Biomedicine & Pharmacotherapy 146 (2022) 112569

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Expression and activity of IL-17 receptor subunits in human cutaneous cells as targets for anti-IL-17 therapeutic antibodies

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ARTICLE INFO

Keywords:
Psoriatic pathogenesis
IL-17 cytokines
IL-17 receptors

ABSTRACT

The key players in different chronic inflammatory skin diseases are cytokines belonging to the IL-17 group, IL-17 receptors and a T helper cell population, Th17 cells. Successful therapeutic strategies that target either IL-17 or the major IL-17 receptor IL-17RA have confirmed the immune-pathogenic pathway. To study the IL-17-ligand – receptor axis at the molecular level, a number of cutaneous cell types from healthy human subjects has been cultured and analyzed for the expression of IL-17 receptors. IL-17RA was the most abundantly expressed receptor type in keratinocytes, epidermal stem cells, fibroblasts, mesenchymal stem cells, hemo- and lymphovascular endothelial cells. IL-17RC and IL-17RD showed moderate expression, while the genes for IL-17RB and IL-17RE were poorly expressed. In none of the investigated cell types, IL-17 ligands caused an increased expression level of the five receptor types in time- and dose-dependent experiments. No evidence for IL-17A, -C, -E or -F induced signal transduction cascades could be obtained by a qRT-PCR and western blot analyses. Further studies are necessary to identify relevant co-stimulating factors from IL-17 subtypes under physiological and pathophysiological conditions.

1. Introduction

The importance of IL-17 as a pro-inflammatory cytokine in various chronic inflammatory diseases is well documented [1–3]. IL-17 signaling involves a family of six IL-17 subtypes (A-F) and five IL-17 receptor (IL-17R) subtypes (A-E) [4]. IL-17A and IL-17F have a 50% sequence homology and, like all IL-17 subtypes, are available as homodimers, but also as IL-17A/F heterodimer [5]. The receptor subtypes IL-17RA and IL-17RC bind IL-17A, IL-17F and IL-17A/F. IL-17RA also binds IL-25, which mediates anti-inflammatory effects [2,6,7]. The structure of the ectodomain of IL-17RA in complexation with homodimer IL-17F could be shown with X-ray crystallography [8]. By means of surface plasmon resonance (SPR) measurements it was also possible to prove that IL-17A binds to IL-17RA with an affinity about 100 times higher than IL-17F [8].

IL-17 is ostensibly produced by T cells that do not produce interferon γ (TH1 cells) and no IL-4 (TH2 cells) and are therefore called Th17 cells [9]. These are integrated into pro-inflammatory cascades to ward off extracellular pathogens and the associated destruction of the extracellular matrix as well as in the induction of epithelial proliferation with synthesis of antimicrobial peptides [2]. The differentiation of the Th17 cell is induced by a transforming growth factor (TGF), IL-6 and IL-1 or tumor necrosis factor (TNF) milieu and by IL-21 and IL-23 amplification as well as stabilization and expansion [10,11]. The IL-17 released by Th17 cells binds to effector cells according to the expression patterns of the respective formulation subtypes, wherein macrophages, endothelial cells, fibroblasts, osteoblasts and chondrocytes are described as pathogenetic significant [2]. This initially results in a functional integration of Th17 cells into the infection defense by activating neutrophilic granulocytes (e.g. mycobacterium tuberculosis, Candida albicans), but also in chronic inflammatory processes as well as autoimmune reactions [12–16]. One of these Th17-cell-mediated autoimmune diseases based on polygenic disposition is psoriasis vulgaris [17–19]. Chronic inflammatory processes of this systemic disease primarily lead to symptoms of disease in the skin, joints, bone close to the joints and enthesis [20]. Secondary, however, co-morbidities may develop depending on the inflammatory load and individual pathological factors [21–23]. In the foreground of the morphological changes in the skin organ are disorders of epidermal regeneration in the form of epidermis hyperplasia (hyperkeratosis and acanthoses) as well as inflammatory phenomena with

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https://doi.org/10.1016/j.biopha.2021.112569
Received 15 October 2021; Received in revised form 11 December 2021; Accepted 19 December 2021
Available online 29 December 2021
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formation of flat, combined papules (plaques). An effective systemic therapy of inflammation leads to phenotypic restitution strategy [24]. The targeted blockade of the IL-23/IL-17 axis shows the best therapeutic effects compared to other therapeutic approaches [25].

