

Alpha-toxin is produced by skin colonizing *Staphylococcus aureus* and induces a T helper type 1 response in atopic dermatitis

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Summary

Background *Staphylococcus aureus* is a well known trigger factor of atopic dermatitis (AD). Besides the superantigens, further exotoxins are produced by *S. aureus* and may have an influence on the eczema.

Objective To explore the impact of staphylococcal α -toxin on human T cells, as those represent the majority of skin infiltrating cells in AD.

Methods Adult patients with AD were screened for cutaneous colonization with α -toxin producing *S. aureus*. As α -toxin may induce necrosis, CD4⁺ T cells were incubated with sublytic α -toxin concentrations. Proliferation and up-regulation of IFN- γ on the mRNA and the protein level were assessed. The induction of t-bet translocation in CD4⁺ T cells was detected with the Electrophoretic Mobility Shift Assay.

Results Thirty-four percent of the patients were colonized with α -toxin producing *S. aureus* and α -toxin was detected in lesional skin of these patients by immunohistochemistry. Sublytic α -toxin concentrations induced a marked proliferation of isolated CD4⁺ T cells. Microarray analysis indicated that α -toxin induced particularly high amounts of IFN- γ transcripts. Up-regulation of IFN- γ was confirmed both on the mRNA and the protein level. Stimulation of CD4⁺ T cells with α -toxin resulted in DNA binding of t-bet, known as a key transcription factor involved into primary T helper type 1 (Th1) commitment.

Conclusion α -toxin is produced by *S. aureus* isolated from patients with AD. We show here for the first time that sublytic α -toxin concentrations activate T cells in the absence of antigen-presenting cells. Our results indicate that α -toxin is relevant for the induction of a Th1 like cytokine response. In AD, this facilitates the development of Th1 cell dominated chronic eczema.

Keywords α -hemolysin, α -toxin, atopic dermatitis, eczema, exotoxin, IFN- γ , *Staphylococcus aureus*, T lymphocyte

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease which is influenced by various external factors [1]. Allergen-specific T cells could be generated from biopsies of spontaneous lesions and patch test lesions [2–5]. Whereas acute patch test lesions and spontaneous eczematous lesions are dominated by a T helper (Th) type 2 like cytokine milieu, Th1 cytokines are predominantly found in chronic patch test lesions [5–7]. The factors driving the naïve T cell to a Th1 phenotype are an area of active investigation. IFN- γ can strongly augment the expression of T-bet, a member of the

T-box family of transcription factors, and the expression of T-bet acts in primary Th1 commitment [8]. IL-12 secreted by antigen-presenting cells seems to be critical for the development of a Th1-type immune response [9].

Over the last decades, *Staphylococcus aureus* has been identified as an important trigger factor of AD. Thirty to sixty percent of the strains isolated from patients with AD have been found to secrete enterotoxins with superantigenic properties [10–13]. An interrelation between *S. aureus* strains producing superantigens and the severity of AD has been demonstrated in a variety of studies [14–16]. Superantigens have the ability to augment antigen-specific Th1 responses by induction of IL-12 in antigen-presenting cells, which might contribute to the chronification of AD [17, 18]. On the other hand, the severity of AD decreased also in patients colonized with non-toxigenic *S. aureus* strains upon antimicrobial treatment [10], which suggests the involvement of other pathogenic factors than superantigens derived from *S. aureus*.

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A distinct percentage of *S. aureus* strains are able to produce α toxin, a potent 33 000 Da cytolyisin which does not belong to the group of enterotoxins (superantigens) [19]. A wide range of human cells has been shown to be prone to lysis by α -toxin with greatly varying susceptibility, among them keratinocytes and lymphocytes [20, 21]. In keratinocytes, TNF- α is released simultaneously to lysis [22]. Besides this well described mechanism of lysis, α -toxin may induce apoptosis in T lymphocytes [20]. In the present study, we detected α -toxin in lesional skin of AD patients. Therefore we investigated the effects of sublytic α -toxin concentrations on human CD4⁺ T cells, as those represent the majority of inflammatory cells in lesional skin of AD. We show here for the first time that low α -toxin concentrations are able to activate T lymphocytes in the absence of antigen-presenting cells, and to induce IFN- γ on both the mRNA and the protein level. Our results indicate that α -toxin represents a further factor for the induction of a Th1 response.

Methods

Isolation of Staphylococcus aureus and identification of α -toxin

Rayon-tipped swabs were taken from the skin of 64 adult patients with mild to severe AD without overt signs of cutaneous infections (mean age 36 years, mean SCORAD 40.1 points). *S. aureus* isolates were screened for α -toxin production using the synergistic hemolysis (CAMP-like) test on Columbia 5% sheep blood agar plates (OXOID) with α -toxin positive and negative *S. aureus* strains as controls.

