

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Macrophages from patients with atopic dermatitis show a reduced CXCL10 expression in response to staphylococcal α -toxin

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atopic dermatitis; chemokines; human macrophages; IP-10/CXCL10; MDC/CCL22; psoriasis; *Staphylococcus aureus*; α -toxin.

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Abstract

Background: Patients with atopic dermatitis (AD) are frequently colonized with *Staphylococcus aureus* (*S. aureus*), one-third of them producing α -toxin, which is correlated with the severity of eczema in AD. *Staphylococcus aureus* colonizes in patients with psoriasis as well. Distinct expression of chemokine (C-C motif) ligand (CCL) and chemokine (C-X-C motif) ligand (CXCL) chemokines has been documented in both diseases. In this study, we investigated the effects of sublytic α -toxin concentrations on human macrophages that accumulate in the skin of patients with AD and psoriasis.

Methods: IFN- γ -induced protein of 10-kDa (IP-10)/CXCL10 and macrophage-derived chemokine (MDC)/CCL22 production were evaluated at the mRNA or at the protein level using qRT-PCR or ELISA, respectively. Cell surface markers' expression and chemotaxis were determined by flow cytometry and Boyden chamber technique, respectively.

Results: Sublytic concentrations of α -toxin strongly induced CXCL10 in macrophages at both the mRNA and the protein levels and significantly up-regulated MHC class II expression. Supernatants of α -toxin-stimulated macrophages induced the migration of human CD4⁺ lymphocytes via the CXCL10 receptor (CXCR3). Macrophages from patients with AD produced lower levels of CXCL10 compared to cells from patients with psoriasis as well as healthy controls in response to α -toxin. α -Toxin did not lead to a large variation in CCL22 production in macrophages from all three groups.

Conclusions: Staphylococcal α -toxin contributes to Th1 polarization by induction of CXCL10 in macrophages. Macrophages from patients with AD and psoriasis responded to α -toxin in the induction of Th1-related chemokine CXCL10 diversely, which could favour the recruitment of distinct leucocyte subsets into the skin.

Abbreviations

AD, atopic dermatitis; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; HLA, human leucocyte antigen; IFN- γ , interferon gamma; IL, interleukin; IP-10, IFN- γ -induced protein of 10 kDa; LPS, lipopolysaccharide; MDC, macrophage-derived chemokine; MHC, major histocompatibility complex; PASI, Psoriasis Area and Severity Index; RT, reverse transcription; *S. aureus*, *Staphylococcus aureus*; SEB, Staphylococcal enterotoxin B; Th, T helper; TNF- α , tumour necrosis factor alpha.

Atopic dermatitis (AD) and psoriasis are common chronic inflammatory skin disorders in which T-cell-mediated mechanisms have a crucial role in pathogenesis (1–3). Patients with AD exhibit exaggerated Th2 responses, and initiation of AD lesions is thought to be mediated by means of early skin infiltration of Th2 lymphocytes releasing high levels of IL-4, IL-5 and IL-13 (1, 4). Subsequently, the accumulation of activated monocytes, mature dendritic cells and eosinophils determines a rise in IL-12 expression and the appearance of a mixed Th2/Th1 cytokine pattern, with

reduced IL-4 and IL-13 and the presence of IFN- γ in the chronic phase (1, 4). By contrast, psoriasis is characterized by numerous activated Th1 cells, focal intra-epidermal collections of neutrophils, and dermal accumulation of macrophages and dendritic cells. IFN- γ -producing Th1 cells dominate psoriatic lesions and are primarily responsible for epidermal changes (3, 5). Recently, the pathogenesis of psoriasis has evolved from a classical type 1 (Th1) disease activated by IFN- γ to include a new T-cell subset, Th17 cells, as well (3).

Recent studies demonstrated that infiltration of inflammatory cells into tissues is regulated by chemokines. Two main subfamilies, recently renamed chemokine (C-C motif) ligand (CCL) and chemokine (C-X-C motif) ligand (CXCL) chemokines, have been distinguished (6). Several studies have suggested a crucial involvement of CCL chemokines in allergic inflammation because they potently attract eosinophils, basophils, monocytes and T-cell subsets (6). By contrast, CXCL chemokines attract primarily Th1 lymphocytes and neutrophils. Chemokines and their receptors are implicated in the development of symptoms of AD and psoriasis (6–8). A subset of chemokines including CCL1, CCL11, CCL13, CCL17, CCL18, CCL22 and CCL27 are highly expressed in AD (6, 7), whereas a distinct chemokine network such as CCL2, CCL3, CCL4, CCL5, CCL17, CCL19, CCL20, CCL27, CXCL7, CXCL8, CXCL9 and CXCL10 plays a role in pathogenesis of psoriasis (8, 9). Patients with AD and psoriasis are frequently colonized with *Staphylococcus aureus* (10, 11).

A distinct percentage of *S. aureus* strains are able to produce α -toxin, a potent 33-kDa cytotoxin which does not belong to the group of enterotoxins (superantigens) (12). In a former study, we found 30% α -toxin-producing *S. aureus* strains isolated from the skin of untreated patients with AD (13). More recently, we investigated 127 patients with AD who were undergoing standard anti-inflammatory and anti-septic treatment, and found a skin colonization of 63% with α -toxin-producing *S. aureus* in these patients (14). In psoriasis, Marples et al. (15) detected *S. aureus* in nonlesional skin of 27% of patients and in lesional skin of 46% of patients. Tomi et al. (11) 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%. It has been shown that staphylococcal toxigenic strains isolated from both lesional skin and nares of patients with psoriasis were significantly higher than those of healthy controls. A significant relationship between PASI scores and toxin production was also demonstrated (11, 16). Macrophage infiltration is a hallmark of inflammation, especially in the skin where they can mediate chronic inflammation by producing cytokines and chemokines. This is seen in several inflammatory dermatoses including psoriasis and AD (17).

The effects of α -toxin on human macrophages in general and in chronic skin inflammation in particular still remain unclear. We show here for the first time that sublytic α -toxin induces the Th1-related chemokine CXCL10 in

human macrophages. Moreover, we studied the effects of α -toxin on Th1- and Th2-related chemokines in macrophages from patients with AD and psoriasis where the intrinsic abnormal and different chemokines production profile is well defined.

Materials and methods

Patients

Peripheral blood samples were taken from healthy donors and patients with AD or psoriasis after obtaining informed consent, respectively, presented to our outpatient and inpatient department, as previously described (18). Atopic dermatitis was determined by the diagnostic criteria described by Hanifin & Rajka (19). Further information is provided in Online Repository (OR).

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep density-gradient centrifugation from healthy donors and from patients with AD and psoriasis as previously described in OR. To culture macrophages, CD14⁺ cells were purified by negative selection according to the manufacturers' instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in 'macrophage medium'. Further information is provided in OR.

Stimulation of macrophages

Macrophages were either unstimulated or stimulated for various periods of time with lyophilized staphylococcal α -toxin (Sigma-Aldrich, Deisenhofen, Germany) in sublytic concentrations, recombinant human IFN- γ (10 ng/ml; ImmunoTools, Friesoythe, Germany) or TNF- α (20 ng/ml; ImmunoTools). Lipopolysaccharide (LPS) was not detected in any reagent, as determined by the Limulus amoebocyte assay (Haemochrom Diagnostika, Essen, Germany).

FACS analysis

Flow cytometric analysis was performed as described previously (18). Further information is provided in OR.

Cell viability assay

Approximately 3×10^5 macrophages were prestimulated with α -toxin in a dose-dependent manner. Cell viability was assessed by flow cytometry by staining with 7-amino-actinomycin D (7-AAD) (BD Biosciences, Heidelberg, Germany) after 10–15 min to determine the percentage of viable cells that were treated with α -toxin in a dose-dependent manner. 7-amino-actinomycin D intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Viability of cells with

<1 μ g/ml α -toxin stimulation was not affected in macrophages. Therefore, we chose <1 μ g/ml for our investigations. Figure S1 shows one representative of five independent experiments.

mRNA isolation, reverse transcription, quantitative RT-PCR and microarray experiments

RNA isolation, reverse transcription and quantitative real-time PCR were performed as previously described (18). Further information is provided in OR.

Microarray experiments were performed with the human inflammation microarray (Ocimum Biosolutions, Corporate Office Europe, IJsselstein, The Netherlands), which contains 136 gene probes for inflammatory genes and 19 gene probes for housekeeping genes (13, 20, 21).

Cytokine assessment by ELISA

Cell-free culture supernatants were harvested and analysed for CXCL10 or CCL22 (each Duo Set; R&D-Systems, Minneapolis, MN, USA), using a commercially available enzyme-linked immunosorbent assay (ELISA) system following the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was analysed as described previously (21). Further information is provided in OR.

Chemotaxis assay

The chemotactic activity of supernatants from α -toxin-stimulated macrophages on lymphocytes (CD4+ T cells) was determined using a Boyden chamber technique as previously described (21). Further information is provided in OR.