Secukinumab and ixekizumab are used to inhibit IL-17A, bimekizumab to inhibit IL-17A/F and brodalumab to block IL-17RA [26,27]. The IL-17RA subtype is activated by the homodimeric IL-17A, IL-17C, IL17-E, IL-17F, viral IL-17 and the heterodimer IL-17A/F. At the same time, however, IL-25 is also bound, for which antiproliferative effects are described [28]. So far, it is unclear whether and what differences there are between the inhibition of IL-17A and the blockade of IL-17RA. One of the reasons for this is that it has not yet been studied which cutaneously relevant cell types express which IL-17R subtypes. Although initial data are available on keratinocytes, other relevant cutaneous cell types have not been studied in this respect [29–32]. The stimulation of IL-17RA by IL-17 ligands activates two signal cascades, the MAP-MEK-ERK/p38/JNK and the PI3K-Act-NFκB pathway [33]. Both pathways merge into the transcriptional activation of IL-17 target genes coding for CXCL1, CXCL2, IL-6, GM-CSF, TNFα, IFNγ [34–36]. Notably, a previous report identified the basic transcription factor C/EBPα as an expression control player in psoriasis, where an IL-17 induced keratinocyte proliferation is accompanied by down-regulation of C/EBPα [37,38]. This study revealed altered mRNA levels of several factors involved in IL-17 modulated keratinocytes such as DEFB4, cathelicidin, KRT16, CCL18, CCL20.

The objective of this study was to analyze the expression of IL-17R subunits A–E in different relevant cutaneous cell types. In addition, we addressed the question whether a dose-response relationship would exist between IL-17 ligands and the regulation of receptor types in a cell-type-dependent manner. The investigations were combined with the analysis of IL-17-induced signal transduction cascades.

2. Materials and methods

2.1. Proteins

The following cytokines were used for the investigations: IL-17A and IL-17F (Immuno Tools GmbH, Friesoythe, Germany), IL-17C (R&D Systems Abingdon, United Kingdom) and IL-17E (ProSpec, East Brunswick, USA).

2.2. Isolation and culture of cutaneous cell types

Postmitotic keratinocytes (NHEK), epidermal stem cells (ESC), cutaneous fibroblasts (NHDF), cutaneous mesenchymal stem cells (MSC) as well as cutaneous micro-hemovascular (HDMEC) and microlymphovascular endothelial cells (LEC) were isolated and cultured from human foreskin tissue according to standardized protocols and with permission of the ethics committee of Medical Faculty of Martin Luther University Halle-Wittenberg. After dispase pretreatment, NHEK and ESC were isolated from the pre-isolated epidermis and the other cell types from the dermis content [39–44]. For this purpose, the cells were cultured in 24 microtiter plates (Greiner) up to a degree of confluence of 85% at 37 °C, 5% CO_2 and 80% humidity. Cell type-specific media and supplements were used for cell culture (NHEK and ESC: Keratinocyte-SFM Gibco (Fisher, Schwerte, Germany) with Supplement: Human Recombinant Epidermal Growth Factor and Bovine Pituitary Extract; NHDF: Fibroblast Basal Medium (Promocell, Heidelberg, Germany) with supplement BFGF/Insulin; MSC: Mesenchymal Stem Cell Basal Medium (Lonza Ltd, Basel, Switzerland) with Supplement: MCGS/L-Glutamine/GA-1000; HDMEC and LEC: Endothelial cell Growth Medium MV2 (Promocell, Heidelberg, Germany) with Supplement: VEGF/MixMV2). The cells were harvested using cell harvesters (Inotech, Wohlen, Switzerland) and standardized for the experiments.

2.3. Cytotoxicological examinations of cutaneous cell types

The cytotoxicological studies were implemented according to established standard protocols in three independent experimental procedures for the therapeutic antibodies secukinumab, ixekizumab and brodalumab. Cells were incubated with therapeutic antibodies (0, 1, 10, 100 nmol/L) for 24, 48, and 72 h. The proof of vitality was carried out using the gentian violet method [45]. The protein-bound cationic triarylmethane dye was measured in the absorption reader at 620 nm. To quantify the proliferative activity, the rate of installation of tritium thymidine was measured [46]. To investigate the apoptosis behavior, an Annexin-V-FITC/propidium iodide staining was performed according to the instructions of the Annexin-V-FITC supplier (BD Pharmingen, Heidelberg, Germany). Cellular fluorescence was detected by FACScan® analysis (Becton Dickinson, San Jose, Calif., USA) using FL1-H (530/30 nm) for Annexin-V-FITC and FL2-H (585/42 nm) for propidium iodide. Percentages of subpopulations classified as vital, apoptotic and late-apoptotic/necrotic cells were calculated by quadrant analysis within the dot plot Annexin-V-FITC vs. propidium iodide.

The IC_{50}/EC_{50} values were calculated using the pharmacokinetics tool XLSTAT V2014.5.02 (Addinsoft, New York, USA) add-on for MS Excel for MAC 2011 (V14.6.3). The antibody concentrations at 50% vitality, proliferative activity, necrosis and apoptosis were given as IC_{50} and EC_{50} values respectively.