Cutaneous 5 mm punch biopsy specimens were taken from three patients with AD colonized with α -toxin producing strains and three healthy controls. Six micrometer serial cryostat sections were blocked with goat serum and thereafter incubated with polyclonal anti-staphylococcal α -toxin antibodies (rabbit, Sigma-Aldrich, Munich, Germany) at 5 μ g/mL for 60 min. Sections were stained using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, USA) according to the supplier's instructions. The negative-staining controls were rabbit IgG antibodies with irrelevant specificity. The local ethics committee had approved taking skin biopsy specimens from adult patients with AD.

Cytokines and reagents

Lyophilized staphylococcal α -toxin was obtained from Sigma-Aldrich. Highly purified staphylococcal enterotoxin B (SEB) was obtained from Toxin Technology (Sarasota, FL, USA).

The endotoxin content of α -toxin and SEB was determined by the Limulus amoebocyte assay (BioWhittaker Inc., Walkersville, MD, USA) and was <200 pg/mL for the concentrations used in cell culture.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) from healthy and atopic donors (i.e. patients with AD or allergic rhinitis) were isolated from heparinized blood samples by density gradient centrifugation on Lymphoprep (Axis Shield PoC AS, Oslo, Norway). Monocytes were isolated by adherence

on Petri dishes (Heraeus, Hannover, Germany). Adherent cells were detached, washed and further purified by negative selection with the MACS Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting cell preparation contained >90% monocytes when assessed by staining with phycoerythrin (PE)-conjugated monoclonal antibodies to CD14 (mouse IgG2a, Coulter-Immunotech, Hamburg, Germany) and flow cytometric analysis (FACS) analysis. Non-adherent cells were washed. CD4⁺ T cells were enriched to >95% purity by negative selection with the MACS CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). Purity was assessed by staining with FITC-conjugated monoclonal antibodies to CD4 (mouse IgG1, Coulter Immunotech) and FACS. In order to determine the cell types of the remaining <5% cells, the cells were stained with FITC-/PE-conjugated anti-CD3, -CD8, -CD14, -CD20 and -CD56 antibodies and a FACS analysis was performed in one patient. In this patient, the purity of CD4⁺ T cells was 96.5%. The remaining cells were CD8⁺/CD3⁺ cells (0.34%), NK-cells (0.68%), B cells (0.16%), monocytes (0.18%) and non-vital cells. The cells were resuspended in Iscove's medium (Biochrom, Berlin, Germany), supplemented with 4% human heat-inactivated AB serum (IAB medium).

T cell lines were generated from the peripheral blood and stimulation assays were performed as described previously [2, 5].

Detection of apoptosis/necrosis

Apoptosis of PBMC was determined by use of the Annexin V-FITC Apoptosis Detections Kit I (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. 10⁵ cells were stained with Annexin V-FITC and Propidium iodide (PI). Percentages of cells undergoing apoptosis/necrosis were determined at different time-points as indicated in results by FACS.

CFSE labelling

Proliferation of PBMC/CD4⁺ T lymphocytes was assessed with the VibrantTM CFDA SE Cell Tracer Assay (Molecular Probes Inc., Eugene, OR, USA). Cells were stained according to the manufacturer's instructions. 3 $\times$ 10⁵ cells/200 μ L were plated in duplicates with different α -toxin concentrations as indicated in results.

At several time-points the percentages of cells with a low CFSE fluorescence which had undergone one or more cell divisions were determined on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

DNA microarray experiments

Microarray experiments were performed by using the Human Inflammation Array (MWG Biotech, Ebersberg, Germany). This microarray contains validated oligonucleotide probes for 110 inflammatory genes as described previously [23].

Cytokine determinations in cell culture supernatants

Detection of IFN- γ in cell culture supernatants was performed using the Human IFN- γ ELISA Ready-SET-Go kit (Biocarta, Hamburg, Germany). IL-4 and IL-5 secretion were measured with the Human IL-4 and IL-5 Ready-SET-

Go kits (Biocarta). IL-13 secretion was measured with the human-13 DuoSet ELISA kit (R&D Systems, Abingdon, UK). The detection limit was 4, 2, 4 and 10 pg/mL for IFN- γ , IL-4, IL-5 and IL-13, respectively.

mRNA isolation and reverse transcription

mRNA was isolated from 1×10^5 cells (PBMC, CD4⁺ T cells) stimulated with 10 ng/mL α -toxin for 24 h using the High Pure RNA Isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). cDNA was synthesised with the First-strand cDNA Synthesis kit (Roche Molecular Biochemicals). Real-time fluorescence PCR was performed using the Light Cycler (Roche Molecular Biochemicals). For quantitative analysis the second derivative maximum method was used [24].

