

# IL-22 and TNF- $\alpha$ represent a key cytokine combination for epidermal integrity during infection with *Candida albicans*

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T cells exercise their full impact on target cells through a combination of secreted cytokines. The recently described T helper cell subset Th22 is characterized by a combinatorial secretion of IL-22 and TNF- $\alpha$ . Here, we demonstrate that IL-22 increases the TNF- $\alpha$ -dependent induction and secretion of several immune-modulatory molecules such as initial complement factors C1r and C1s, antimicrobial peptides S100A7 and HBD-2 (human  $\beta$  defensin 2), and antimicrobial chemokines CXCL-9/-10/-11 in primary human keratinocytes. The synergism of IL-22 and TNF- $\alpha$  is transmitted intracellularly by MAP kinases and downstream by transcription factors of the AP-1 family. The induction of innate immunity is relevant in an in vitro infection model, where keratinocytes stimulated with Th22 supernatants or recombinant IL-22 plus TNF- $\alpha$  effectively inhibit the growth of *Candida albicans* and maintain survival of epithelia. Accordingly, the combinatorial stimulation of keratinocytes with IL-22 and TNF- $\alpha$  most efficiently conserves the integrity of the epidermal barrier in a three-dimensional skin infection model as compared with IFN- $\gamma$ , IL-17, IL-22 or TNF- $\alpha$  alone. In summary, we demonstrate that IL-22 and TNF- $\alpha$  represent a potent, synergistic cytokine combination for cutaneous immunity.

**Keywords:** *Candida albicans* · Epidermal integrity · IL-22 · TNF- $\alpha$

## Introduction

The T helper cell family was recently expanded by the discovery of the so-called Th22 cells by five independent groups [1–5].

Th22 cells belong to a new class of leukocytes with little or no direct impact on other immune cells, but selective effects on epithelia. This characteristic functional profile of Th22 cells is mediated by distinct cytokines. Th22 cells lack production of IFN- $\gamma$ , IL-4 and IL-17, but they secrete TNF- $\alpha$  and their lead cytokine IL-22 [4]. IL-22 is a glycoprotein belonging to the IL-10 family [6], which binds to a heterodimer of the IL-10 receptor  $\beta$

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(IL-10R $\beta$ ) and the IL-22 receptor (IL-22R) [7]. While IL-10 R $\beta$  is widely expressed, IL-22R expression is limited to epithelial cells, thus ensuring tissue-specific effects of IL-22. Highest expression of IL-22R has been reported on epithelial cells of the gastrointestinal system and the skin [8]. In line with that observation, Th22 cells are enriched in the skin of inflammatory disorders such as atopic eczema and psoriasis [1, 4]. However, the functional role for Th22 cells in the skin is unknown to date. Recombinant IL-22 inhibits differentiation, induces migration and enhances proliferation of keratinocytes [9, 10]. Furthermore, IL-22 induces antimicrobial peptides such as  $\beta$  defensin 2 and S100 proteins [11]. In the context of the discovery of Th22 cells, we have recently shown first evidence for a further important functional property of IL-22. Th22 cells induce genes belonging to the innate immune response in primary human keratinocytes, and this induction is dependent on the synergistic action of TNF- $\alpha$  and IL-22 [4].

The aim of this study was to investigate the molecular mechanisms underlying the synergism of TNF- $\alpha$  and IL-22 and the functional impact of this synergistic effect. It is demonstrated that IL-22 and TNF- $\alpha$  act on primary human keratinocytes via synergistic induction of MAP kinases and transcription factors of the AP-1 family, and that this induction results in an effective protection of the epidermal barrier after infection with *Candida albicans*.