2.4. qRT-PCR analysis

All cell types originated from three individual donors. Cutaneous primary cell types were NHEK, NHDF, MSC, HDMEC and LEC. For basal expression analysis, cells ($0.5–1 \times 10^6$) were cultured for 7–10 days in cell-specific medium. For incubation assay, 200,000 cells were seeded in 2 mL medium in 6-well plates. 48 h after seeding, the medium was exchanged and the individual IL-17 subtypes were added at concentrations of 10 ng/mL, 50 ng/mL and 100 ng/mL. A sample with water served for control. After three different incubation intervals (24 h, 48 h and 72 h), cells were harvested via Accutase (Sigma Aldrich, St. Louis, USA) or trypsin treatment. Cells were subsequently processed for RNA and protein extractions. RNA isolation was performed via the Trifast reagent according to the suppliers' instructions. 500 ng RNA were transcribed into cDNA with M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, USA). For expression analysis, quantitative real-time PCR (qPCR) was performed with the Quantstudio 5 Real-time PCR System using PowerUP-SYBR Green (Thermo Fisher Scientific, Waltham, USA) according to the suppliers' instructions. Primers (5′–3′ ends) used for IL-17 receptor subunits: (receptor subunit, forward primer, reverse primer) IL-17RA, GCTTCACCCTGTGGAACGAAT, TATGTGGTGCATGTGCTCAAA; IL-17RB, ATGTCGCTCGTGCTGCTAAG, AGCCACATTGAACGGTCGG; IL-17RC, CCGCAGATCATTACCTTGAACC, ATGTTCGTCCTAACGGAGTCA; IL-17RD, AGGCATCTATGACTCGTCTGT, TGAAGAGGAGGACACGCT; IL-17RE, CTGCTGTCAGGTGGCTCA, GGAAGACTTTTTGGATTTCTGC. Primers (5′–3′ ends) used for heatmap analysis: (cytokine, forward primer, reverse primer) CCL-2, CTCTCGCCTCCAGCATGAAA, CTTGAAGATCACAGCTTCTTTGG; IFN-α1, GCTTTACTGATGGTCCTGGTGG, CTGGAACTGGTTGCCATCAAAC; IFNγ, TCAGCTCTGCATCGTTTTGG, GTTCCATTATCCGCTACATCTGA; IL-1b, TGATGGCTTATTACAGTGGCAATG, GTAGTGGTGGTGGGAGATTCG; IL-4, CCAACTGCTTCCCCCTCTG, TCTGTTACGGTCAACTCGGTG; IL-6, GACAGCCACTCACCTCTTCAGA, GTGCCTCTTTGCTGCTTTCAC; IL-8, GCTAAAGAACTTCGATGTCAGTGC, CTCAGCCCTCTTCAAAAACTTCTC; IL-10, GAGGCTACGGCGCTGTCA, TCCACGGCCTTGCTCTTG; IL-12A, CCTTGCACTTCTGAAGAGATTGA, ACAGGGCCATCATAAAAGAGGT; IL-12B, CCAGCAGTTGGTCATCTCTTGG, TTGCCAGAGCCTAAGACCTCAC; IL-18, CCTGGAATCAGATTACTTTGGC, CCATACCTCTAGGCTGGCTATC; IL-23, GTGCCAGCAGCTTTCACAGAAG, AGACCCTGGTGGATCCTTTGC; IL-33, CCTGTCAACAGCAGTCTACT, TTGGCATGCAACCAGAAGTC; TNFα, CTTCTGCCTGCTGCACTTTG, GGCCAGAGGGCTGATTAGAGA.

2.5. Western blot analysis

Cell pellets of three samples of a specific cell type were re-suspended in RIPA buffer and the protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). After separation of the samples by 4–12% Bis-Tris polyacrylamide gels, proteins were transferred to nitrocellulose membranes. Membranes were incubated for 1 h in PBS containing 5% milk powder (Carl Roth, Karlsruhe, Germany). Subsequently, membranes were incubated over night at 4 °C with the primary antibodies that had been diluted 1:1000 in PBS containing milk powder. Membranes were washed 3 times and the transferred protein was made to react with secondary peroxidase-coupled antibodies for 1 h at 37 °C. After another washing cycle, the samples were detected by the ECL Plus System (Amersham Biosciences, Little Chalfont, UK) according to the suppliers´ instructions. Controls were performed with an antibody against GAPDH.

The catalog numbers of the antibodies were as follows: Il-17RA: #12661 (Cell Signaling), IL-17RB: HPA005482 (Sigma Aldrich), IL-17RC: LS-C384096 (LS Bio), IL-17RE: PA5-34499 (Thermo Fisher), Akt (Pan): #9272 (Cell Signaling), Phospho-Akt (Ser473): #4060 (Cell Signaling), Phospho-Akt (Thr308) (D25E6): #13038 (Cell Signaling), p44/42 MAPK (Erk1/2): #9102 (Cell Signaling), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (20G11): #4376 (Cell Signaling), p38 MAPK (D13E1): #8690 (Cell Signaling), Phospho-p38 MAPK (Thr180/Tyr182) (D3F9): #4511 (Cell Signaling), and GAPDH: #51332 (Cell Signaling). All of them were used in 1:1000 dilution.