Western blot

A total of 10^6 macrophages were prestimulated with α -toxin (100 ng/ml), IFN- γ (10 ng/ml), TNF- α (10 ng/ml) or LPS, which was derived from *Escherichia coli* serotype 055:B5 (500 ng/ml; Sigma-Aldrich) for various periods of time (5, 15, 30, 60 min or 2, 4, 6, 24 h). Cell extracts were subjected to Western blot analysis as described previously (21). Further information is provided in OR.

Statistical analysis

Data were analysed with either the Student's t-test (normality test passed) or the Mann-Whitney rank sum test (normality test failed). *P*-values below 0.05 were regarded as significant. *P* < 0.05 is depicted with *, *P* < 0.01 with ** and *P* < 0.001 with ***. The program GraphPad prism version 3.02 (GraphPad Software Inc., San Diego, CA, USA) and the software Sigma stat for Windows (Systat Software, San Jose, USA) were used for statistical analysis.

Results

Induction of IP-10/CXCL10 by staphylococcal α -toxin in human macrophages

To screen for the potential effects of sublytic α -toxin concentrations that did not induce cell death (Fig. S1), we investigated the expression of a selected set of 136 genes with known relevance to inflammation (13, 20, 21), such as cytokines, chemokines and matrix metalloproteinases in purified macrophages derived from healthy controls which had been treated with α -toxin (100 ng/ml) for 24 h. Microarray analysis revealed that CXCL10 was the most strongly up-regulated gene in macrophages (215.1-fold induction) (Table S1). In the same experimental set-up, the mean ratio for CXCL9, CCL8 and CCL15 were 11.6, 8.8 and 3.8, respectively. An increased expression was not observed for other chemokines (including CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL20, CCL27, CXCL3, CXCL5 and CXCL8). The mean ratio for all these chemokines was between 0.2 and 2 when comparing not stimulated with α -toxin-stimulated macrophages (Table S1). CXCL10 expression and secretion following α -toxin stimulation were investigated at the mRNA level as determined by quantitative RT-PCR (Fig. 1) as well as at the protein level as determined by ELISA (Fig. 2). As shown in Fig. 1A, an efficiency-controlled quantitative real-time PCR for CXCL10 confirmed the microarray results. A marked up-regulation of CXCL10 was observed both after 6 and 24 h following α -toxin stimulation at the mRNA level in macrophages derived from healthy controls (Fig. 1). Low concentrations of α -toxin (10 ng/ml) also up-regulated CXCL10 in macrophages derived from healthy controls (data not shown). Higher concentrations of α -toxin (10, 20 μ g/ml) led to reduction in the expression of CXCL10 dramatically, which was because of

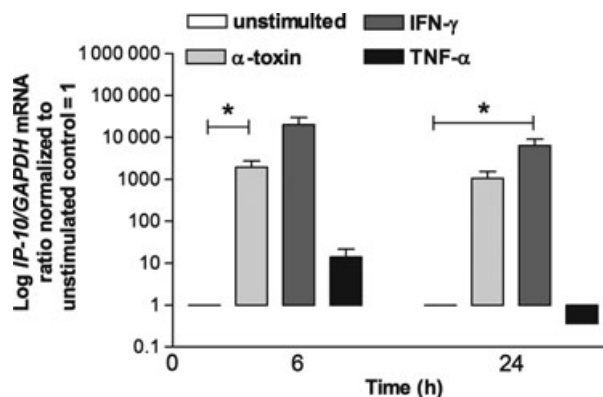


Figure 1 Induction of chemokine (C-X-C motif) ligand (CXCL)10 following α -toxin stimulation in human macrophages at the mRNA level. Cells were stimulated with α -toxin (100 ng/ml), IFN- γ (10 ng/ml) or TNF- α (20 ng/ml) for 6 and 24 h respectively. qRT-PCR was employed to determine CXCL10 mRNA expression. Data are shown as mean CXCL10/GAPDH ratio + SEM of *n* = 9 independent experiments. **P*-value less than 0.05 was compared with that in the unstimulated control.

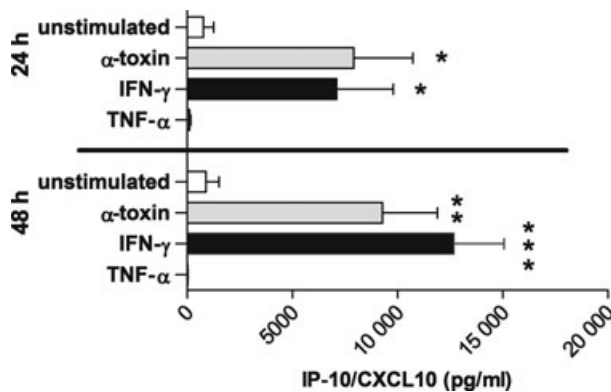


Figure 2 Induction of chemokine (C-X-C motif) ligand (CXCL)10 following α -toxin stimulation in human macrophages at the protein level. Cells were left either unstimulated or stimulated with α -toxin (100 ng/ml), IFN- γ (10 ng/ml) or TNF- α (20 ng/ml) for 24 and 48 h. Cell culture supernatants were measured for CXCL10 secretion, using an ELISA. The mean value $\pm$ SEM of $n = 10$ experiments is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ were compared with those in the unstimulated control.

cell death (data not shown). IFN- γ and TNF- α , which have been shown previously to induce CXCL10 in human monocytes (22), were used as positive controls. α -Toxin was a stronger inducer of CXCL10 compared with TNF- α but not with IFN- γ at mRNA level (Fig. 1). As shown in Fig. 2, our mRNA data could also be reproduced by ELISA at the protein level: A higher amount of CXCL10 was already observable after 24 h stimulation and with α -toxin, which was stable after 48 and 120 h (Fig. S2A). This induction was observable in a dose-dependent manner (10, 50, 100 ng/ml, 1 μ g/ml) in macrophages from healthy controls (Fig. S2B). α -Toxin induced CXCL10 secretion in a similar manner to IFN- γ , whereas TNF- α was ineffective in induction of CXCL10 at the protein level (Fig. 2). To compare *in vitro* data with *ex vivo* findings, we used 3-mm punch biopsies from healthy individuals and cultured them in keratinocyte growth medium with or without α -toxin (100 ng/ml) or IFN- γ (100 ng/ml). After 24 h of incubation, formalin-fixed and 5- μ m paraffin-embedded biopsy sections were stained for CXCL10 as well as CD68. CXCL10 staining was more prominent in the dermis in α -toxin-stimulated biopsies as compared to unstimulated biopsies. IFN- γ was used as a positive control for CXCL10 staining. CD 68 staining points to infiltration of macrophages in the dermis (Fig. 3).

α -Toxin-stimulated macrophages could induce the migration of human CD4 $^{+}$ T cells via CXCR3

The biological activity of CXCL10 induced by staphylococcal α -toxin was examined by means of a chemotaxis assay (Boyden chamber). As shown in Fig. 4A, supernatants of α -toxin-stimulated macrophages induced significantly the migration of CD4 $^{+}$ T cells. CD4 $^{+}$ T lymphocytes were pretreated with a blocking anti-CXCR3 antibody known to

neutralize the biological activity of surface CXCR3, or IFN- γ -induced protein of 10-kDa (IP-10) secretion was directly blocked using an anti-IP-10 antibody. Both inhibitors abrogated the migration induced by α -toxin-treated macrophage supernatants (supernatants from unstimulated macrophages attracted a mean of 267 000 T cells, and supernatants from α -toxin [100 ng/ml]-stimulated macrophages attracted a mean of 360 000 cells compared with 193 000 or 237 000 cells when pretreated with neutralizing CXCR3 or IP-10 antibodies, respectively) (Fig. 4B). α -Toxin in cell culture medium without macrophages did not directly induce the migration of human CD4 $^{+}$ lymphocytes. Medium free of cells was used as a negative control (Fig. 4B). IL-27-stimulated keratinocytes that were shown to induce CXCL10 were used as a positive control (data not shown) (21). Isotype controls of neutralizing antibodies did not abrogate the migration induced by α -toxin-treated macrophages (data not shown).

α -Toxin could up-regulate HLA-DR (MHC class II) expression in macrophages

α -Toxin induced a moderate up-regulation of MHC class II on the cell surface of macrophages (Fig. S3). This effect was visible on cells from healthy donors and from patients with AD or psoriasis (data not shown). No effect was detected on CD14 $^{+}$, CD64 $^{+}$, CD80 $^{+}$, and CD86 $^{+}$ expression (data not shown).

Low effect of α -toxin on CXCL10 induction (Th1-related chemokine) in macrophages from patients with AD

Macrophages from patients with AD and psoriasis as well as from healthy controls were either left unstimulated or were stimulated with 10, 50, 100 ng/ml or 1 μ g/ml α -toxin, respectively, for the indicated time points. To compare α -toxin effects in CXCL10 production with other previously described inducers, cells were stimulated with IFN- γ or TNF- α as well.