## Results

### IL-22 and TNF- $\alpha$ synergistically induce an innate immune profile in primary human keratinocytes

In our original description of Th22 clones we have shown first evidence of mRNA induction of genes via a functional interplay of TNF- $\alpha$  and IL-22 on primary human keratinocytes [4]. Table 1 confirms the synergism of TNF- $\alpha$  and IL-22 in the induction of some innate immunity genes in primary keratinocytes obtained from healthy individuals. At protein level, TNF- $\alpha$  induced CXCL-10 secretion in primary keratinocytes ( $n = 6$ ) by ten-fold (Fig. 1A), CXCL-11 by six-fold (Fig. 1B) and HBD-2 by 21-fold (Fig. 1C). In contrast, IL-22 only marginally induced CXCL-10, CXCL-11 and HBD-2. Co-stimulation with IL-22 and TNF- $\alpha$  consistently and significantly enhanced the secretion over the level of an additive effect by 20-fold ( $p \leq 0.001$  versus IL-22/ $p \leq 0.01$  versus TNF- $\alpha$ ) 8,7-fold ( $p \leq 0.001$  versus IL-22/ $p \leq 0.01$  versus TNF- $\alpha$ ) and 41-fold ( $p \leq 0.001$  versus IL-22/ $p \leq 0.001$  versus TNF- $\alpha$ ), respectively. To estimate the biological relevance of this synergistic induction, we also stimulated keratinocytes with known inducers of these proteins. IL-22 and TNF- $\alpha$  stimulation lead to an upregulation of CXCL-10, CXCL-11 and HBD-2 in the same dimension as IFN- $\gamma$  and IL-17 respectively. This synergistic CXCL-10 induction and secretion becomes significant after 36 h (four-fold;  $p \leq 0.05$  versus IL-22/ $p \leq 0.05$  versus TNF- $\alpha$ ) and is maintained over three days (17-fold after 48 h  $p \leq 0.005$  versus IL-22/ $p \leq 0.05$  versus TNF- $\alpha$ ; 42-fold after 72 h;  $p \leq 0.001$  versus

**Table 1.** Synergistic induction of mRNA expression of genes belonging to the innate immune system in primary human keratinocytes by TNF- $\alpha$  and IL-22<sup>a)</sup>

Gene	Fold induction compared with control after stimulation with:		
	IL-22	TNF- $\alpha$	IL-22+TNF- $\alpha$
CXCL-9	1 $\pm$ 0.2	98 $\pm$ 211	238 $\pm$ 19
CXCL-10	2 $\pm$ 0.2	1848 $\pm$ 778	7410 $\pm$ 3232
CXCL-11	1 $\pm$ 0.1	309 $\pm$ 116	1797 $\pm$ 741
C1r	3 $\pm$ 0.4	40 $\pm$ 9	399 $\pm$ 151
C1s	3 $\pm$ 0.1	48 $\pm$ 14	238 $\pm$ 76
S100A7	3 $\pm$ 0.1	3 $\pm$ 0.4	8 $\pm$ 0.7
HBD-2	6 $\pm$ 0.8	359 $\pm$ 74	826 $\pm$ 99

<sup>a)</sup> Keratinocytes ( $n = 10$ ) were stimulated for 12 h with 50 ng/mL of indicated cytokines. Fold induction to unstimulated control keratinocytes is shown.

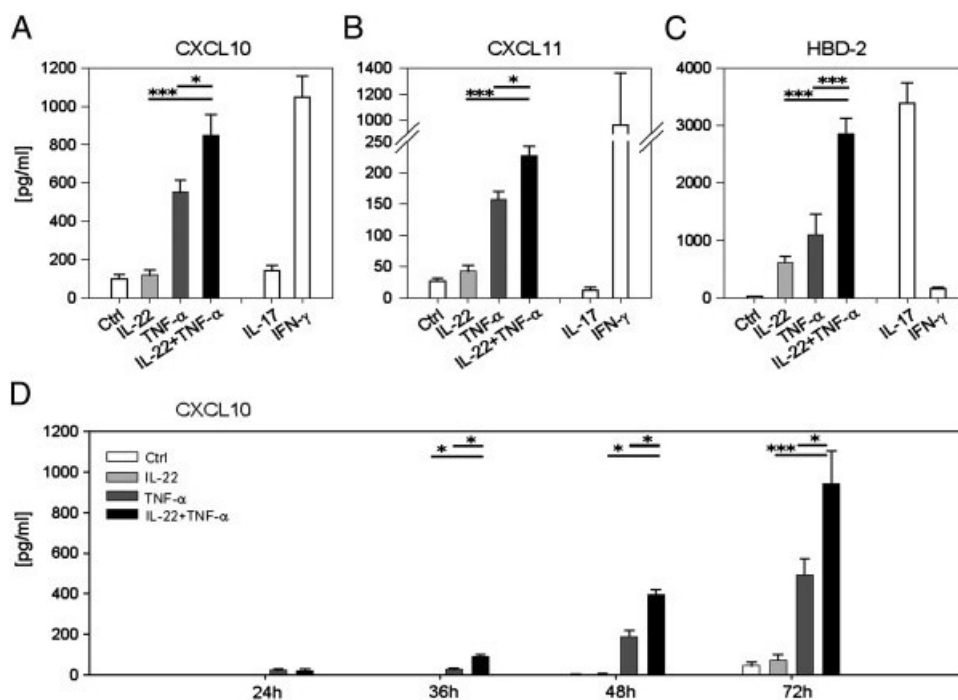
IL-22/ $p \leq 0.01$  versus TNF- $\alpha$ ) (Fig. 1D). Similar results have been obtained for CXCL11 and HBD-2 (data not shown).