For the quantification of IFN- γ (forward 5'gcatcggtttgggttccttggtgttactgc3', reverse 5'ctccttttcgcttcctgttttagctgctgg3') an efficiency-adjusted relative quantification [25] was performed.

Electrophoretic Mobility Shift Assay

Isolated CD4⁺ T cells were incubated for 2 h with 10 ng/mL α -toxin or with stimulating anti-CD3 (1 μ g/mL) and anti-CD28 (0.2 μ g/mL) antibodies (Peli Cluster, Amsterdam, Netherlands) as positive control. As a second signal, 100 ng/mL IL-12 was added for further 4 h. Nuclear extracts of 2×10^6 CD4⁺ T cells per sample were prepared using the NE-PERTM Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Bonn, Germany). Protein content was determined according to Lowry (Bio-Rad protein assay, Bio Rad, Munich, Germany). Electrophoretic mobility shift assay (EMSA) for T-bet was performed with equal amounts of protein. For the binding reaction 1 μ L of NP40, glycerol, MgCl₂, Poly (dI:dC) and 2 μ L binding buffer were added to the nuclear extracts. For the detection of T-bet binding activity double stranded, biotin labelled oligonucleotides (5'-iotin-AAT TTC ACA CCT AGG TGT GAA ATT-3') comprising the t-bet binding site of the IFN- γ gene were added to the reaction. Mutant oligonucleotides (5'- Biotin-AAT TTC ACG TCT AGG TAC GAA ATT-3') were used for specificity control. To resolve the complexes, native polyacrylamide gels were loaded in $0.5 \times$ TBE for 1 h at 100 V. Thereafter the complexes were blotted onto a nylon membrane for 30 min at 20 V. The oligonucleotides were crosslinked to the membrane using UV light. The membranes were incubated in blocking buffer with horseradish peroxidase labelled streptavidin, washed and incubated with luminol (Pierce LightShift[®] Chemiluminescence EMSA Kit, Perbio Science, Bonn, Germany). Chemiluminescence was detected using the ChemiImagerTM 4400 (Biozym, Hess. Oldendorf, Germany). The AlphaEase Software Version 5.5 (AlphaInnotech, San Leandro, CA, USA) was used to analyse the band intensities.

Results

Detection of α -toxin producing *Staphylococcus aureus* strains on the skin of patients with atopic dermatitis

α -toxin producing *S. aureus* strains were isolated from 22/64 (34%) adult patients with AD. Patients who harboured

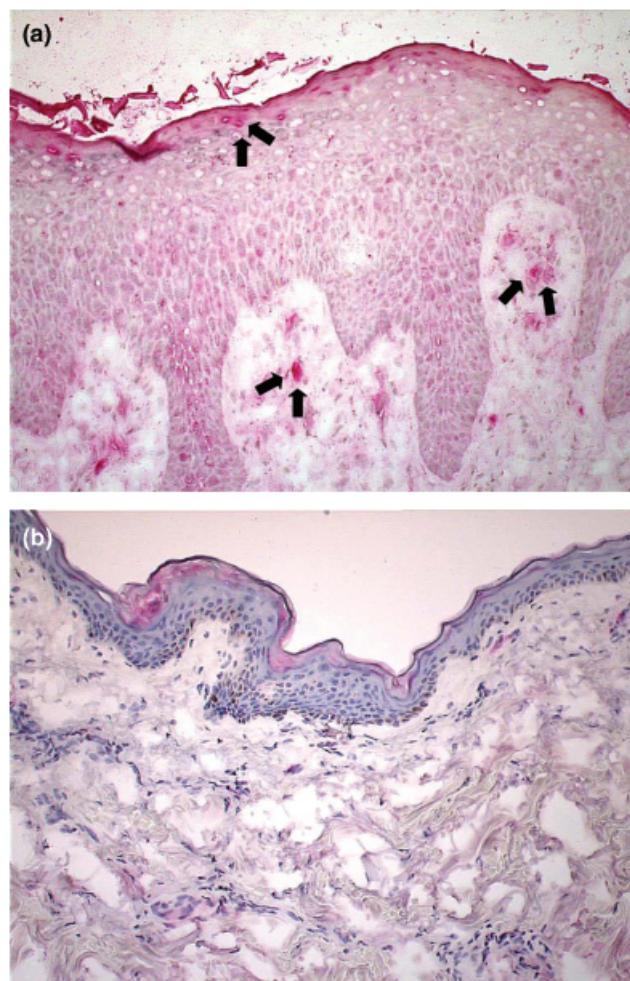


Fig. 1. Immunohistological staining of skin sections with anti- α -toxin antibodies. (a) Skin from an adult patient with atopic dermatitis (AD) colonized with α -toxin producing *Staphylococcus aureus*. Staining of stratum corneum and of the upper dermis is indicated by arrows. Similar staining could be shown in further two patients. (b) Skin from a healthy individual. Negative staining of healthy skin was found in further two individuals.