CXCL10 expression and secretion following α -toxin stimulation were investigated at the mRNA level as determined by quantitative RT-PCR as well as at the protein level as determined by ELISA. α -Toxin increased CXCL10 expression as well as secretion in macrophages from patients with AD and psoriasis and from healthy controls compared with the appropriate medium control both at the mRNA (Figs 5A and S4) and at the protein levels (Fig. 5B–D). However, α -toxin induced significantly lower levels of CXCL10 expression (Figs 5A and S4) or secretion (Fig. 5B–D) in patients with AD compared with healthy controls as well as psoriasis at all time points and doses tested. There was no significant difference in CXCL10 induction between psoriasis and healthy controls following α -toxin stimulation (Fig. 5A–D). In contrast to α -toxin, IFN- γ strongly enhanced CXCL10 secretion in AD as well (Fig. 5C and D).

IFN- γ that was used as a positive control stimulus was a stronger inducer of CXCL10 in macrophages from patients with psoriasis compared to healthy controls and patients with

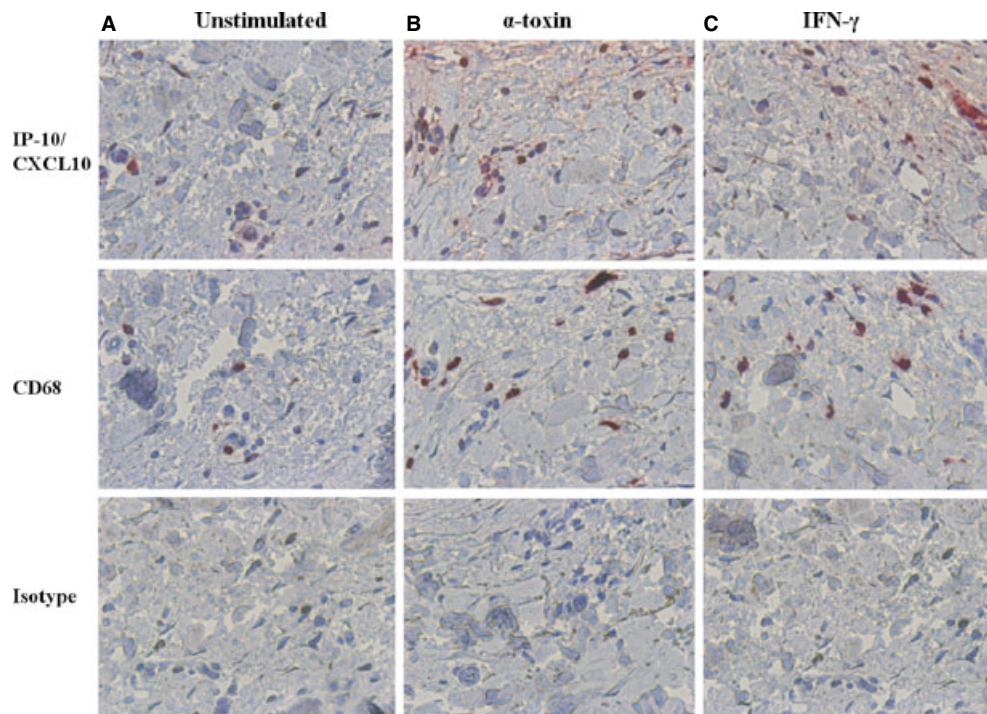


Figure 3 IFN- γ -induced protein of 10-kDa/chemokine (C-X-C motif) ligand (CXCL)10 expression in macrophages from the skin of healthy controls. Punch biopsies (3 mm) from healthy individuals were left either unstimulated (A) or stimulated with α -toxin (100 ng/ml) (B) or IFN- γ (100 ng/ml) (C) for 24 h at 37°C. 5- μ m paraffin sec-

tions were stained for CXCL10 along with appropriate isotype as well as CD68. A representative set of CXCL10 and CD68 staining of two independent experiments is depicted. Original magnification was X400.

AD by trend (Fig. 5C and D). No significant effect of TNF- α on CXCL10 production was found in macrophages from all three groups (Fig. 5C and D).

Effect of α -toxin on MDC production (Th2-related chemokine) in macrophages from patients with chronic inflammatory skin diseases

Based on the effect of α -toxin on Th1-related chemokine (IP10/CXCL10) production in macrophages, we investigated the Th2-related chemokine (macrophage-derived chemokine, MDC/CCL22) production.

We chose CCL22 that plays a crucial role in pathogenesis of AD. We recently showed that IL-13-stimulated human primary keratinocytes (HPKs) express CCL22, which preferentially attract CD4⁺ CCR4⁺ T cells. Furthermore, CD4⁺ CCR4⁺ T cells from patients with AD showed higher chemotactic indices towards IL-13-stimulated HPKs than those from healthy individuals (23).

Macrophages from patients with AD as well as patients with psoriasis showed an intrinsically higher CCL22 production compared to healthy controls by trend. On the whole, α -toxin did not regulate CCL22 secretion. A slight increase of 17% of CCL22 was observed in patients with AD and psoriasis, while there was a slight decrease (significant) in CCL22 secretion in cells from HC (Fig. 6).

There was no significant difference in CCL22 production following α -toxin stimulation between patients with AD and psoriasis (Fig. 6).

Discussion

Atopic dermatitis and psoriasis are the most common immune-mediated chronic inflammatory skin diseases with frequent colonization with *S. aureus* which correlates with disease severity (10, 11). More recently, we described a skin colonization of 63% with α -toxin-producing *S. aureus* in patients with AD undergoing anti-inflammatory and antiseptic treatment (14). In higher concentrations, α -toxin may lead to necrosis in a wide range of human cells by forming small pores (1–2 nm diameter) into cell membranes (13). Besides this well-described mechanism of α -toxin lysis (24), subcytotoxic concentrations of α -toxin seem to induce pro-inflammatory (IL-1 β and TNF- α production) effects in a variety of cells involved in inflammatory processes, for example monocytes (25), polymorphonuclear cells (26) and endothelial cells (27), presumably because of the alteration in the cellular ion balance following pore formation, specifically because of calcium influx (28). Previously, we showed that sublytic concentrations of α -toxin were able to effectively activate T lymphocytes, which led to proliferation and induction of IFN- γ (13).

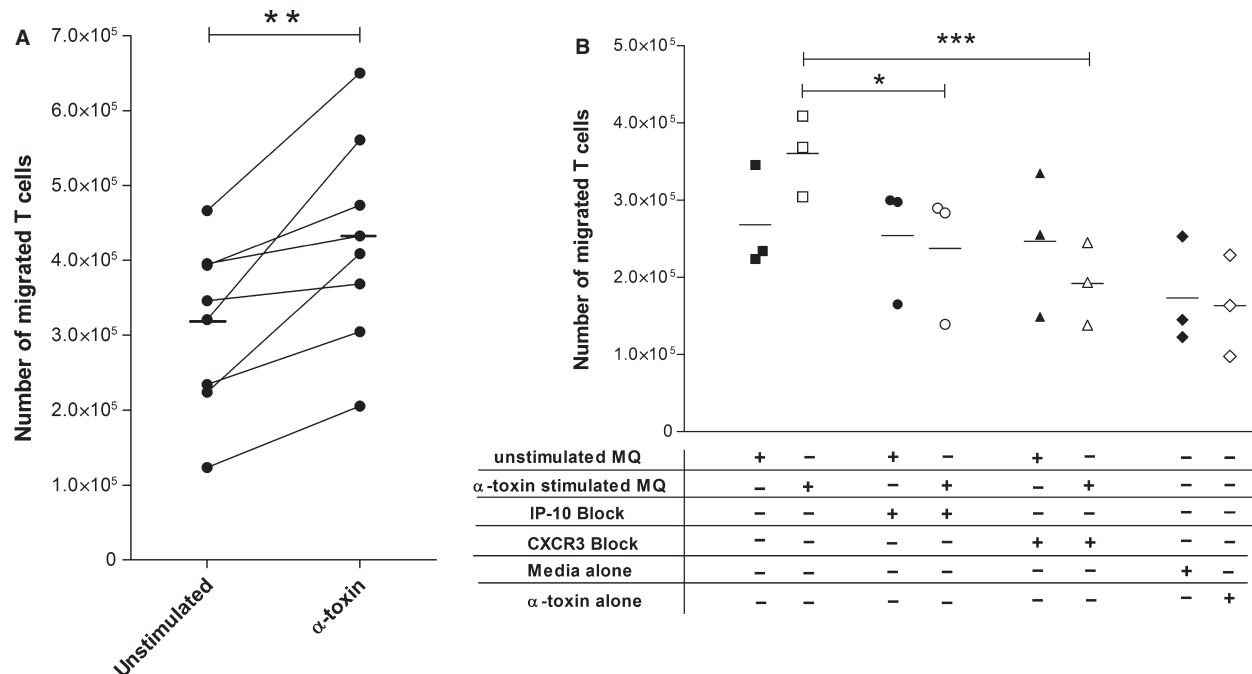


Figure 4 Chemotactic activity of α -toxin-stimulated macrophages and inhibition of chemokine (C-X-C motif) ligand (CXCL)10-induced chemotaxis using a neutralizing anti-CXCL10 or anti-CXCR3 Ab. (A and B) Macrophages were left either unstimulated or stimulated with α -toxin (100 ng/ml) for 24 h, and supernatants of unstimulated or α -toxin-stimulated macrophages were used in chemotaxis assays