### IL-22 and TNF- $\alpha$ synergistically induce MAP kinases in keratinocytes

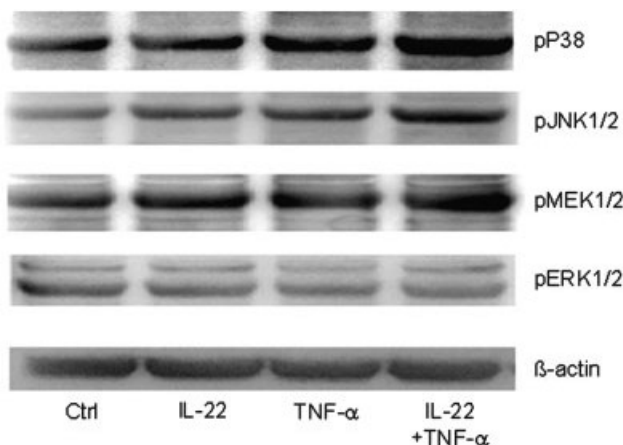
To investigate intracellular mechanisms underlying the synergism in the induction of innate immune genes, key signal transduction in primary keratinocytes was investigated. Since both IL-22 and TNF- $\alpha$  are known to induce several MAP kinases, we investigated the phosphorylation of p38, MEK1/2, ERK1/2 and JNK1/2 in primary human keratinocytes by Western Blot after stimulation with IL-22, TNF- $\alpha$  or a combination of both. TNF- $\alpha$  and IL-22 alone weakly induced the phosphorylation of p38, JNK1/2 and MEK1/2 at 5 min incubation (Fig. 2). ERK1/2 phosphorylation was not altered. The combination of both cytokines synergistically induced the phosphorylation of the investigated MAP kinases with the strongest effect on p38.

### IL-22 and TNF- $\alpha$ promote activation of AP-1 family transcription factors, but not of STAT3 or NF- $\kappa$ B

Since phosphorylation of p38 and other MAP kinases results in activation and translocation of transcription factors belonging to the AP-1 family, we investigated the impact of IL-22 and TNF- $\alpha$  on these transcription factors in primary human keratinocytes. In line with our previous results, sole stimulation with IL-22 or TNF- $\alpha$  weakly induced AP-1 (1.30  $\pm$  0.08 relative luminescence or 1.33  $\pm$  0.1 relative luminescence), as measured by a dual luciferase system. In contrast, co-stimulation with IL-22 and TNF- $\alpha$  resulted in a significant activation of AP-1 (1.84  $\pm$  0.17 relative luminescence, Fig. 3A). To identify single members of the



**Figure 1.** IL-22 reinforces TNF- $\alpha$ -induced innate immunity in keratinocytes. Primary human keratinocytes ( $n = 6$ ) were stimulated with TNF- $\alpha$ , IL-22, IL-17 (50 ng/mL each) and IFN- $\gamma$  (300 U/mL) or a combination of TNF- $\alpha$  and IL-2. Content of (A) CXCL10, (B) CXCL11 and (C) HBD-2 after 72 h, (D) content of CXCL10 over time course in cell-free supernatant was quantified using commercially available ELISA kits. Bars show the mean and SEM of six independent experiments. Asterisks indicate statistical significance. Statistical differences were determined by One-way ANOVA and Bonferroni's Multiple Comparison Test as post test and defined as \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ .



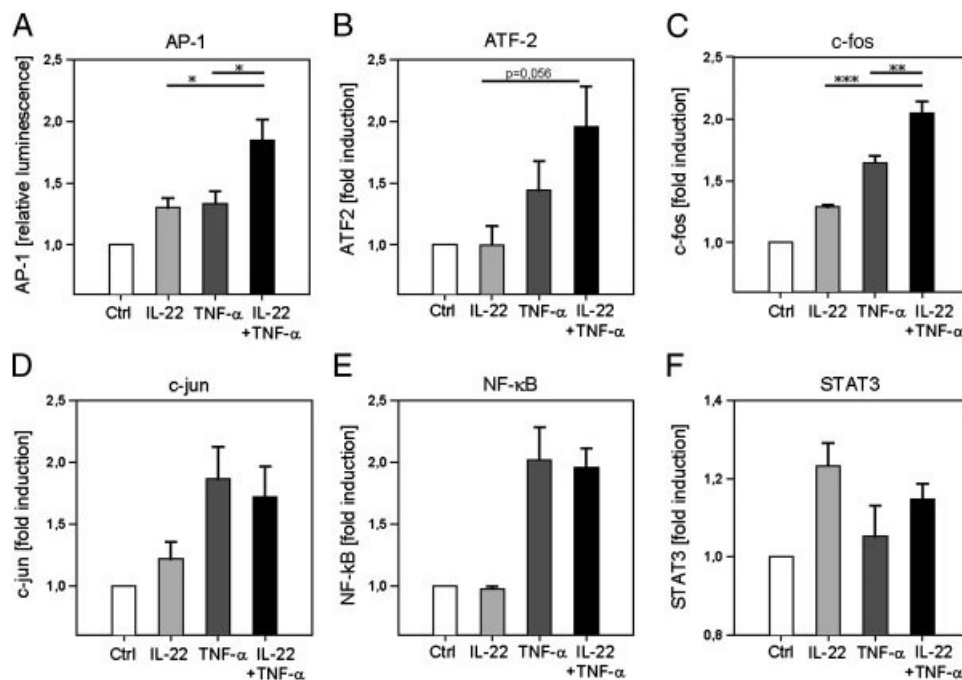
**Figure 2.** The synergism of TNF- $\alpha$  and IL-22 is mediated by the MAP kinase p38. Primary human keratinocytes stimulated with 50 ng/mL TNF- $\alpha$ , 50 ng/mL IL-22 or a combination of both for 5 min were lysed and analyzed by Western blot for phosphorylated p38, JNK1/2, MEK1/2 and ERK1/2 in relation to a housekeeping protein ( $\beta$ -Actin). The Western blot shown is representative of six independent experiments.

AP-1 family, TransAM ELISA systems were used to detect nucleus translocation. TransAM experiments demonstrated that *c-fos* (Fig. 3C) was synergistically induced by IL-22 and TNF- $\alpha$  ( $1.89 \pm 0.17$  fold induction,  $p \leq 0.001$  versus IL-22/ $p \leq 0.01$  versus TNF- $\alpha$ ). ATF-2, another AP-1 family member, showed a non-

significant trend of induction by interaction of both cytokines ( $1.95 \pm 0.33$  fold induction) (Fig. 3B). STAT3 (Fig. 3F) was only induced by IL-22 ( $1.23 \pm 0.06$  fold induction), whereas *c-jun* (Fig. 3D) and NF- $\kappa$ B (Fig. 3E) were only activated by TNF- $\alpha$  ( $1.83 \pm 0.16$  fold induction,  $p \leq 0.001$  versus control;  $2.22 \pm 0.18$  fold induction,  $p \leq 0.001$  versus control).

### The synergism of TNF- $\alpha$ and IL-22 is of functional relevance for the innate immune response

To verify the functional impact of the observed synergistic innate immune induction, we analyzed effects of TNF- $\alpha$  and IL-22 in an *in vitro* *Candida* infection model. *Candida* growth was inhibited by supernatant of keratinocytes stimulated with TNF- $\alpha$  plus IL-22 or Th22 supernatant respectively (Fig. 4A). In contrast, IL-22 alone had no effect and TNF- $\alpha$  only a weak inhibitory effect on *Candida* growth. Furthermore, both TNF- $\alpha$  plus IL-22 (Fig. 4B upper graph) and Th22 supernatant (Fig. 4B, lower graph) protected epithelial cells from cytotoxic cell death after infection with *Candida*, as measured by significantly lower lactate dehydrogenase (LDH) release 20 h after infection ( $62.45 \pm 6.16\%$ ,  $p \leq 0.01$  and  $66.12 \pm 8.55\%$ ,  $p \leq 0.01$ , respectively). Again, TNF- $\alpha$  and IL-22 alone had little or no protective effect ( $90.55 \pm 7.2\%$  and  $104.79 \pm 5.31\%$ ). These results indicate that a Th22-like combination of cytokines synergistically induces an effective innate immune response of epithelial cells.



**Figure 3.** Transcription factors of the AP-1 family are synergistically activated by TNF- $\alpha$  and IL-22. (A) Primary human keratinocytes stimulated with 50 ng/mL TNF- $\alpha$ , 50 ng/mL IL-22 or a combination of both were transfected with a dual vector system. Binding of transcription factors of the AP-1 family (B) ATF-2, (C) c-fos, and (D) c-jun, (E) NF- $\kappa$ B and (F) STAT-3 in nuclear extracts of keratinocytes was quantified using TransAM kits. Bars show the mean and SEM of six independent experiments. Asterisks indicate statistical significance. Statistical differences were determined by One-way ANOVA and Bonferroni's Multiple Comparison Test as post test and defined as \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