α -toxin producing *S. aureus* strains on lesional skin had a significantly higher SCORAD than patients who did not (SCORAD 48.9 ± 20.2 and 35.7 ± 18.4 points, mean $\pm$ SD, $P = 0.01$).

α -toxin was detected by means of immunohistochemistry in sections of skin biopsy specimens obtained from lesional skin of patients with AD who were colonized with α -toxin-producing *S. aureus* strains (Fig. 1). Specimens from healthy individuals did not stain with anti- α -toxin antibodies. Isotype-specific rabbit antibodies used as primary antibodies did not lead to significant staining of skin sections.

Low α -toxin concentrations do not induce apoptosis/necrosis of peripheral blood mononuclear cells

PBMC were incubated with α toxin (10 ng/mL–10 μ g/mL) and 1% saponin as positive control, cell culture medium served as negative control. At various time-points (30 min., 1 h, 4 h, 24 h), samples were stained with Annexin V-FITC and PI. Percentages of cells in early stages of apoptosis (single annexin V positive) and late stages of apoptosis/necrotic cells (double

annexin V and PI positive) were measured by FACS analysis. 10–100 ng/mL α -toxin did not induce necrosis whereas incubation with 1–10 μ g/mL α -toxin for 30 min. and longer resulted in increasing numbers of necrotic cells. After 24 h, the proportions of necrotic/late apoptotic cells were $4.9 \pm 4.2\%$, $64.5 \pm 6.4\%$, $43.6 \pm 23.0\%$, $7.0 \pm 4.5\%$ and $90.5 \pm 11.0\%$ (mean $\pm$ SD) for medium control, α -toxin 10, 1 μ g/mL, 100 ng/mL and 1% saponin, respectively ($n = 3$). The proportions of early apoptotic cells were $7.3 \pm 7.8\%$, $29.6 \pm 7.2\%$, $29.8 \pm 4.5\%$, $9.9 \pm 2.6\%$, and $4.9 \pm 5.2\%$ (mean $\pm$ SD) after 24 h for medium control, α -toxin 10, 1 μ g/mL, 100 ng/mL and 1% saponin, respectively, and the number of early apoptotic cells rose slower than the number of necrotic cells. Lower concentrations did not induce apoptosis. No differences between atopic and normal donors were observed.

Sublytic α -toxin concentrations induce proliferation of $CD4^+$ T lymphocytes

Proliferation of PBMC incubated with 1×10^{-6} – 10^{-8} g/mL α -toxin was measured with the Vibrant™ CFDA SE Cell Tracer Assay. One microgram per millilitre of α -toxin and higher did not lead to proliferation because of cell death, similarly no proliferation was detected at 1 pg/mL and lower (data not shown). Time kinetic experiments revealed a concentration-dependent proliferation of PBMC after 2 days which peaked at day 5/6. The percentages of divided cells after 5 days were 87.8 ± 1.9 , 60.0 ± 37.6 , 50.7 ± 17.1 , 20.7 ± 8.8 , and 1.5 ± 1.3 (mean $\pm$ SD) for α -toxin 100, 10, 1 ng/mL and 100 pg/mL, and medium control, respectively ($n = 4$). Proliferation induced by 10–100 ng/mL α -toxin was comparable with that induced by 100 ng/mL SEB. There was no significant difference between cells derived from atopic and non-atopic individuals (data not shown).

Furthermore, also incubation of isolated $CD4^+$ T cells with α -toxin resulted in a concentration-dependent proliferation which was less pronounced than that induced in PBMC (Fig. 2). Similarly, proliferation was induced in five T cell lines which were derived from the peripheral blood of a healthy individual and were virtually free of monocytes. The mean stimulation index (ratio of mean counts per minute of stimulated to unstimulated cultures) after stimulation of T cell lines with 10 ng/mL α -toxin for 4 days was 7.2 (5.0 SD).