($n = 8$) (A). (B) α -toxin-induced chemotaxis was completely abolished using a neutralizing anti-CXCL10 or anti-CXCR3 Ab that was incubated with CD4⁺ T cells for 30 min and at 2 μ g/ml or 10 μ g/ml, respectively. The number of migrated CD4⁺ T cells is depicted. α -Toxin and media free of cells alone were used as negative controls ($n = 3$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

In this study, we demonstrate for the first time an inflammatory effect of α -toxin on induction of the Th1-related chemokine CXCL10 both *in vitro* and *ex vivo* and up-regulation of HLA-DR expression in human macrophages. The biological activity of α -toxin-induced CXCL10 which was measured by means of a chemotactic assay and blocking experiments was confirmed by resulting in the attraction of CXCR3⁺ Th1 cells as well. Moreover, the lack of up-regulation of the Th2-related chemokine CCL22 following α -toxin stimulation is in favour of a role of α -toxin in human Th1 responses. This indicates that besides pore-forming abilities, sublytic concentrations of α -toxin have immunological properties and therefore may serve as a pathogenic factor in chronic inflammatory skin diseases.

CXCL10 is secreted by activated T cells, monocytes, endothelial cells, keratinocytes and fibroblasts (22, 29). It belongs to the CXC chemokine subfamily and acts through CXCR3 receptors that attract T lymphocytes (22, 29, 30).

Very few studies have investigated the production pattern of CXCL10 in skin-homing cells other than keratinocytes (29, 30), such as macrophages, or their role in chronic inflammatory skin diseases such as AD and psoriasis.

One study investigated the mechanisms regulating CXCL10 production in the human monocytic cell line (THP-1 cells) following treatment with IFN- γ and TNF- α alone or in combination (22). They showed that TNF- α has more

potential than IFN- γ to induce CXCL10 production in human THP-1 monocytes. In addition, IFN- γ synergistically enhances TNF- α -induced production of CXCL10 by activating signal transducer and activator of transcription factor (STAT)-1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) through Janus-activated kinases (JAKs) pathways (22).

In contrast, we showed that IFN- γ and α -toxin are indeed stronger inducers of CXCL10 production compared to TNF- α both at the mRNA and at the protein levels in human macrophages. Based on the strong induction of CXCL10 following α -toxin stimulation, we investigated possible signalling pathways that are involved in CXCL10 production including STAT-1, extracellular signal-regulated kinase (ERK) and NF- κ B (22). However, we failed to detect activation of these molecules following α -toxin stimulation compared with not treated macrophages or using positive controls such as IFN- γ , TNF- α or LPS.

α -Toxin could not activate STAT-1, ERK 1/2 or NF- κ B compared with unstimulated macrophages, whereas IFN- γ that was used as a positive control for STAT-1, and TNF- α or LPS which was used as a positive control for ERK1/2 or NF- κ B activated clearly ERK1/2 and NF- κ B, respectively (Fig S5A–C). Several previous studies have shown that TNF- α induced phosphorylation and degradation of I κ B- α , thereby induced phosphorylation and nuclear translocation of NF- κ B

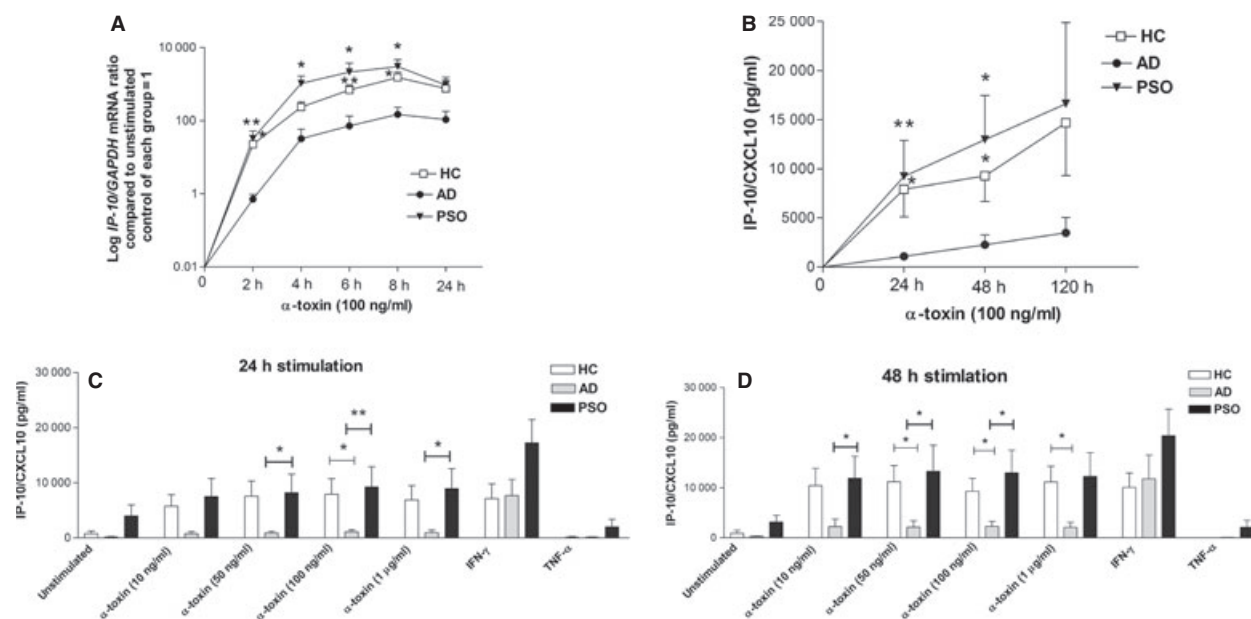


Figure 5 Comparison of chemokine (C-X-C motif) ligand (CXCL10) induction in macrophages from patients with chronic inflammatory skin diseases following α -toxin stimulation. Macrophages from patients with atopic dermatitis (AD) ($n = 7$) and psoriasis (PSO) ($n = 6$) as well as from healthy controls (HC) ($n = 7$) were left either unstimulated (medium control) or stimulated with α -toxin as indicated or with IFN- γ (10 ng/ml) or TNF- α (20 ng/ml), respectively, for

the indicated periods of time. (A) qRT-PCR was employed to determine CXCL10 mRNA expression. (B–D) Cell-free culture supernatants were quantified for CXCL10 secretion by ELISA. (A) Data are shown as mean CXCL10/GAPDH ratio $\pm$ SEM compared to unstimulated control of each group and (B–D) are shown the mean CXCL10 value in pg/ml $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. P values are compared with those in patients with AD.

depending on the cell type (22). However, we failed to detect α -toxin-induced phosphorylation and degradation of I κ B- α in macrophages compared with unstimulated or TNF- α -treated cells as well (data not shown). Moreover, the c-JUN–JNK complex has been implicated in the regulation of CXCL10 gene expression (31). We also investigated the possible involvement of c-Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) signalling pathway in CXCL10 induction following α -toxin stimulation. In our experiments, staphylococcal α -toxin did not activate c-JUN as well as JNK phosphorylation in human macrophages (data not shown). The intracellular mechanism of cell activation by sublytic doses of proteins forming small pores such as α -toxin is not clear yet. Both direct actions on toll-like receptors on cell membranes and intracellular activities such as the activation of caspase-1 through NLRP-3 inflammasomes have been discussed (32, 33).

Chemokines and their receptors are implicated in the pathogenesis and maintenance of AD. The imbalance in serum concentration of Th1- and Th2-derived chemokines is one of the factors involved in pathogenesis of AD.

However, discrepancies obtained in many studies regarding the imbalance in chemokine serum levels in patients with AD and relatively small number of the patients included did not allow drawing equivocal conclusions (29, 34, 35). Narbutt et al. (34) found significant lower serum concentration of CXCL9, CXCL10 and CCL17 and higher concentrations

of CXCL12, CCL22 and CCL27 in patients with AD compared to healthy controls.

These findings are in line with our observations that show the alteration of the Th1 axis in AD following α -toxin stimulation and might support Th2 responses.