### The combination of TNF- $\alpha$ and IL-22 protects the epidermal integrity in a 3D skin infection model

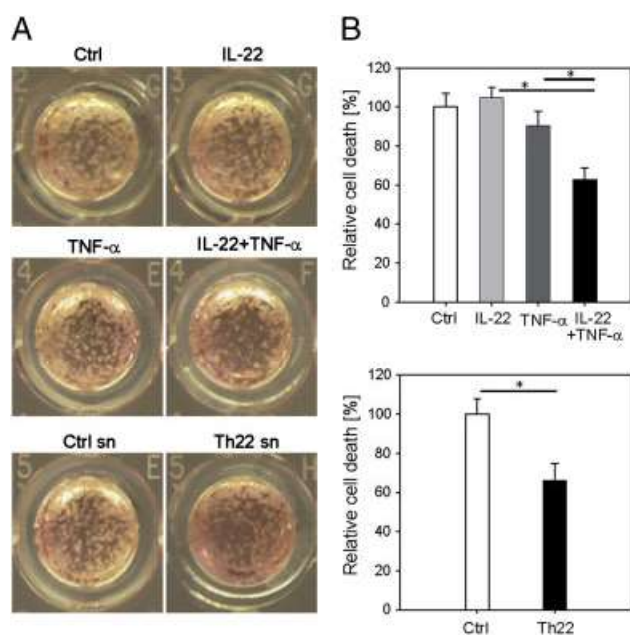
To estimate the impact of the observed innate immune response on the epidermal integrity, we established a three-dimensional skin infection model. A reconstituted epidermis was stimulated with recombinant cytokines or Th22 supernatant and subsequently infected with live *C. albicans*. Stimulation with TNF- $\alpha$  or IL-22 in the absence of *C. albicans* resulted in a mild hyper-proliferation of the three-dimensional skin models (Fig. 5, left pictures). While *C. albicans* completely destroyed the epidermal structure of skin models stimulated with medium, IL-22, or TNF- $\alpha$ , a weak protective effect was observed after stimulation with IFN- $\gamma$  or IL-17. The only condition that conserved integrity of the epidermal structure was TNF- $\alpha$  plus IL-22 (Fig. 5, right pictures). Similarly, stimulation of the skin models with Th22 supernatant protected the epidermal structure from *Candida* infection (Fig. 5, right pictures).

## Discussion

Increasing evidence suggests that impact of T cells on epithelial cells is determined rather by a combination than by single cytokines. In this study we demonstrate a strong functional synergism of TNF- $\alpha$  and IL-22, two key cytokines secreted by Th22 cells. TNF- $\alpha$  and IL-22 synergistically induce an innate immune response in primary human keratinocytes, suggesting that this combination warrants epidermal barrier integrity during infection with *C. albicans*.

IL-22 belongs to the new class of tissue signaling cytokines with little or no impact on immune but major effects on epithelial cells [12]. A functional synergism of IL-22 and IL-17 leads to the effective induction of HBD-2 in human keratinocytes [13]. The importance of this interaction and its restriction to epithelial cells is obvious in patients suffering from chronic mucocutaneous candidiasis and Hyper IgE syndrome. Both diseases result from a lack of IL-17 and IL-22 – either through an impaired secretion by T cells [14–18] or auto-antibodies directed against these cytokines [19, 20] – which leads to severe and recurrent infections of skin and mucosal membranes; however IL-17 and IL-22 appear dispensable in systemic infections. Therefore, the tissue-signaling cytokines IL-17 and IL-22 appear to be essential gate keepers at barrier organs of the human organism. However, not only the interplay between IL-22 and IL-17 is important for epithelial immunity as both cytokines can also functionally interact with pro-inflammatory cytokines. An IL-17/IFN- $\gamma$  axis synergistically induces the expression of ICAM-1 on keratinocytes [21], which enhances leukocyte-mediated keratinocyte apoptosis and consecutively leads to an unspecific amplification cascade of cutaneous inflammation [22]. While IL-17 and IFN- $\gamma$  form this acute inflammatory axis, first evidence for a functional interplay of TNF- $\alpha$  and IL-22 has been reported recently. TNF- $\alpha$  enhances IL-22-induced expression of keratin16 and CXCL-8. Furthermore, a positive feedback loop in terms of receptor expression for both TNF- $\alpha$  and IL-22 on keratinocytes has been observed [23–25]. Here, we expand the functional relevance of the IL-22/TNF- $\alpha$  axis to skin immunity by demonstrating that IL-22 reinforces the