Microarray analysis of $CD4^+$ T cells/peripheral blood mononuclear cells stimulated with α -toxin

To screen for further potential effects of sublytic α -toxin concentrations, we investigated the expression of a selected set of 110 genes with known relevance to inflammation [23], such as cytokines, chemokines, and matrix metalloproteinases in purified $CD4^+$ T cells and PBMC derived from healthy donors which had been incubated with 10 ng/mL α -toxin for 24 h. Microarray analysis revealed that IFN- γ was the most strongly up-regulated gene, in both $CD4^+$ T cells (22.3-fold induction) and PBMC (16.7-fold induction). Additionally, a small number of other genes was induced in $CD4^+$ cells, namely IL-9 (6.8-fold induction), CXCL10 (3.7-fold induction), IL-10 (3.2-fold induction) and IL-2 (2.0-fold induction). In PBMC, the same genes were expressed as follows with the following ratios: IL-9 (6.0-fold induction), CXCL10 (2.3-fold

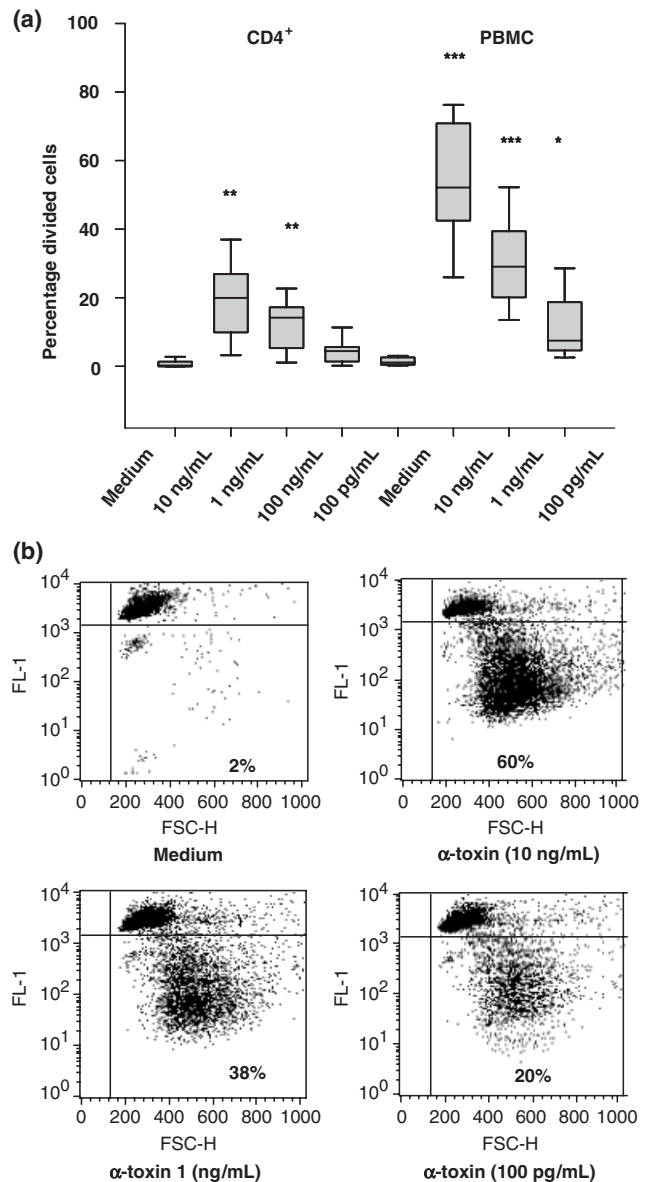


Fig. 2. α -toxin induces concentration-dependent proliferation of lymphocytes. $CD4^+$ cells or peripheral blood mononuclear cells were incubated with α -toxin for 4 days. Percentages of cells having undergone one or more cell divisions are shown in comparison with the medium control. Rank Sum test, boxes show median, 25th and 75th percentiles, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 24$). (b) FACS analysis of one representative experiment. Vertical: intracellular fluorescence of CFSE is shown, horizontal: forward scatter. The percentages of cells having undergone one or more cell divisions (right lower quadrant) are shown. Populations of cells having undergone a certain number of cell divisions can clearly be discriminated as clouds indicating that low CFSE fluorescence is caused by mitosis leading to the dilution of intracellular dye and not to the efflux of dye via transmembrane pores.

induction), IL-10 (4.9-fold induction) and IL-2 (2.5-fold induction). Low α -toxin concentrations did not exert significant effects on monocytes as judged by microarray analysis of purified human monocytes incubated with 10 ng/mL α -toxin for 24 h in two independent experiments.