An interesting observation of this study is that macrophages from patients with AD produced similar amounts of CXCL10 in response to IFN- γ but reduced amounts of CXCL10 in response to α -toxin when compared with macrophages from healthy controls or from patients with psoriasis. In contrast, macrophages from patients with psoriasis responded to the same stimuli with an exaggerated expression of CXCL10 production. These results may indicate that in addition to the general existence of genetically determined defects in the constitutive and induced chemokine production in patients with AD and psoriasis, this abnormal production appeared under a stimulus-specific control rather than complex chemokine-associated. Giustizieri et al. showed that CXCL10 was markedly expressed in the epidermis of patients with psoriasis vulgaris but only weakly and limited to some areas in AD lesions. They concluded that the higher expression of CXCL10 in the epidermis of patients with psoriasis compared with that of patients with AD likely reflects the presence of more numerous Th1 cells in the former, because IFN- γ is the most potent inducer of CXCL10 (30). In addition to this explanation, our data indicate that different responses to the staphylococcal exotoxin α -toxin in

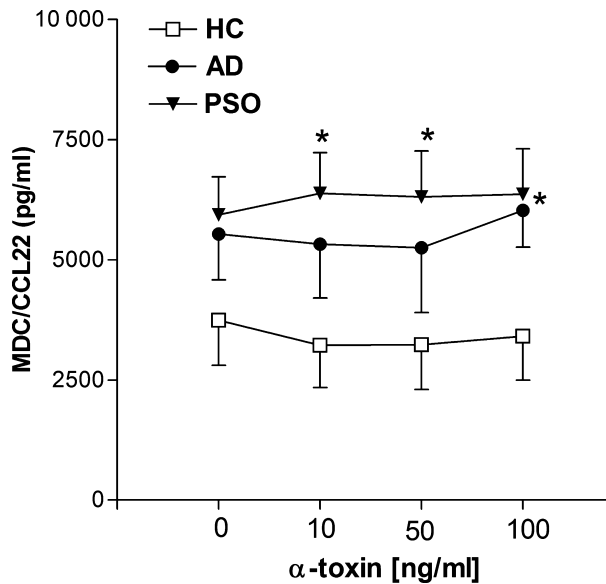


Figure 6 Comparison of chemokine (C-C motif) ligand (CCL)22 secretion in macrophages from patients with chronic inflammatory skin diseases following α -toxin stimulation. Macrophages from patients with atopic dermatitis (AD) ($n = 7$) and psoriasis (PSO) ($n = 6$) as well as from healthy controls (HC) ($n = 7$) were left either unstimulated or stimulated with α -toxin (10, 50 and 100 ng/ml) for 24 h. Cell culture supernatants were measured for CCL22 secretion, using an ELISA. Data are shown as mean values $\pm$ SEM. * $P < 0.05$. P values are compared with those in HC.

macrophages could be another factor that may play a role in the distinct pathogenesis of AD and psoriasis in CXCL10 induction.

Staphylococcal α -toxin contributes to Th1 polarization by induction of CXCL10 in macrophages. Thereby, it provides pathways linking adaptive and innate immune functions. In conclusion, our data support the hypothesis that the contribution of macrophages in the pathogenesis of AD and psoriasis is linked to the presence of distinct alterations in their capacity to respond to the staphylococcal exotoxin α -toxin and that these abnormalities can modulate the amplification

and persistence of chronic skin inflammation. Further studies should be performed to clarify the mechanisms of macrophage activation by staphylococcal α -toxin.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Data S1. Methods.

Figure S1. Cell viability in macrophages is not impaired following sublytic α -toxin ($< 1 \mu\text{g/ml}$) stimulation.

Figure S2. Induction of CXCL10 following α -toxin stimulation in human macrophages at the protein level.

Figure S3. Expression of HLA-DR following α -toxin stimulation. Cells were either left unstimulated or stimulated with α -toxin (100 ng/ml) for 24 h.

Figure S4. Comparison of CXCL10 induction in macrophages from patients with chronic inflammatory skin diseases following α -toxin stimulation at the mRNA level.

Figure S5. Signalling pathways induced by α -toxin in human macrophages.

Table S1. Induction of chemokine genes in α -toxin-treated macrophages.

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Conflict of interest

The authors state no conflict of interest.

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Figure S1:

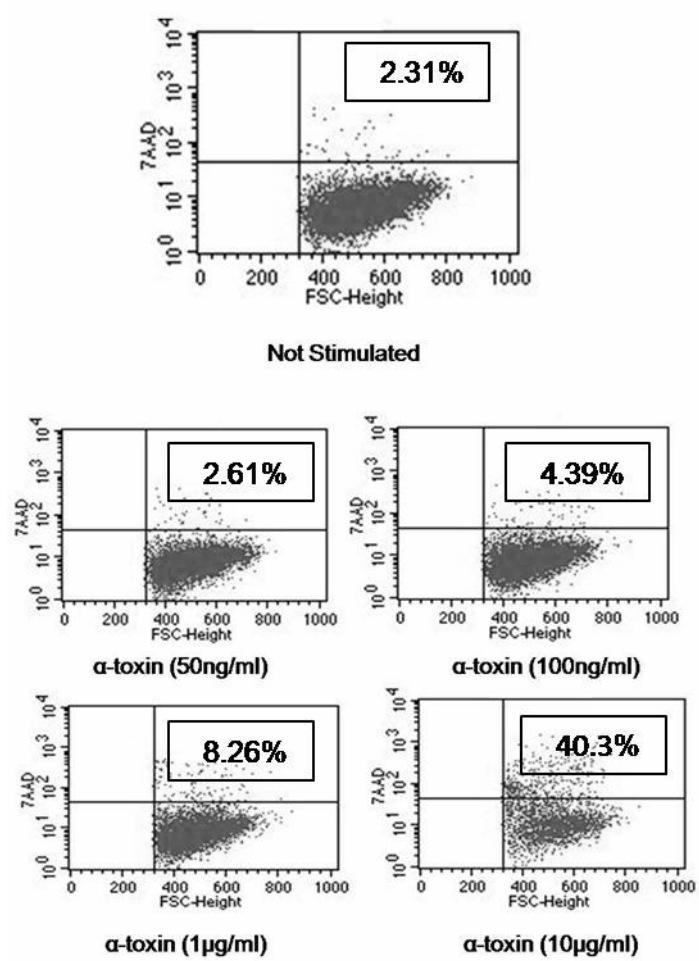


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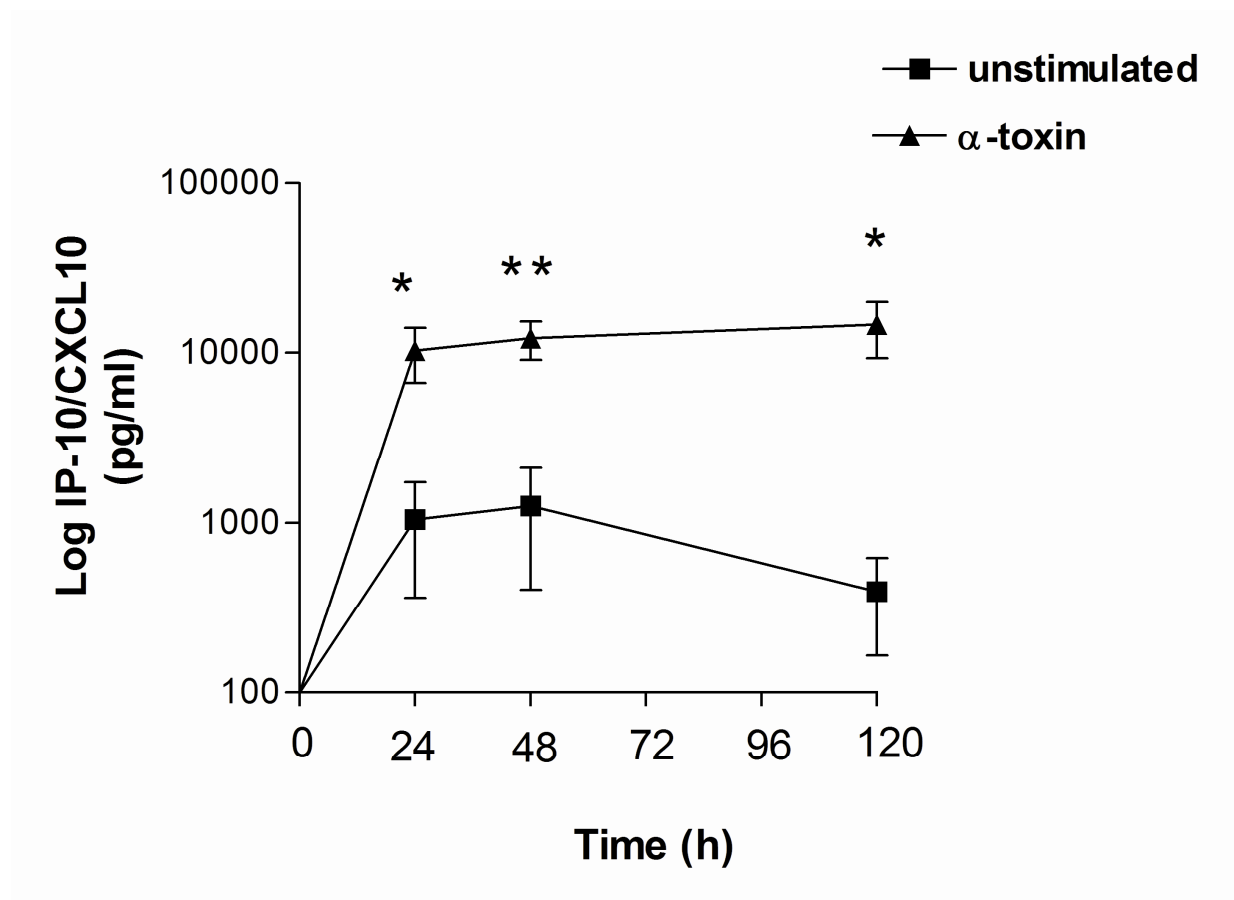