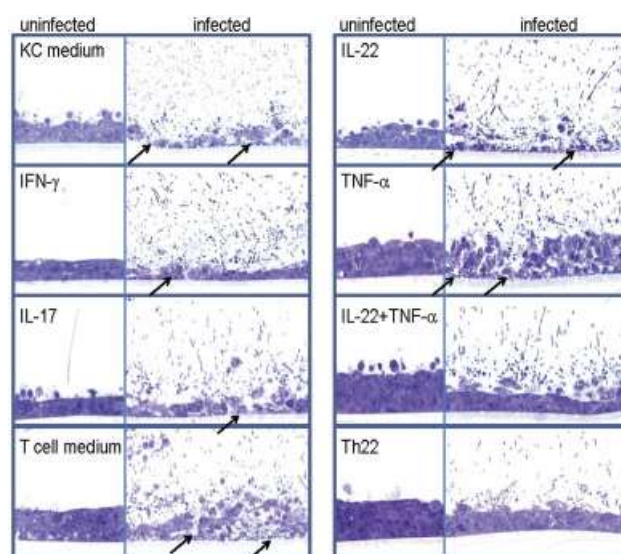




**Figure 4.** TNF- $\alpha$  and IL-22 co-operate to protect keratinocytes against extracellular pathogens. (A) Growth of *C. albicans* was investigated in the presence of supernatant obtained from primary human keratinocyte cultures stimulated for 72 h with 50 ng/mL TNF- $\alpha$ , 50 ng/mL IL-22 or a combination of both cytokines ( $n = 3$ ) (upper two rows) and Th22 supernatant (third row). (B) Human oral keratinocytes (cell line TR146) were stimulated with recombinant cytokines or Th22 supernatant and infected with *C. albicans* after 30 min in a two-dimensional skin infection model. *C. albicans*-mediated cytotoxicity of human oral keratinocytes was measured 20 h after infection by quantification of LDH release ( $n = 3$ ). Statistical differences were determined by One-way ANOVA and Bonferroni's Multiple Comparison Test as post test and defined as  $*p < 0.05$ ; error bars indicate SEM.

TNF- $\alpha$ -induced regulation of multiple innate defense genes such as complement cascade proteins, antimicrobial peptides and antimicrobial chemokines.

The interaction of IL-22 and TNF- $\alpha$  is mediated through the IL-22R heterodimer and tumor necrosis factor receptor I [26] and intracellularly by MAP kinases, in particular p38, which leads to downstream activation of AP-1 family transcription factors. The combination of IL-22 and TNF- $\alpha$  strongly induced the phosphorylation and translocation of MAP kinases to the nucleus whereas the single cytokines only weakly contributed to MAP kinase activation. It is known that both IL-22 [27] and TNF- $\alpha$  [28] activate MAP kinases; however, main signaling pathways for IL-22 are mediated through the transcription factor STAT-3 and other STAT molecules [6, 24], while TNF- $\alpha$  strongly induces the NF- $\kappa$ B signaling cascade in keratinocytes [29]. Since NF- $\kappa$ B is not synergistically activated by the combination of TNF- $\alpha$  and IL-22, the observed synergism does not cover the whole functional spectra of TNF- $\alpha$  and IL-22, but is rather limited to aspects such as innate immunity. This may explain functional diversity of TNF- $\alpha$  and IL-22 as well as a dual role for IL-22: alone it has protective effects and enhances wound healing [30], in combination with TNF- $\alpha$  it becomes immune-stimulatory and arms epithelia for innate responses.



**Figure 5.** The combination of TNF- $\alpha$  and IL-22 protects the epidermal integrity during infection with *C. albicans*. The oral keratinocyte cell line TR146 was used to reconstitute an epidermal cell layer, which was stimulated with recombinant cytokines (50 ng/mL each) or Th22 supernatant, respectively, for 12 h (left panels). Half of the three-dimensional skin models were infected with live *C. albicans* (right panels), and integrity of the epidermal barrier and penetration of *C. albicans* (arrows) to the basal membrane were investigated. KC: keratinocyte (original magnification  $\times 40$ )

The stimulation of the epithelial immune system by the IL-22/TNF- $\alpha$  axis is important for defense against extracellular pathogens like *C. albicans*. Supernatant of keratinocytes pre-incubated with the combination of both cytokines or Th22 clone supernatant most effectively reduced *C. albicans* growth, protected keratinocytes from apoptosis and conserved the epidermal structure in an in vitro *Candida* infection model. Interestingly, common side effects of an anti-TNF- $\alpha$  therapy (Infliximab) are serious respiratory and skin infections [31], which could be explained by the missing interaction of IL-22 with TNF- $\alpha$ . Therefore, the IL-22/TNF- $\alpha$  axis itself is protective and important for the homeostasis of the human organism and its environment; if not tightly regulated, however, this strong synergism might turn pathologic and cause severe and chronic inflammatory skin diseases like psoriasis.

In summary, the discovery of the IL-22/TNF- $\alpha$  axis as an essential combinatorial key for cutaneous immunity gives a first insight into the function of Th22 cells and could lead to new therapeutic approaches of chronic inflammatory skin diseases like atopic eczema and psoriasis.