α -toxin induces the expression of interferon- γ mRNA

An efficiency controlled quantitative real time PCR for IFN- γ confirmed the microarray results. The amount of IFN- γ was

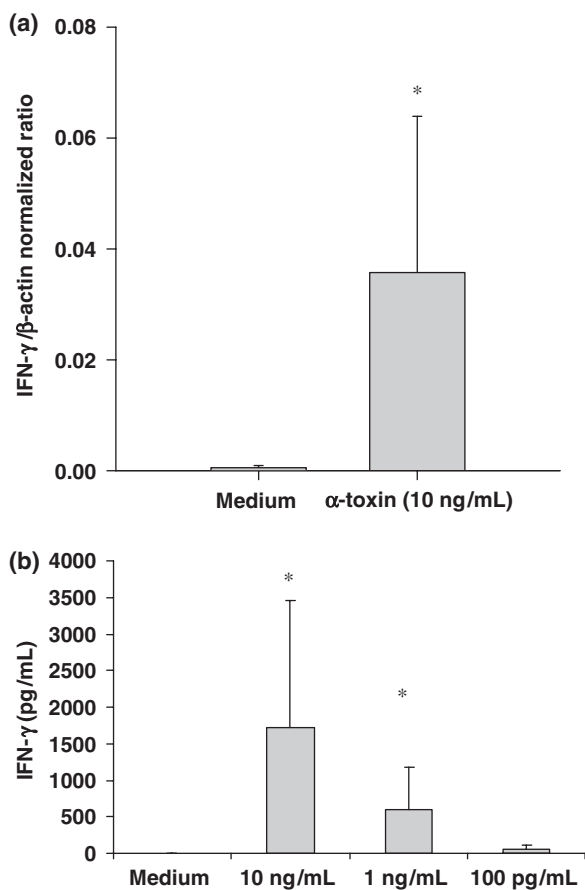


Fig. 3. Up-regulation of IFN- γ after incubation with α -toxin. (a) CD4⁺ T cells were incubated with 10 ng/mL α -toxin for 24 h. An efficiency controlled real time RT-PCR was performed for IFN- γ . Target concentration relative to the reference gene (beta-actin) are given. Mean $\pm$ SD are shown. *Student's *t*-test, $P = 0.025$ ($n = 5$). (b) CD4⁺ T cells were incubated with α -toxin for 4 days. IFN- γ concentrations in the cell culture supernatants were measured by ELISA. Mean $\pm$ SD are shown. *Student's *t*-test, $P < 0.05$ ($n = 6$).

normalized by the relative quantification software to the same beta-actin. 24 h after stimulation of CD4⁺ T cells with 10 ng/mL α -toxin, an up-regulation of IFN- γ on the mRNA level as compared with unstimulated cells was detected (Fig. 3a). Similarly, IFN- γ was significantly up-regulated in PBMC after incubation with 10 ng/mL α -toxin for 24 h (data not shown).

α-toxin induces a secretion of IFN-γ in CD4⁺ T lymphocytes

PBMC were incubated with 100 pg–100 ng/mL α -toxin for 1–6 days, and IFN- γ in the cell culture supernatants was measured by ELISA. Time-kinetic experiments revealed that incubation with 10–100 ng/mL α -toxin induced marked IFN- γ secretion which was detected on day 1 and peaked on day 3–4 (Fig. 4). The amount of IFN- γ was very high and comparable with that induced by 100 ng/mL SEB. Median IFN- γ concentrations in the cell culture supernatants of PBMC which had been incubated with medium and α -toxin at 10, 1 ng/mL and 100 pg/mL for 4 days were 0.0, 14910.0, 2638.5 and 575.5 pg/mL, respectively ($P < 0.001$ for the differences between medium control and α -toxin, Rank Sum test, $n = 21$). No significant difference was observed between atopic and

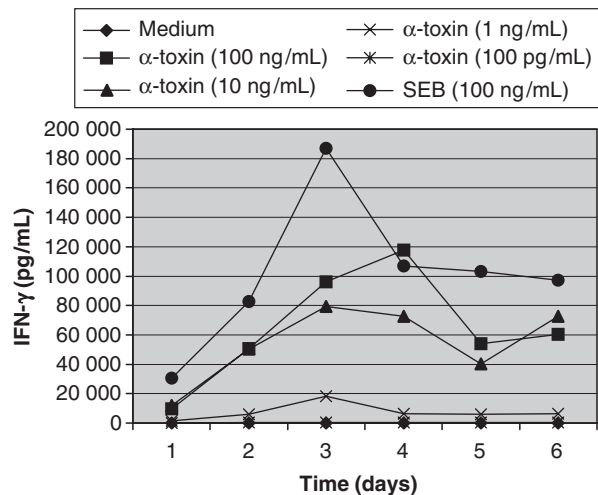


Fig. 4. Time-dependent secretion of IFN- γ upon incubation with α -toxin. Peripheral blood mononuclear cells (PBMC) of a healthy individual were stimulated with different concentrations of α -toxin and staphylococcal enterotoxin B (SEB) for 1–6 days. Representative experiment out of two.

non-atopic individuals indicating that α -toxin may induce these effects also in AD patients. α -toxin induced also a concentration-dependent secretion of IFN- γ in isolated CD4⁺ T cells (Fig. 3b), but the IFN- γ secretion induced in PBMC from the same donors was higher. In contrast, incubation of PBMC with α -toxin lead to only marginal secretion of IL-4, IL-5 and IL-13 which was in the pg/mL range as measured in the cell culture supernatants by ELISA and which was not significantly higher than the medium control (data not shown).