2.6. Immuno staining analysis

Cells were cultured as described above. After detachment by Accutase 100,000 cells were seeded into cell-specific culture medium in 12-well plates and kept for two days under standard conditions. Subsequently, IL-17A, IL-17C, IL-17E and IL-17F were added to reach final concentrations of 100 ng/mL. Cells were incubated with the cytokines for three days. After removal of the medium, cells were washed with 1 mL PBS and fixed for 15 min with 4% para-formaldehyde at 37 °C. The samples were washed with PBS three times at room temperature. Permeabilization of cell membranes was achieved by incubation with 0.1% Triton X-100 in PBS for 15 min at 37 °C. The cells were subsequently washed twice for 5 min at room temperature and incubated at room temperature with 1% BSA for 1 h. For immuno-fluorescence cell staining an overnight reaction with antibodies against the NFκB subunit p65 at 4 °C was initiated with a 1:200 dilution in PBS. After three washing steps with PBS at room temperature, the material was incubated with a secondary, fluorescence-labeled antibody (Alexa fluor 555, Thermo Fisher Scientific, Waltham, USA) diluted 1:1000 in PBS for 2 h at 37 °C. After 3 washing steps with PBS buffer, 250 µL PBS containing DAPI at a 1:500 dilution was added, and samples were incubated for 30 min at room temperature. Subsequently, the samples were washed three times with PBS and finally covered with 1 mL PBS. The results of the immunolabeling were recorded with an Olympus IX81 microscope (Olympus Europa SE & Co. KG, Hamburg, Germany). Recommended standard filters for DAPI and Alexa fluor 555 fluorescence were applied.

3. Results

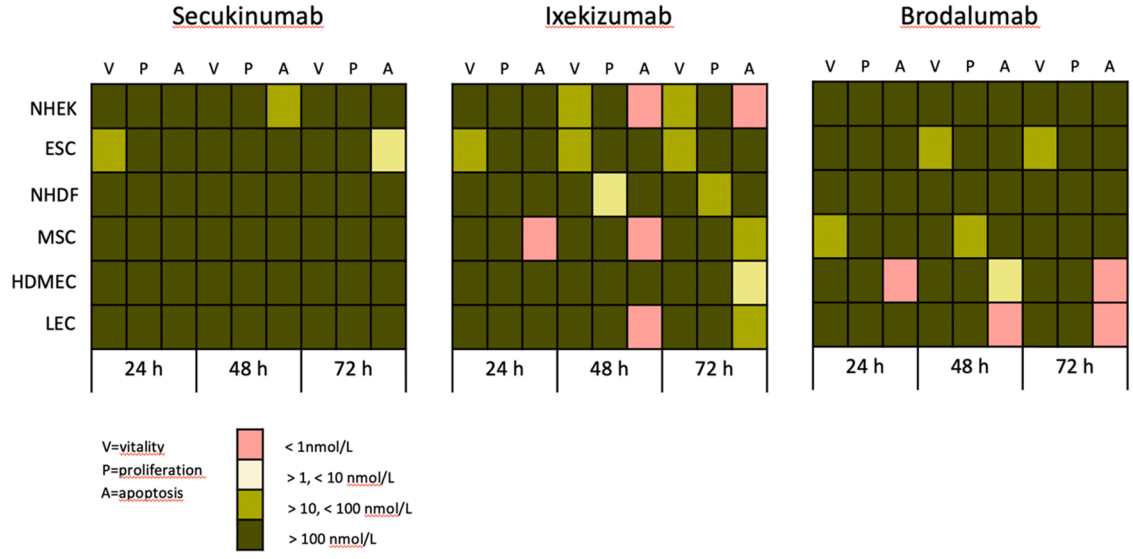
3.1. Cytotoxicological examinations

The IC_{50} and ED_{50} values of secukinumab, ixekizumab and brodalumab in the investigated cutaneous cell types are given in Table 1. There was no evidence for toxicologically relevant effects of the antibodies studied.

3.2. Transcriptional quantification of IL-17 receptor subunits

In order to determine expression levels of all IL-17 receptor subunits, quantitative real time PCR was performed. Expression was quantified in primary cells isolated from human foreskin tissue. Expression was monitored in NHEK, NHDF, MSC, HDMEC and LEC. With one exception, all receptor subunits were amplified in the tested cell types (Fig. 1). However, amplification levels of IL-17RB subunit were low and at the detection limit. A control experiment with monocytes (THP-1), which are known to express IL-17RB, confirmed expression of the receptor subunit under the applied experimental system. The comparison of receptor subunit expression levels between the various cell types showed the highest expression for IL-17RA in NHEK, ESC, MSC, HDME and LEC. Only in NHDF, the strongest amplification was observed for IL-17RD. The comparison of the expression levels of the individual receptor subunits in the cell types revealed that IL-17RA and IL-17RD subunits were most strongly expressed in NHDF and MSC. The expression of IL-17RB subunit was moderate in NHDF and MSC, while low expression of these receptor types was observed in NHEK, ESC, HDMEC and LEC. IL-

Table 1
IC50/ED50 data for secukinumab, ixekizumab and brodalumab in NHEK, ESC, NHDF, MSC, HDMEC, and LEC.