## Materials and methods

### Patients

Primary human keratinocytes were obtained from human foreskin (Western blot analysis) or healthy adult volunteers ( $n = 10$ ).

Before samples were taken, each participant gave his informed consent. The study was approved by the ethical committee of the Technical University Munich and was conducted according to the declaration of Helsinki.

### Isolation and expansion of primary human keratinocytes

Keratinocytes were isolated using the method of suction blister as described previously [32]. Briefly, blisters were induced by generating a vacuum on normal skin of the forearms. Epidermal sheets were obtained from blister roofs, treated with 0.05% trypsin (Invitrogen) to obtain single-cell suspension and seeded on a feeder layer of irradiated 3T3/J2 fibroblasts in modified Green's medium. At 70–80% confluence, keratinocytes were detached with 0.05% trypsin, aliquoted and cryopreserved in liquid nitrogen. Keratinocytes of second and third passage were used in experiments.

### Stimulation of keratinocytes

In total, 70–80% confluent keratinocytes were stimulated with 50 ng/mL TNF- $\alpha$ , 50 ng/mL IL-22 (both R&D Systems) or a combination of both. For some experiments,  $10^6$  cells of human Th22 clones obtained from lesional skin of atopic eczema or psoriasis patients were stimulated for 48 h with anti-CD3 and soluble anti-CD28 in a 24-well plate. Supernatant was obtained and tested for content of cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-4, IL-17, IL-22) by commercially available ELISA systems (all R&D systems). Incubation time varied depending on the readout (5 min for Western Blots, 1 h for TransAM, 12 h for real-time PCR, 24 h for dual luciferase assay, 12–72 h for ELISA).

### Real-time PCR

Total RNA was isolated from fresh human primary keratinocyte cultures with the RNeasy Mini kit (Qiagen) and reversely transcribed using oligo (dT) primers and avian myeloblastosis virus reverse transcriptase (Roche Applied Sciences). The cDNA was amplified with SYBR Green Mastermix (Applied Biosystems) using the following primer sequences: S100A7 (forward 5'-GCTGACGATGATGAAGGAGAACT-3', reverse 5'-GTAATTTGTGCCCTTTTGTGACA-3'); HBD2 (forward 5'-CTCCTCTTCTCGTTTCTCTCATATT-3', reverse 5'-AGGATCGCTATACCACCAAAA-3'); CXCL-9 (forward 5'-TCACATCTGCTGAATCTGGG-3', reverse 5'-CCTTAAACAATTTGCCCAA-3'); CXCL-10 (forward 5'-GCTGATGCAGGTACAGCGT-3', reverse 5'-CACCATGAATCAAAGTCCGA-3'); CXCL-11 (forward 5'-ATGCAAAGACAGCGTCTCT-3', reverse 5'-CAAACATGAGTGTGAAGGGC-3'); C1s (forward 5'-CAAAGGGTTCTCTGGGACT-3', reverse 5'-TGGGGAGTATCACTGTGCTG-3'); C1r (forward 5'-TCCCAGGCTTTTCTTATCA-3', reverse 5'-GAAGCTCGTCTTCAGCAGT-3'). The comparative  $\Delta\Delta C_t$  method was used to calculate the relative quantification and the range of confidence.

### Cell lysis, gel electrophoresis and immunoblotting

Primary human keratinocytes were lysed for 20 min at 4°C in radioimmunoprecipitation assay buffer containing 1  $\times$  PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL PMSF, 50 kIU aprotinin, 100 mM sodium orthovanadate and 10  $\mu$ L/mL rotease inhibitor cocktail (Sigma). Cell lysates were collected in a microfuge for 15 min at 15 000  $\times$  g. Supernatant was collected and utilized for SDS-PAGE. After cell lysis, the supernatant was titrated in reducing SDS-PAGE loading buffer (Invitrogen), treated at 70°C for 10 min, separated in a 10% Bis-Tris gel (Invitrogen) with MOPS or MES Buffer, according to the manufacturer's instructions and transferred to a PVDF membrane (Immobilon P, Millipore, MA, USA) for 60 min using transfer buffer (Invitrogen). Membranes were blocked for 30 min at room temperature (Blocking buffer: 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.05% Tween20, 0.5% BSA), incubated at 4°C overnight with the following primary antibodies: anti- $\beta$ -Actin (Sigma) (0.25  $\mu$ g/mL), anti-JNK1/2 (1  $\mu$ g/mL), anti-phospho-JNK1/2 (1  $\mu$ g/mL), anti-P38 and anti-phospho-P38 (1  $\mu$ g/mL) (all diluted in Tris-buffered saline containing 0.5% BSA and 0.05% Tween20). Blots were washed repeatedly in washing buffer (15 mM NaCl, 50 mM Tris-HCl, 0.05% Tween20; pH 7.6) and incubated for 1 h at room temperature with 0.1  $\mu$ g/mL peroxidase-conjugated donkey anti-mouse IgG in blocking buffer. Peroxidase activity was detected using chemiluminescence substrate (Pierce) and recorded with a chemiluminescence detector (Vilber Lourmat). Mouse anti-MEK1/2 (phosphorylated and non-phosphorylated), mouse anti-JNK (phosphorylated and non-phosphorylated) and mouse anti-p38 (phosphorylated and non-phosphorylated) were obtained from Cell Signaling Technology, Danvers, MA, USA