IL-12R signalling is a well-known stimulus for IFN- γ induction in T cells. To clarify whether the higher α -toxin-induced up-regulation of IFN- γ in PBMC compared with isolated CD4⁺ T cells was indirectly caused by IL-12 from monocytes, we stimulated isolated human monocytes with different α -toxin concentrations after pre-incubation with 20 ng/mL IFN- γ for 2 h. This stimulation did not induce intracellular IL-12 production as assessed by flow cytometry (three independent experiments, data not shown). As expected, 50 ng/mL lipopolysaccharide used as a second signal instead of α -toxin induced a marked expression of IL-12 as previously described [26]. In addition, no significant amounts of IL-18 could be detected by ELISA in the cell culture supernatants of PBMC stimulated with α -toxin (3 independent experiments, data not shown).

α-toxin induces t-bet DNA binding

Isolated CD4⁺ T cells were assessed for the induction of t-bet binding by α -toxin with the EMSA. Figure 5 shows the induction of t-bet binding to the binding site of the IFN- γ gene in response to a treatment with α -toxin at 10 ng/mL for 6 h. The specificity of the induced complexes was confirmed by using mutant oligonucleotides, which did not lead to t-bet binding.

Discussion

S. aureus is a potent trigger factor of cutaneous inflammation in patients with AD and is known to produce a variety of

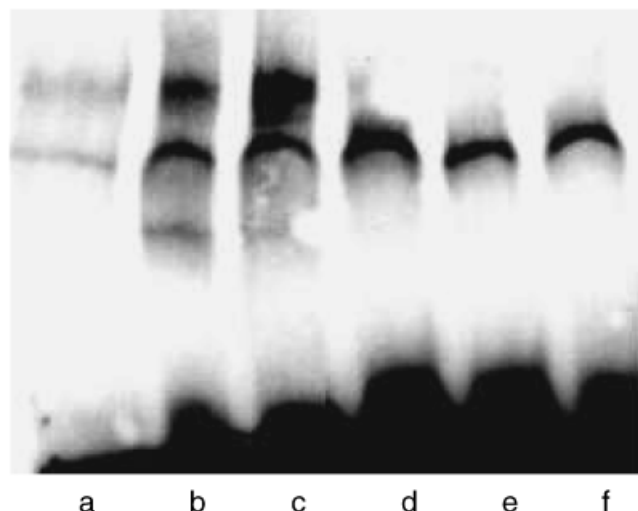


Fig. 5. α -toxin induces T-bet DNA binding activity in $CD4^+$ T cells. DNA binding of t-bet in $CD4^+$ T cells. One representative experiment out of 3. Lane a, d, medium control; lane b, e, anti-CD3/anti-CD28; lane c, f, α -toxin 10 ng/mL; lane a–c, t-bet binding oligonucleotides; lane d–f, mutant oligonucleotides.

exotoxins, among them staphylococcal superantigens able to act also as allergens [11–16, 27, 28]. The high susceptibility to *S. aureus* colonization in these patients might be partly because of a deficiency in the production of antimicrobial peptides such as the human defensins, a component of the innate immune system [29, 30]. The fact that an antimicrobial treatment results in an improvement of eczema in AD patients who are colonized with *S. aureus* strains unable to produce superantigens [10] suggests that other factors than superantigens derived from *S. aureus* may contribute to the development and maintenance of the eczema.

S. aureus α -toxin (α -hemolysin), a cytotoxin which does not belong to the group of enterotoxins (superantigens) is produced by a high percentage of human *S. aureus* isolates [19, 31]. We detected α -toxin producing *S. aureus* strains on the skin of more than 30% of our adult patients with AD, who exhibited no overt evidence of cutaneous infection. Five out of 10 *S. aureus* strains isolated from patients with AD were found to secrete α -toxin in a recent study [22]. Furthermore α -toxin staining of skin sections derived from patients colonized with α -toxin producing *S. aureus* strains showed a distribution of α -toxin in the epidermis and the upper dermis, a pattern which suggests penetration of α -toxin into the skin. There it may come into contact with various inflammatory cells, including T cells, professional and non-professional antigen-presenting cells. The distribution of α -toxin in skin sections resembles that of SEB upon cutaneous application [16]. As $CD4^+$ T cells comprise 80% of inflammatory cells in lesional skin of AD, we were interested in the effects of α -toxin on T cells.