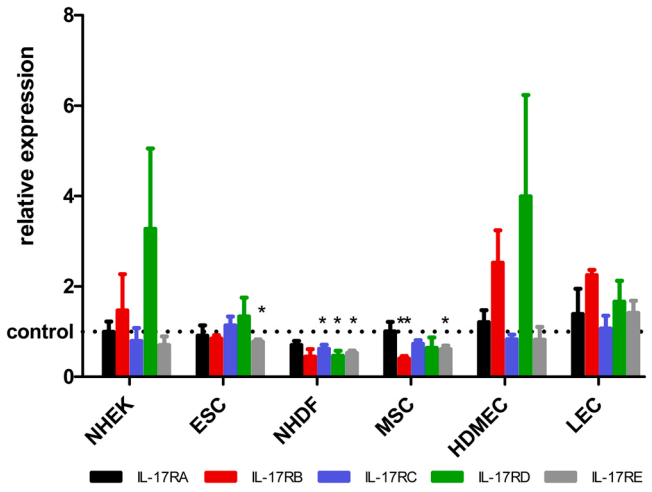


Fig. 1. Expression of the IL-17 receptor subunits in NHEK, ESC, NHDF, MSC, HDMEC, and LEC after stimulation with 100 ng/mL IL-17A. The data represent the gene expression relative to vehicle treated control (dotted line). Bars represent average ± standard deviation of at least three independent experiments. Student T-test in comparison to control, * $p \leq 0.05$; ** $p \leq 0.01$.

17RE showed the highest expression in NHEK, ESC and NHDF.

Protein level analysis by Western Blot showed a high concentration of IL-17RC in all tested cell types (Fig. 2A). Very poor IL-17RE subunit protein levels were exclusively found in HDMECs and LECs. IL-17RB was detected in NHEK, ESC, HDMEC and LEC. A Western Blot for IL-17RD was not viable, because no antibody was available.

The results indicate divergent RNA and protein expression profiles. IL-17RA as well as IL-17RC are predominantly expressed in the tested cell types. IL-17RD shows a moderate protein level, IL-17RE and IL-17RB show a rather low gene expression.

3.3. Influence of IL-17A on IL-17R subunits expression

In order to explore a potential induction of IL-17R subunits by IL-17A, expression of the receptor subunits was monitored upon incubation with three concentrations of IL-17A. The various cell types were incubated with 10 ng/mL, 50 ng/mL and 100 ng/mL IL-17A. After 24 h, 48 h and 72 h, transcription of the receptor subunits was studied as described before. Since the evaluation of the real time was neither dose nor time dependent, we only show the expression results after 72 h at the highest Il-17A concentration (Fig. 1). For NHEK we could observe an increase in IL-17RD expression after treatment with IL-17A. ESCs show no obvious ligand dose- nor time-dependent expression changes for all five receptor subunits. Similar results were obtained with NHDF and MSC, which show in tendency a slightly decreased expression of nearly all receptor subunits. In HDMEC for IL17-RB and IL-17RD, a tendency towards increased transcription was visible upon prolonged incubation periods with the elevated ligand concentrations of 50 ng/mL and 100 ng/mL. LECs show a slight up-regulation of the IL-17RB, IL-17RD and IL-17RE subunits.

Taken together, the expression of the different IL-17 receptor subunits is not relevantly affected by IL-17A. All investigated cell types show no significant change in the expression of IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RF. These results are independent of time (24 h, 48 h, 72 h) and concentration (ctrl., 10 ng/mL, 50 ng/mL, 100 ng/mL).

3.4. IL-17 dependent signal transduction pathways

IL-17 leads to the activation of two signal cascades, the MAP-MEK-ERK alias p38 pathway and PI3-Act-mediated NFκB activation [2]. The potential of IL-17 ligands to activate signal transduction pathways in the studied cell types was investigated. In a first experiment, the translocation of the NFκB subunit p65 into the nucleus was analyzed by immuno-fluorescence cell staining with primary antibodies against p65 and a dye-coupled secondary antibody (Alexa 555). Nuclear localization was assessed by DAPI staining. After a 3d-incubation of the cells with 100 ng/mL IL-17A, -C, -E and -F, cellular responses were monitored. Surprisingly, in none of the cell types the NFκB subunit p65 could be detected in the nucleus (Fig. 3).