Figure S2B:

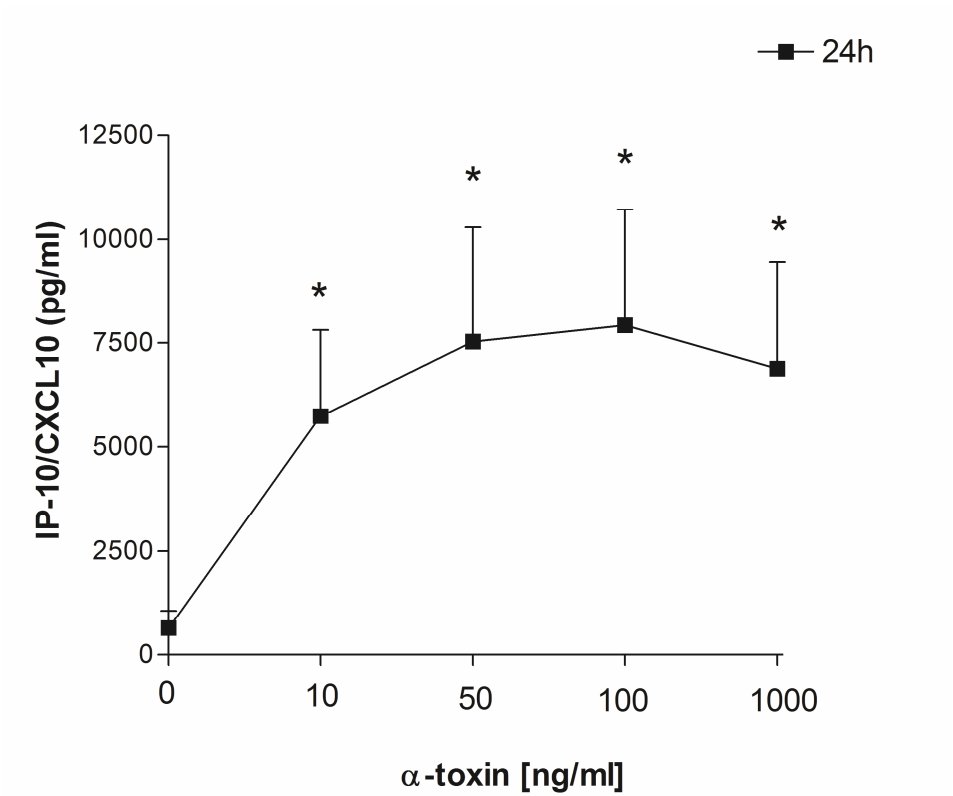


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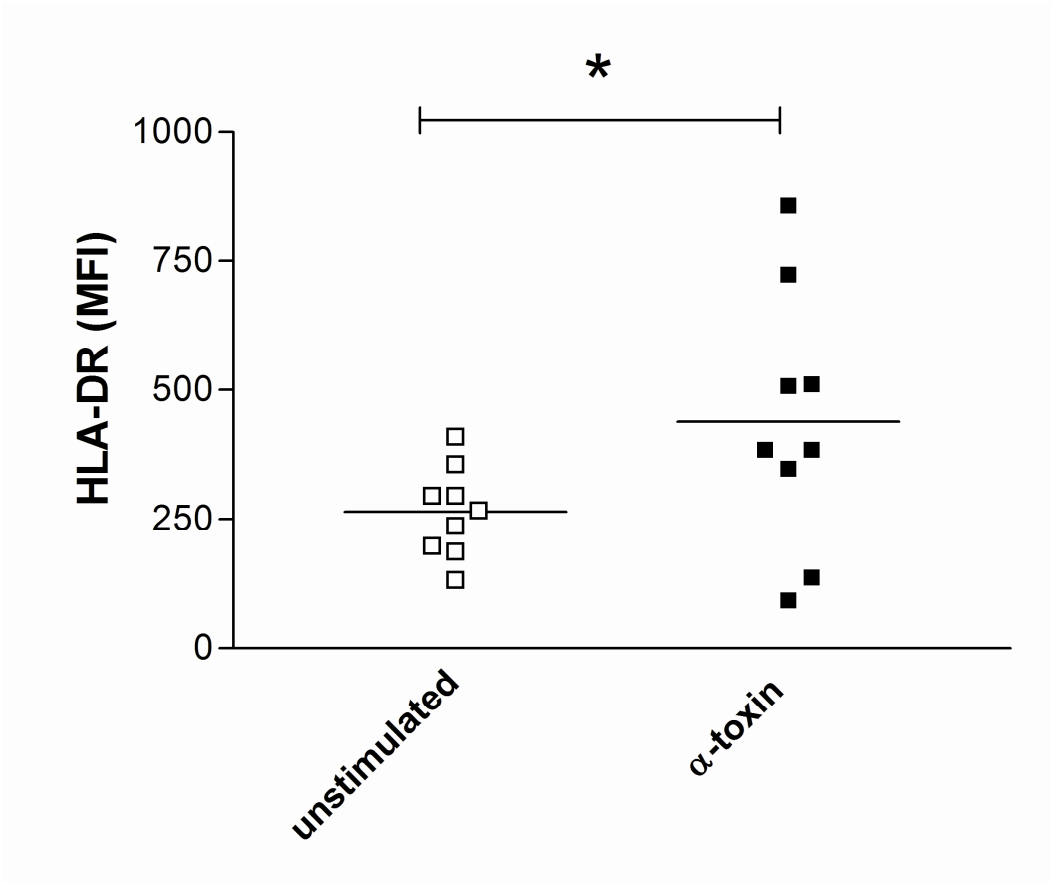


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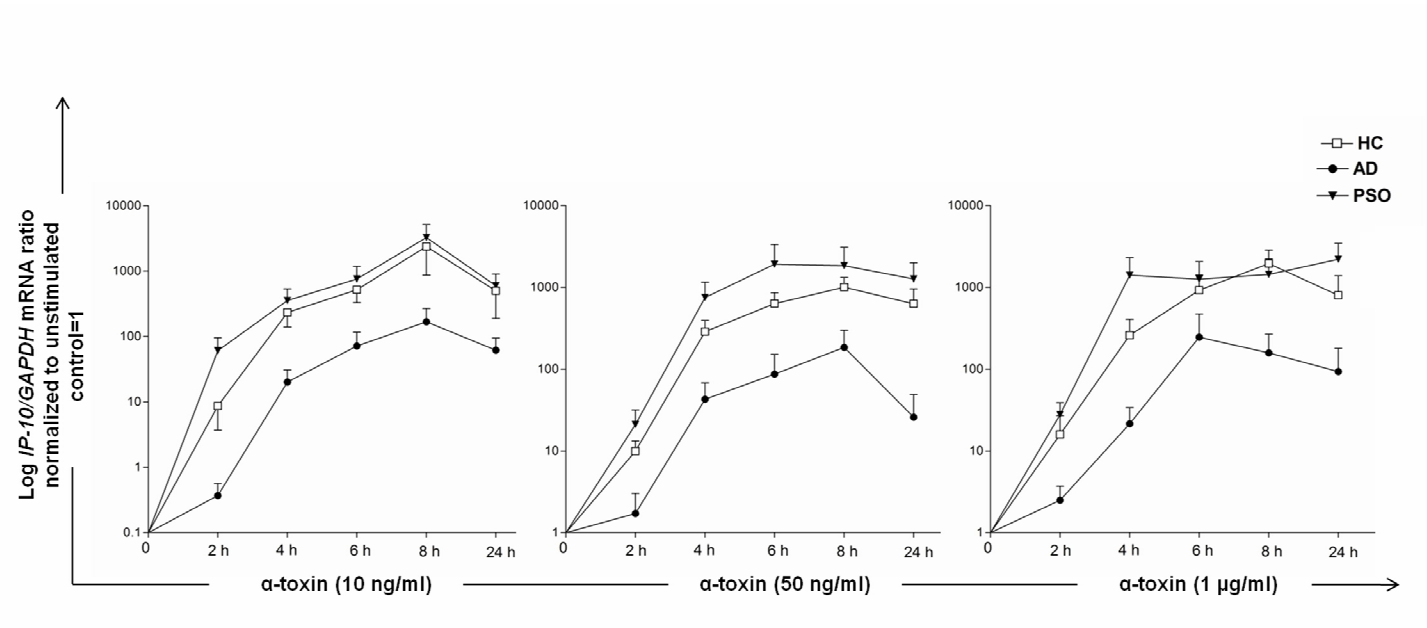