Specifically rabbit erythrocytes are prone to lysis by α -toxin and levels of α -toxin as low as 1 μ g are lethal when injected into rabbits intravenously [19, 31]. Monomers of α -toxin have been shown to bind to the membrane and form functional hexameric transmembrane pores [32, 33], resulting in the influx of Na^+ ions, subsequent osmotic swelling and rupture of the cell. Besides these well described mechanism of lysis

caused by high α -toxin concentrations, subcytotoxic concentrations seem to induce proinflammatory effects in a variety of cells involved in inflammatory processes, e.g. monocytes [34], polymorphonuclear cells [35] and endothelial cells [36], presumably because of the alteration of the cellular ion balance following pore formation, specifically because of calcium influx [37].

We found that a high proportion of cells incubated with moderate concentrations (i.e. 1–10 μ g/mL) of α -toxin for 0.5–24 h were in late-phases of apoptosis or necrotic whereas low concentrations (i.e. 100 pg–100 ng/mL) of α -toxin did not lead to cell death, which suggests that low concentrations of α -toxin do either not lead to the formation of pores in T cells or the cells are able to recover from the attack of α -toxin as it has been shown for human fibroblasts [38, 39]. The induction of apoptosis in T cells by α -toxin has been described earlier. Consistent with our results, T cells underwent apoptosis after incubation with subcytotoxic α -toxin doses exceeding 100 ng/mL [20, 40].

In this study, we show for the first time that α -toxin may not only induce apoptosis and necrosis, but may also activate human T cells in the absence of antigen-presenting cells when used at concentrations lower than those required for the induction of apoptosis or necrosis. After incubation of isolated $CD4^+$ T cells with low α -toxin concentrations a strong concentration and time-dependent proliferation could be detected indicating activation of T cells to an extent comparable with that induced by SEB. We used microarray analysis to screen for potential proinflammatory effects of α -toxin and found that IFN- γ was induced in $CD4^+$ T cells and PBMC. This up-regulation was markedly higher in relation to other genes, which were only marginally up-regulated when compared with IFN- γ . Induction of IFN- γ was confirmed at the mRNA level using quantitative real time PCR. Further, we could show that IFN- γ secretion in $CD4^+$ T cells was induced in a time and concentration-dependent manner. In contrast, only marginal secretion of Th2 cytokines like IL-4, IL-5 or IL-13 was induced by sublytic α -toxin concentrations. In addition, we found a strong T-bet binding activity upon incubation of human T cells with α -toxin. The transcription factor T-bet is critically involved into the regulation of Th1 like cytokine responses and controls the expression of the Th1 cytokine IFN- γ by direct trans-activation of the ifng promoter and induction of chromatin remodelling of the endogenous ifng locus [8]. Incubation of $CD4^+$ T cells with α -toxin was followed by DNA binding of T-bet protein, comparable with TCR signalling through anti-CD3/anti-CD28 binding. The induction of IFN- γ by sublytic concentrations of α -toxin has not been described yet and indicates that this toxin might play a critical role in chronification of eczema.

The mechanisms underlying the described effects in T cells induced by subcytotoxic α -toxin concentrations are not clear. The presence of antigen-presenting cells was not a prerequisite for the activation of $CD4^+$ T cells as firstly, our $CD4^+$ cell preparation contained less than 0.2% monocytes, and secondly, proliferation was also induced in T cell lines which were virtually free of monocytes. This points to a direct effect of α -toxin on T cells. Pore formation and subsequent ion flux may activate signalling events and lead to proliferation and secretion of IFN- γ .

Proliferation of cells incubated with α -toxin and also up-regulation of IFN- γ were more pronounced in PBMC than in isolated CD4⁺ T cells, which points to the fact that factors derived from monocytes, B cells, NK cells or CD8⁺ cytotoxic/helper cells may increase the response of CD4⁺ T cells to α -toxin. As we were not able to detect effects on monocytes by a microarray focusing on genes with known relevance to inflammation and direct analysis of IL-12 and IL-18, other lymphocyte populations besides T helper cells may be involved in the stronger induction of IFN- γ . Moreover, we could rule out that an indirect effect caused by monocytes present in the PBMC preparation is responsible for the strong up-regulation of IFN- γ .

Taken together, sublytic concentrations of α -toxin are able to activate T lymphocytes to divide and to induce IFN- γ on both the mRNA and the protein level by involvement of t-bet DNA binding, thus representing a possible factor for the induction of a TH1 response and chronification of eczema. Further studies have to clarify the mechanism of T cell activation by *S. aureus* α -toxin.

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