Induction of the MAP-MEK-ERK signal cascade was tested by Western blot analysis with antibodies against phosphorylated signal

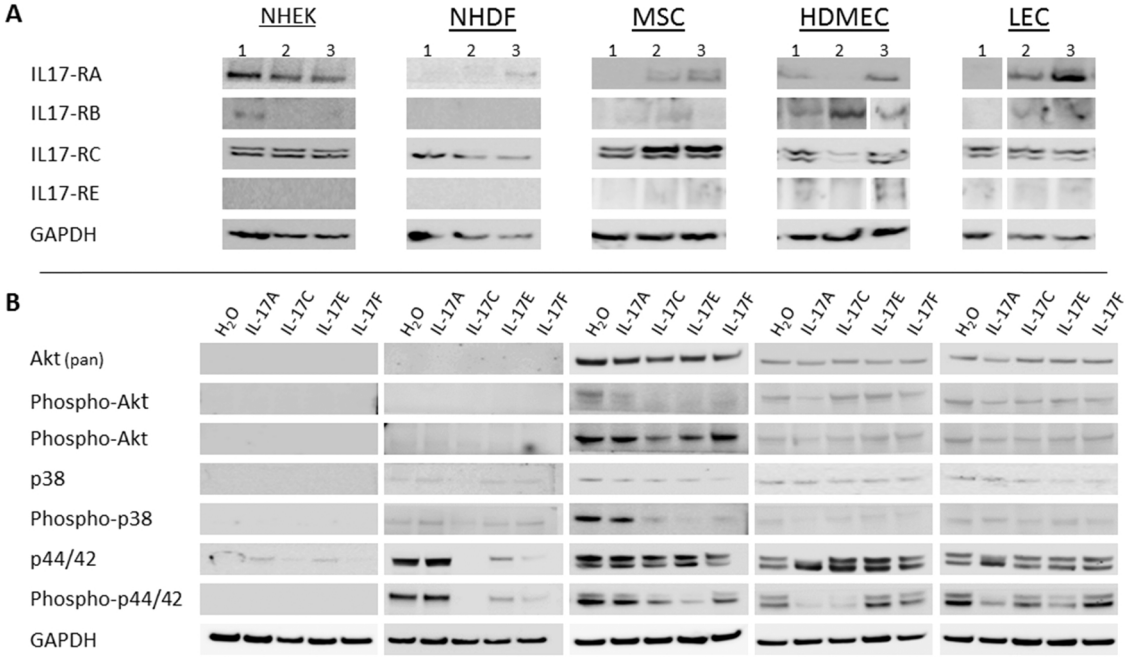


Fig. 2. Western blot analysis in NHEK, ESC, NHDF, MSC, HDMEC, and LEC **A:** basal expression levels of IL-17 receptor subunits (3 different donors) **B:** activation of MAP-MEK-ERK/p38 after stimulation by different ligands of IL-17.

transduction components Akt, p38 and p44/42. In NHEK, hardly any signal transduction components could be detected, while the control with GAPDH confirmed transfer and comparable amounts of proteins on the membranes. In MSC, the addition of IL-17A, -C, -E or -F had not led to any increased phosphorylation of the signal transduction components by comparing the signals with the water control. In contrast we could observe a decreased phosphorylation for pAkt (Ser473) by all ligands, a weaker p38 phosphorylation by IL-17C, IL-17E and IL-17F treatment as well as a reduced p44/p42 phosphorylation by IL-17E in MSCs. Likewise, no enhanced phosphorylation states of the three signal transduction factors were detectable in NHDF. Unexpectedly, here the signals of the non-phosphorylated proteins were low and only strong p44/42 signals could be discovered with IL-17A, however, comparable signals were also present in the water control. Similarly, in LEC and HDMEC, addition of the IL-17 ligands did not result in an increased phosphorylation of signal transduction factors, however the treatment with IL-17A and IL-17E for LECs, and IL-17A and IL-17C for HDMECs reduced the p44/42 phosphorylation (Fig. 2B).

IL-17 ligands have been reported to increase transcription of several IL-17 target genes [32]. In order to investigate whether IL-17A, -C, -E and -F would elicit such responses in the cutaneous cell lines, the ligands were added to the cells at concentrations of 10 nmol/L, 50 nmol/L and 100 nmol/L. qRT-PCR analyses were carried out in triplicate to exclude statistical outliers. Expression levels of the following cytokines were determined: CCL2, IFNγ, IL-1b, IL-4, Il-6, IL-8, TNFα, IFNα1, IL-10, IL-12a, IL-12b, IL-18, IL-23 and L-33. As Fig. 4 demonstrates, an IL-17-dependent increased transcription could not be detected in NHEK, NHDF, LEC or HDMEC. However, variations in expressions were high, taking into account that even in the absence of added cytokine, transcription of IL-8 in NHEK, for example, was higher than upon addition of IL-17E or IL-17F. In MSC, a tendency towards elevated expression of CCL2, IL-6 and IL-8 could be observed with 50 nmol/L and 100 nmol/L IL-17A.

4. Discussion

Even if non-specific reactions occurred at the injection site during the application of a variety of therapeutic antibodies, direct skin toxicity is very unlikely [47]. The events are explained as immunological or allergic reaction patterns, which depend individually on the immunogenicity of the antibody, its specificity, the excipients contained and individual pathological factors of the patient [48,49]. The reactions involved are categorized according to the possible patho-mechanisms [50–52]. It is most probable that cutaneous cell types, in particular keratinocytes, are involved in various signal cascades and are thus functionally involved in possible adverse reactions [37,53]. For therapeutic antibodies of the anti-IL-17A group (secukinumab and ixekizumab) as well as for IL-17RA blockers (brodalumab) reactions at the injection site are described occasionally [48]. However, here too the above-named causes are assumed to be predominantly probable, so that toxic effects may also be produced by high cutaneous concentrations of the therapeutic antibodies. The available cytotoxicity results support this assumption and ensure the safety of high antibody concentrations with respect to toxic interactions.