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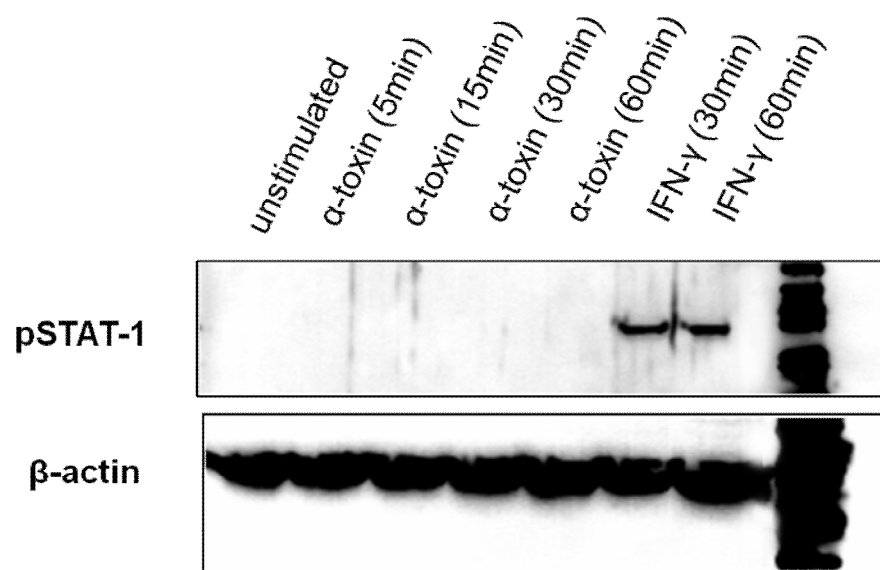


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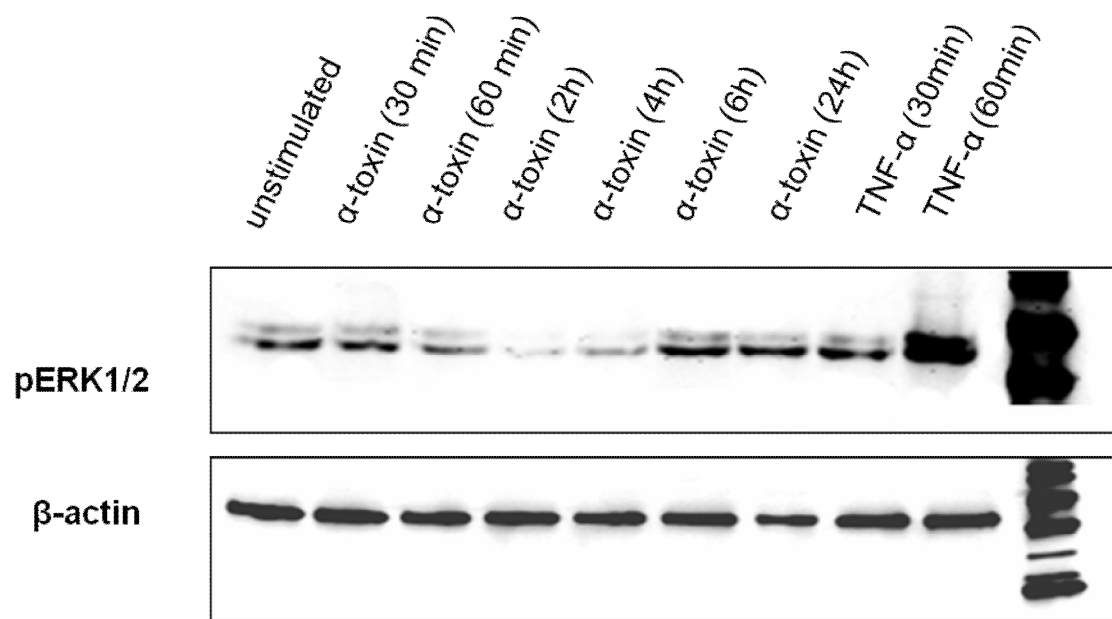
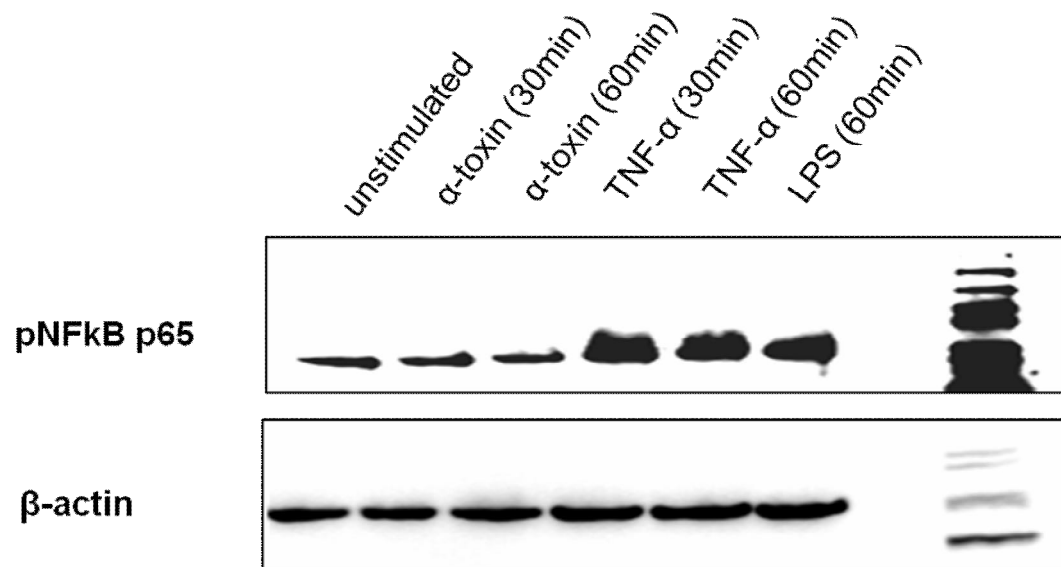


Figure S5C:



ONLINE SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1:

Cell viability in macrophages is not impaired following sublytic α -toxin ($< 1 \mu\text{g/ml}$) stimulation. Cell viability was accessed with 7-Amino-Actinomycin D (7-AAD) by flow cytometry. Cells were either left unstimulated or were stimulated with 50 ng/ml, 100 ng/ml, 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ α -toxin, respectively. One representative 7-AAD staining following stimulation with α -toxin out of 5 independent experiments is shown

Supplementary Figure S2:

Induction of CXCL10 following α -toxin stimulation in human macrophages at the protein level. Cells were left either unstimulated or were stimulated with α -toxin (100 ng/ml) (**A**) over period of 24h, 48h or 120h or with (**B**) different doses of α -toxin (10 ng/ml, 50 ng/ml or 1 $\mu\text{g/ml}$). Cell culture supernatants were measured for CXCL10 secretion, using an ELISA. The mean value $\pm$ SEM of $n = 7$ (**A**), $\pm$ SEM of $n = 10$ (**B**) experiments is shown. * $P < 0.05$, ** $P < 0.01$ are compared with those in the unstimulated control.

Supplementary Figure S3:

Expression of HLA-DR following α -toxin stimulation. Cells were either left unstimulated or stimulated with α -toxin (100 ng/ml) for 24h. The mean fluorescence intensity (MFI) of HLA-DR positive cells ($n = 9$) is shown. * $P < 0.05$ compared to medium control.

Supplementary Figure S4:

Comparison of CXCL10 induction in macrophages from patients with chronic inflammatory skin

diseases following α -toxin stimulation at the mRNA level. Macrophages from patients with atopic dermatitis (AD) (n = 3) and psoriasis (PSO) (n = 6) as well as healthy controls (HC) (n = 7) were left either unstimulated (medium control) or were stimulated with α -toxin (10 ng/ml, 50 ng/ml or 1 μ g/ml). Data are shown as mean CXCL10 / GAPDH ratio + or - SEM compared to unstimulated controls.

Supplementary Figure S5:

Signalling pathways induced by α -toxin in human macrophages. Phospho-STAT (pSTAT) 1 (**A**), phospho-ERK1/2 (pERK1/2) (**B**), phospho-NF κ B (pNF κ B) (**C**) and β -actin levels were determined by means of Western blot analysis after α -toxin, IFN- γ or TNF- α stimulation. Approximately 10^6 macrophages were left either unstimulated or were prestimulated with α -toxin (100 ng/ml) (**A-C**), IFN- γ (10 ng/ml) (**A**), TNF- α (20 ng/ml) (**B and C**) or LPS (500 ng/ml) (**C**) as indicated. Cellular extracts were normalized on the basis of protein determination. One representative experiment of 3 independent experiments from different donors is shown.

ONLINE SUPPLEMENTARY TABLE LEGEND

Supplementary Table 1:

Induction of chemokine genes in α -toxin-treated macrophages. Gene expression in macrophages (n = 3) subjected to α -toxin (100 ng/ml) treated cells was compared to that of unstimulated cells and is expressed by the indicated ratios of normalized fluorescence intensities. The complete set of data can be obtained from the corresponding author. ¹ Fold induction equals α -toxin signal relative to the signal generated by not stimulated (NS) control.

