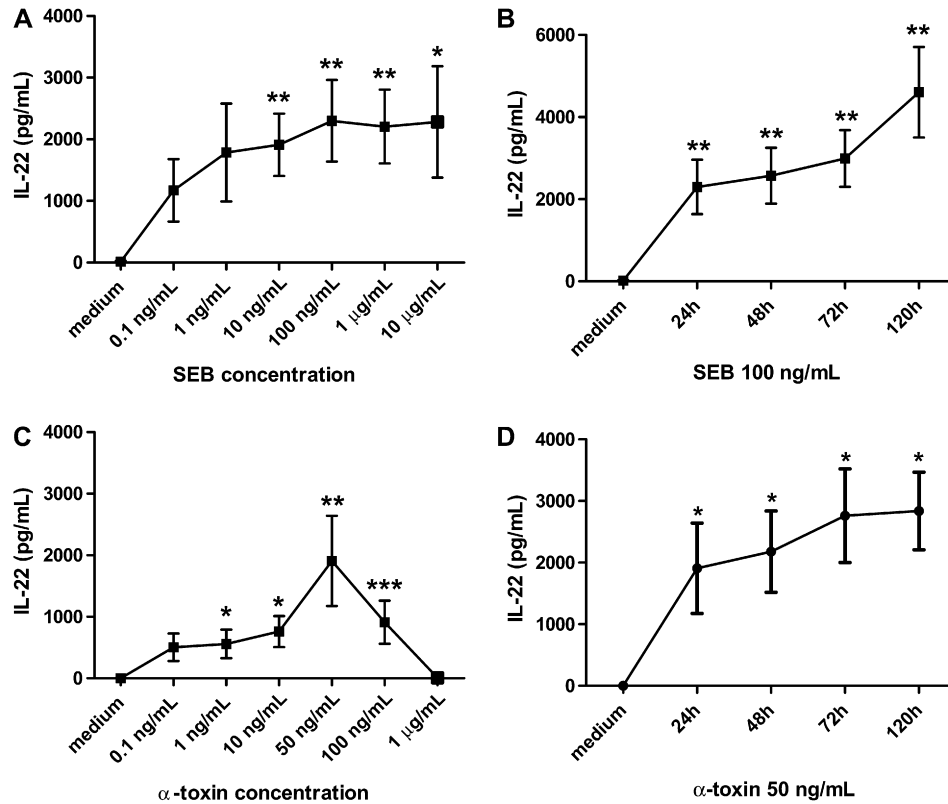


FIG E2. PBMCs secreted significantly more IL-22 on stimulation with SEB as well as α -toxin compared with medium control. PBMCs ($n = 9$, except for SEB 0.1 ng/mL and 1 ng/mL, $n = 3$) were either left unstimulated or stimulated with SEB (**A and B**) or α -toxin (**C and D**) in a dose-dependent manner for 24 hours (**A and C**) or in a time-dependent manner as indicated (**B and D**), and supernatants were analyzed for IL-22 secretion by ELISA. Data are shown as the mean IL-22 value (pg/mL) $\pm$ SEM. * $P < .05$; ** $P < .01$; *** $P < .001$.

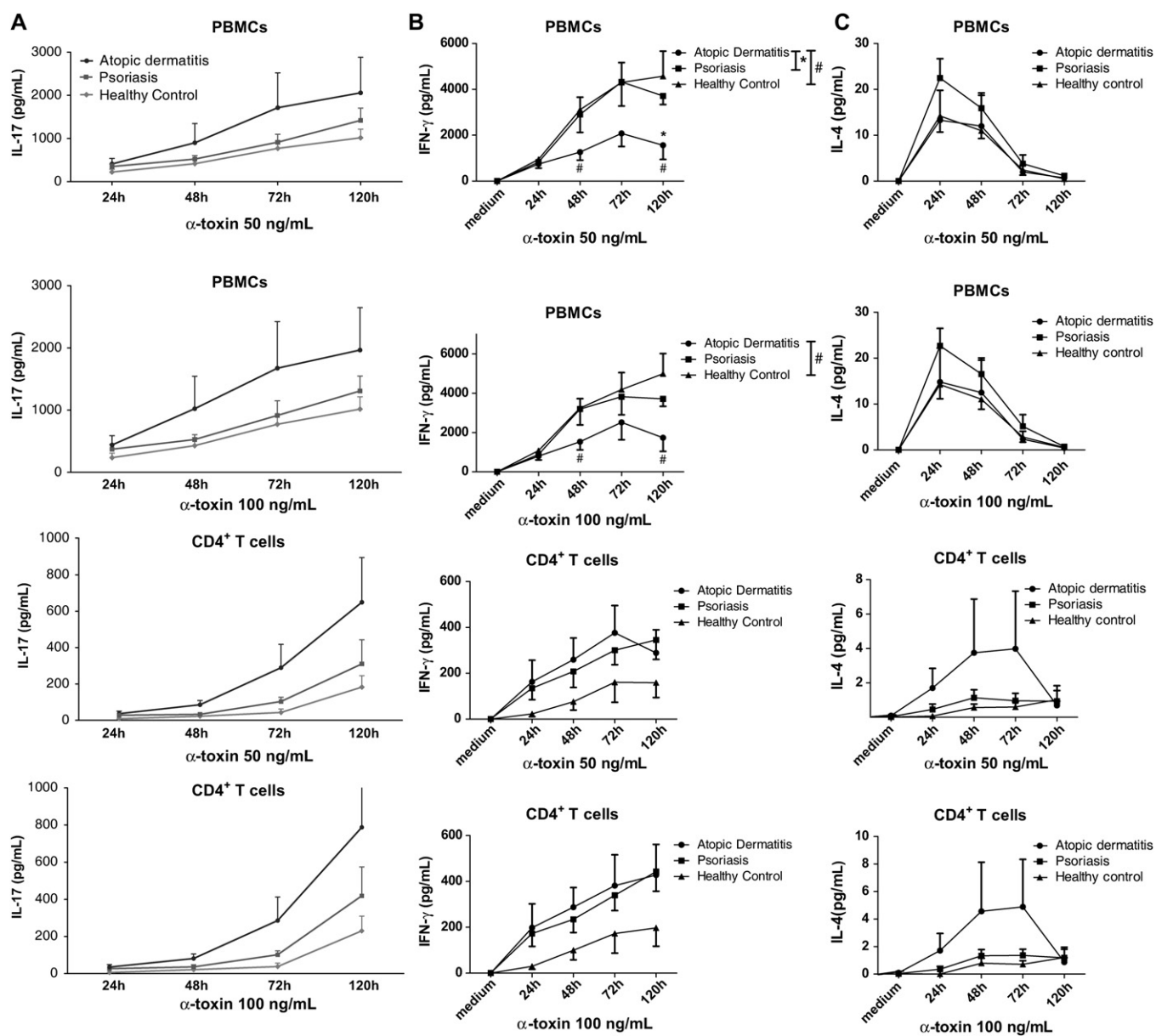


FIG E3. IL-17, IFN- γ , and IL-4 production in PBMCs and CD4⁺ T cells from AD compared with psoriasis and healthy controls on α -toxin stimulation. PBMCs and CD4⁺ T cells from patients with AD ($n = 10$) and psoriasis ($n = 8$) as well as healthy controls ($n = 8$) were either left unstimulated or stimulated with α -toxin as indicated. Supernatants were quantified for IL-17 (**A**), IFN- γ (**B**), and IL-4 (**C**) by ELISA. Data are shown as mean values $\pm$ SEM. *Differences between AD and psoriasis, $P < .05$. #Differences between AD and healthy control, $P < .05$.