The importance of IL-17 receptors in keratinocytes, especially in psoriatic skin, has already been described [30,54]. However, neither on keratinocytes nor other relevant cutaneous cell types the expression of all known IL-17R subunits has been systematically investigated. As already known from the data for psoriatic skin, the present studies corroborated the special significance of IL-17RA. IL-17RC and IL-17RD, however, show a subordinate significance. In comparison, the IL-17RB and IL-17RE subunits are expressed only very little in the cell types studied. We were able to confirm these results at the protein level. The detection of IL-17RB and IL-17RE was at the lower limit. The high expression rates of IL-17RA could be confirmed for NHEK and ESC using Western blot analysis.

Although the ostensible significance of IL-17RA is represented by the strong expression in all cutaneous cell types, the present studies show that the expression of neither IL-17RA nor the other IL-17R subunits were affected by IL-17A regardless of time and concentration. Because T cell-fibroblast co-cultures overexpressed IL17A and IL17RA, there must be co-stimulatory effectors, which we did not have in our experimental setting [55].

It is noteworthy that in all investigated cell types, activation by IL-17A, IL-17C, IL-17E or IL-17F cannot be detected experimentally in the MAP-MEK-ERK/p38/JNK or in the PI3K-Act-NFκB pathway. A possible explanation could be the lack of pre-treatment with an ERK inhibitor. In this context, renal fibroblasts after administration of IL-17A showed an activation of the extracellular signal-regulated kinase (ERK) 1/2, which had previously been reduced by treatment with the ERK inhibitor U0126 [56]. Thus, there is no evidence for an induction of the genes of pro-inflammatory cytokines. This indicates that co-stimulant molecules are missing in the experimental setting and all IL-17 subtypes alone do not cause activation of sessile cutaneous cells with respect to the synthesis of pro-inflammatory factors. An alternative explanation could be that a receptor-ligand ratio necessary for activation was not present under the conditions studied. Further studies are necessary to identify relevant co-stimulating factors from IL-17 subtypes

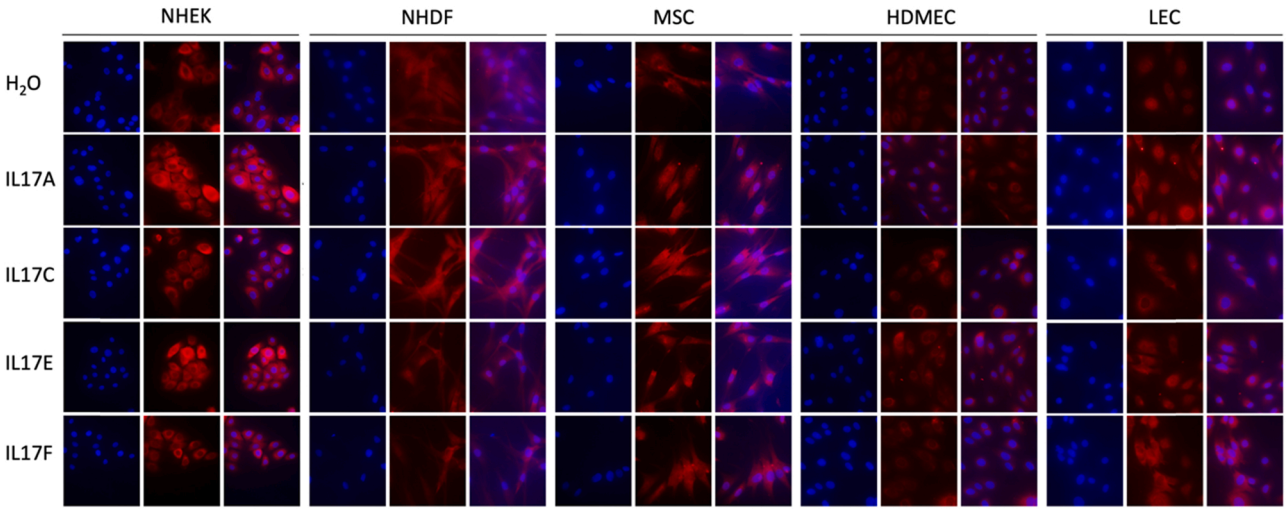


Fig. 3. Analysis of NF-κB subunit translocation into the nucleus upon addition of IL-17 ligands (IL-17A, IL-17C, IL-17E, IL-17F) in NHEK, NHDF, MSC, HDMEC, and LEC. (left = DAPI, center = α p65, right = merge).