### TransAM and dual luciferase assay

For TransAm analysis, primary human keratinocytes were stimulated for 2 h with recombinant cytokines. Nuclear extracts were generated with the Nuclear Extract Kit (Active Motif) and analyzed for activated transcription factors using TransAm Kits (Active Motif) according to the manufacturer's protocols.

For dual luciferase assays, primary human keratinocytes were grown to 70% confluence and transfected with two plasmids, one containing the "Firefly Luciferase" under control of an AP-1-dependent promoter and a control plasmid expressing the "Renilla Luciferase" under the CMV promoter. The transfection was performed in presence of DMRIE-C (1, 2 -Dimyristyloxypropyl-3 - Dimethyl - Hydroxy - Ethyl-Ammoniumbromide plus Cholesterol) (Dual-Luciferase-Reporter Assay System, Promega). Eighteen hours after transfection, keratinocytes were stimulated for 48 h with recombinant cytokines.

### ELISA

Concentration of CXCL-10, CXCL-11 and HBD-2 in cell-free supernatant of primary human keratinocytes stimulated with

50 ng/mL IL-22, 50 ng/mL TNF- $\alpha$  or a combination of both were measured using commercially available sandwich ELISA kit according to the manufacturer's instructions (CXCL-10, CXCL-11: R&D Systems, HBD-2: Phoenix Pharmaceuticals).

### In vitro *Candida* infection model

*C. albicans* wild-type strain SC5314 was used for the infection of human oral keratinocytes (TR146, buccal carcinoma cell line) as described previously [33]. *C. albicans* was grown on Sabouraud's dextrose agar (Difco) followed by two pre-cultures in 10 mL YPG (1% yeast extract, 2% peptone, 2% glucose) medium (Difco), first for 16 h at 25°C and then for 24 h at 37°C through orbital shaking. Human oral keratinocytes were cultured in DMEM medium supplemented with 10% FCS and 0.1% gentamicin solution (50 mg/mL) at 37°C and 5% CO<sub>2</sub>.

For two-dimensional skin infection models, 30 000 human oral keratinocytes (TR146) were plated per well in 96-well plates in antibiotic and antimycotic free culture medium. Twenty-four hours after plating, cells were treated with 50 ng/mL TNF- $\alpha$  and IL-22 or Th22 supernatant. Each treatment was performed in triplicate. Keratinocytes were infected 30 min after treatment with a total amount of 3000 yeast cells (MOI 0.1). In all experiments, the release of LDH from epithelial cells into medium was measured after 20 h as indicator of epithelial cell damage. LDH activity was analyzed using the commercially available Cytotoxicity Detection Kit (Roche).

For three-dimensional skin models,  $1 \times 10^6$  human oral keratinocytes (TR146) were seeded on inert filter substrates (Nunc, polycarbonate filter, 0.4  $\mu$ m pore size, 0.5 cm<sup>2</sup>) in antibiotic/antimycotic-free defined keratinocyte growth medium (Lonza) for 9 days. After 5 days inert filter substrates were lifted to the air-liquid interface and basal cells were fed through the filter substratum. Epithelium was treated with IFN- $\gamma$  (300 U/mL), IL-17, IL-22, TNF- $\alpha$  (50 ng/mL each), IL-22/TNF- $\alpha$  combination or Th22 supernatant directly before infection with  $2 \times 10^6$  *Candida* yeasts for 12 h. Light microscopical studies were performed as previously described using paraffin-embedded oral epithelium specimens [34, 35].

### Statistical analysis

Statistical analysis was done using One-way ANOVA and Bonferroni's Multiple Comparison Test as post test. Statistically significant differences were defined as \* $p \leq 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

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**Abbreviation:** HBD-2: human  $\beta$  defensin 2 · IL-10R $\beta$ : IL-10 receptor  $\beta$  · IL-22R: IL-22 receptor · LDH: lactate dehydrogenase

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