ONLINE SUPPLEMENTARY METHODS

METHODS

Patients

For healthy controls, total IgE levels as well as specific IgE against the common aeroallergens (sx-1: birch, elder, hazel, grass, rye, cat and dog epithelia, Cladosporium sp. and Dermatophagoides sp.) were measured (CAP system; Pharmacia, Freiburg, Germany) and were below the normal limits of 100 and 0.35 kU/I, respectively.

Patients did not receive any systemic treatment for AD and psoriasis, respectively, during the two weeks before blood drawing. The study was approved by the local ethics committee and was in accordance with the protocols of the Declaration of Helsinki.

Cell Culture

Macrophages were cultured in macrophages medium which included RPMI medium 1640 supplemented with 10% heat-inactivated FCS (Gibco, Eggenstein, Germany), 2 mM glutamine (Seromed, Berlin, Germany), 1% penicillin/streptomycin (Seromed, Berlin, Germany) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (10 ng/ml; Berlex Pharmaceutical Company, Montville, NJ, USA) for 5-7 days. Before initiation of further experiments, cells were analysed by two-colour flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany) for contaminating CD3⁺ T cells, CD20⁺ B cells and CD56⁺ natural killer (NK) cells. CD14⁺, CD64⁺ and CD68⁺ macrophages had a purity of at least 88%. In order to isolate CD4⁺ T cells from PBMCs in indicated experiments, they were purified by

negative selection kit according to the manufacturers' instructions (Miltenyi Biotec). Isolated T cells had a purity of at least 97%.

mRNA isolation, reverse transcription, quantitative RT-PCR

mRNA was isolated from 1×10^5 macrophages either unstimulated or stimulated for 2, 4, 6, 8 and 24 h with α -toxin (20, 10, 1 μ g/ml, 100, 50 and 10 ng/ml), 6, 24 h with IFN- γ (10 ng/ml) or 6, 24h with TNF- α using the High Pure mRNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Reverse transcription was performed using the First Strand cDNA Synthesis Kit (MBI Fermentas, St Leon-Rot, Germany). Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals) using SYBR Green with Quantitect primer assays for CXCL10 (QT01003065) and glyceraldehyde-3-phosphate dehydrogenase GAPDH (QT00079247), according to manufacturer's instructions (Qiagen, Hilden, Germany). The following settings were used: an initial activation step of 15 min at 95° C with ramp 20° C/s was followed by three-step cycling (45 cycles): denaturation 15 s, 94° C; annealing 20 s, 55° C; extension 20 s, 72° C (all three with ramp 2° C/s). Melting curve analysis was performed from 60–90° C with ramp 20° C/s. The amount of CXCL10 relative to the reference GAPDH was quantified using the Relative Quantification Software (Roche Molecular Biochemicals).

FACS Analysis

Macrophages (approximately $2-3 \times 10^5$ per well) were washed and resuspended in phosphate-buffered saline (PBS) containing 0,2% gelatine, 20mM sodium azide and 10 μ g/ml heat-

aggravated human immunoglobulin G (IgG) (Sigma, Deisenhofen, Germany) to block FcR for 15 minutes. Subsequently, cells were incubated with the following directly conjugated fluorescent-labelled monoclonal antibodies, on ice for 30 min: CD3-fluorescein isothiocyanate (FITC) (BD Biosciences, Heidelberg, Germany), CD20-FITC (Immunotech, Marseille, France), CD80-FITC (Immunotech, Marseille, France), CD11b-FITC, CD14-FITC, CD36-FITC, CD16-phycoerythrin (PE), CD54-PE, CD56-PE, CD64-PE, CD86-PE (BD Biosciences, Heidelberg, Germany) and CD68-FITC (Biolegend, San Diego, USA) and human leucocyte antigen (HLA)-DR-PE (BD Biosciences) or isotype-matched controls (Sigma, Deisenhofen, Germany). Stained cells were washed three times, fixed in PBS with 1% paraformaldehyde and analysed by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany).

Immunohistochemistry

The *ex vivo* 3 mm punch biopsies from healthy skin were incubated overnight in keratinocytes culture medium alone or were stimulated with α -toxin (100 ng/ml) or IFN- γ (100 ng/ml) at 37° C. The next day, biopsies were embedded in paraffin, thereafter they were embedded in paraffin and 5 μ m sections were prepared for CXCL10 staining, the fixed sections were treated with Target Retrieval Solution (DakoCytomation, Hamburg, Germany); monoclonal mouse anti-human IP-10 (1) (Santa Cruz Biotechnology, Heidelberg, Germany) at 2 μ g/ml, dilution 1:100 and as isotype control mouse IgG2b (Sigma-Aldrich, Munich, Germany) at 2 μ g/ml were used. For CD68 staining, the fixed sections were treated with Target Retrieval Solution (DakoCytomation) as well; monoclonal mouse anti-human CD68 (DakoCytomation) at 1:70 dilution was used. Staining was performed according to the manufacturer's instructions using the EnVision G/2 Singlestain System (DakoCytomation).

Chemotaxis assay

In brief, Boyden chambers (Nucleopore, Tübingen, Germany) were filled with the supernatant of α -toxin-stimulated or unstimulated macrophages, α -toxin or media alone and covered with polycarbonate filters (pore size, 8 μ m; Nucleopore). A 100- μ L volume of a lymphocyte suspension containing 10^6 cells was added to each chamber. After incubation for 3 hours at 37°C, migrated cells in the lower part of the Boyden chambers were lysed by adding 0.1% Triton-X-100, and β -glucuronidase activity in the lysates was determined photometrically by using p-nitrophenyl β -glucuronide as a substrate (all from Sigma-Aldrich). Readings were performed with the FLUOstar Optima instrument (BMG Labtech, Offenburg, Germany) with a 405-nm emission wavelength. For calculation of the number of migrated cells, which is equivalent to β -glucuronidase activity determined in the lower parts of the Boyden chambers, values were calculated from a standard curve by using known numbers of lymphocytes. The assays were performed in triplicates. For blocking experiments where indicated, lymphocytes were pre-treated for 30 minutes with an anti-CXCR3 antibody at 10 μ g/ml (clone 49801, R&D Systems) known for its neutralization capacity of human cell-surface CXCR3-mediated bioactivity or were pre-treated with monoclonal anti-CXCL10 antibody at 2 μ g/ml (clone 33036, R&D Systems) which neutralize 50% of the bioactivity due to 0.2 μ g/mL of CXCL10. IgG1 (R&D Systems) at 10 μ g/ml and 2 μ g/ml was used as isotype control of neutralizing antibodies respectively.

Western blot

Cells were homogenized in M-Per Mammalian Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor (complete mini and

PhosSTOP, Roche Molecular Biochemicals, Mannheim, Germany) After centrifugation (3000g for 10 min at 4°C), the protein concentration in the supernatants was determined with a protein assay (Bio-Rad, Munich, Germany). A total of 250 µg of protein per sample were separated on a 4–20% SDS-polyacrylamide gel (Precise Protein Gels, Thermo Fisher Scientific) under reducing condition by using SDS-PAGE running conditions (Tris-HEPES-SDS Running Buffer, Thermo Fisher Scientific). The semidry system was used to transfer proteins to a nitrocellulose membrane (30 min at 18V and room temperature). Membranes were incubated in blocking buffer composed of Tris-Buffered Saline (Bio-Rad); 0.1% Tween, and 5% non fat milk powder (2 hours at room temperature; Roth GmbH, Karlsruhe, Germany). First antibody incubation was followed with TBS/Tween 0.1% containing 5% BSA (overnight at room temperature, Sigma-Aldrich). Rabbit anti-phospho-STAT-1 (Tyr70I) (1:1000), rabbit anti-phospho-ERK1/2 (p44/42) (Thr202/Tyr204) (1:1000), rabbit anti-phospho-NF-κB p65 (Ser536) (1:1000), rabbit anti-phospho-IκB-α (Ser32) (1:1000), rabbit anti-phospho-c-JUN (Ser63) (1:1000) and rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1000) were used to detect phosphorylated STAT-1, ERK1/2, NF-κB p65, IκB-α, c-JUN and SAPK/JNK respectively. β-actin mouse mAb (1:1000) was used as a reference protein, followed by a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:2000). All antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA. Second antibody incubation was performed in blocking buffer (see above) for 1 hour at room temperature. Immunoreactivity was detected using a chemiluminescence kit according to the instructions of the manufacturer (Roche Applied Science, Mannheim, Germany).

Supplementary Table 1:

Encoded protein	Systematic name	Fold induction (NS/ α -toxin) ¹
MCP-1	CCL2	1.2
MIP-1 α	CCL3	1.2
MIP-1 β	CCL4	1.5
RANTES	CCL5	2
MCP-3	CCL7	1.8
MCP-2	CCL8	8.8
Eotaxin	CCL11	1
MIP-5	CCL15	3.8
LARC	CCL20	0.9
CTACK	CCL27	1
GRO γ	CXCL3	1
ENA-78	CXCL5	0.2
IL-8	CXCL8	0.9
MIG	CXCL9	11.6
IP-10	CXCL10	215.1