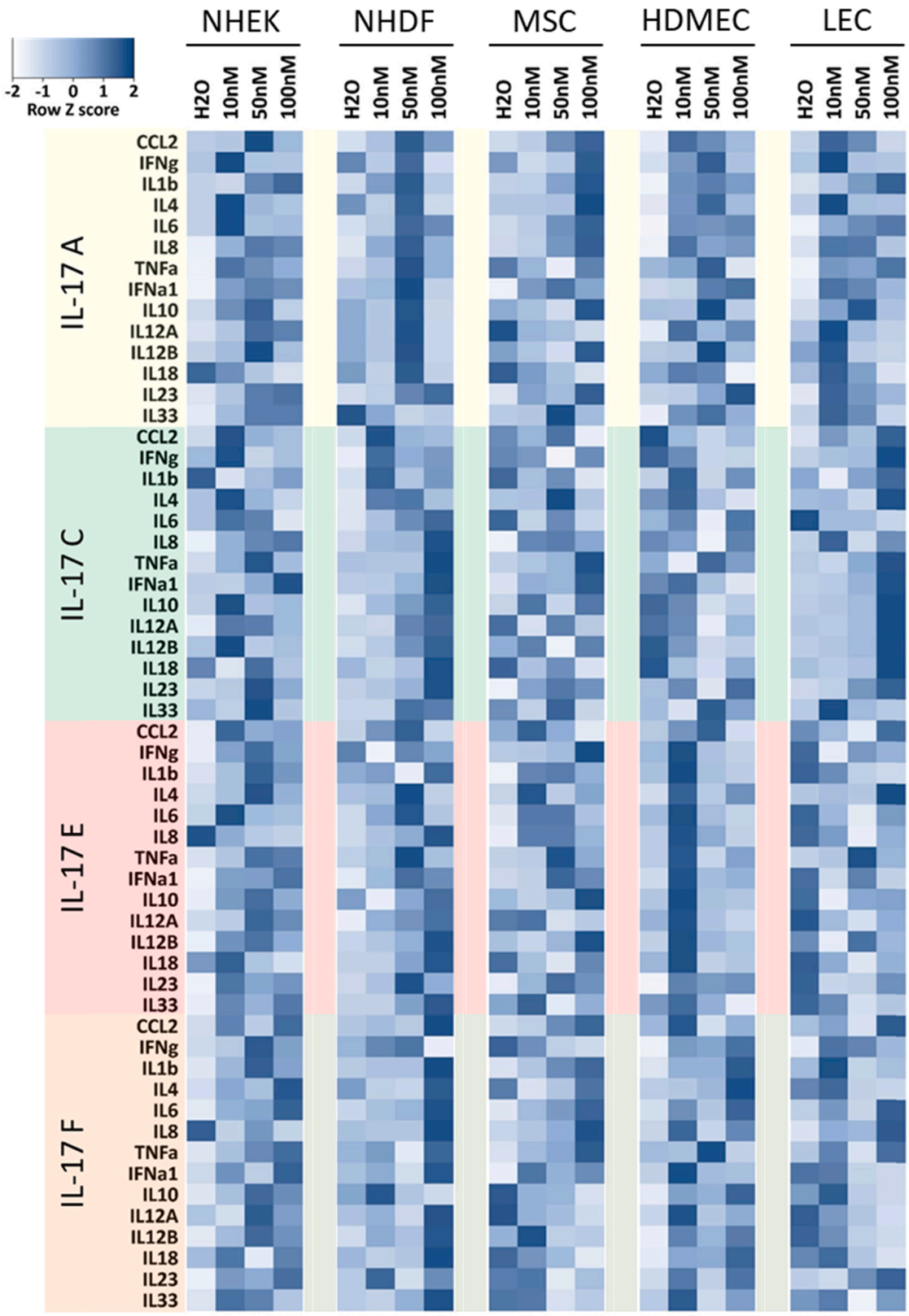


Fig. 4. qRT-PCR analysis of cytokine expression levels upon addition of IL-17A, IL-17C, IL-17E and IL-17F in NHEK, NHDF, MSC, HDMEC, and LEC. Heatmap shows the expression level of analyzed genes.

under physiological and pathophysiological conditions.

Acknowledgments

The authors wish to thank Sylke Fasshauer and Claudia Bruhne for excellent technical assistance. The investigations have been partly funded by Novartis Pharma GmbH (Nürnberg, Germany), (Grant ID: MAIN457_FVKH002). Medical writing support under the direction of the authors was provided by Elisabeth Schwarz, PhD (Halle/Saale, Germany).

Disclosures

J.W. has received fees for lecturing and/or consulting, and/or received funding for scientific projects and clinical studies from Abbvie, Almirall, Amgen, Biogen-Idec, Celgene, Galderma, GSK, Janssen-Cilag, Leo, Lilly, MSD, Mylan, Novartis, Pfizer, Sanofi, and UCB. K.G. was temporarily employed in the project through sponsors' funds. D.G. declares no conflicts of interest.

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