Interleukin-17 and Interleukin-22: key cytokines in tissue inflammation

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1. Summary

IL-17 and IL-22 are so called signature cytokines of T helper (Th) 17 and Th22 cells and link adaptive and innate immune responses via modulation of epithelial cell functions. In the present thesis, I could demonstrate that the function of Interleukin (IL)-17 and IL-22 depends from a local, inflammatory microenvironment in the tissue.

The effects of IL-17 and IL-22 often overlap or synergize in the instruction of epithelial cells in defense against pathogens. However, besides promoting innate immune responses, IL-17 and IL-22 also show distinct functions. IL-17 induces the expression of pro-inflammatory mediators and tissue damage in the epithelium, while IL-22 limits T cell-mediated epithelial damage and promotes tissue regeneration.

This thesis clearly demonstrates that the contribution of IL-17 and IL-22 to the outcome of human diseases depends from the inflammatory environment in the tissue. Here, IL-17 amplifies Th1 mediated immune responses in the epithelium while its’ effects are inhibited by a Th2 dominated environment. This ambivalence influences the outcome of allergic contact dermatitis (ACD) and atopic eczema (AE). Similarly, IL-22 reinforces TNF-α mediated innate immune responses, but inhibits pro-inflammatory effects of IFN-γ on the epithelium showing an overall tissue protective function of IL-22. Depending from the inflammatory environment in which it is expressed, IL-22 might turn pathological. This could be the case in diseases characterized by an exaggerated proliferation of epithelial cells such as psoriasis, cystic fibrosis, and asthma. Therefore, patients affected by chronic remodeling disorders could benefit from a therapeutic targeting of IL-22.

Besides Th17 and Th22 cells, other T cell subsets such as Th1/IL-17, Th2/IL-17, and Th1/IL-22 produce IL-17 and/or IL-22 could be defined. These T cell subpopulations co-produce more than one signature cytokine together. Synergistic and antagonistic effects of these cytokines in the epithelium determine the overall “unique tissue signature” of a T cell subset.
composition and relevance of these subtypes is different depending from the disease investigated.

Influencing the local tissue microenvironment of complex diseases, rather than targeting single T cell subsets or single cytokines, could promote the development of more efficient therapeutical approaches.
2. General Introduction

Innate and adaptive immune responses are two distinct defense mechanisms against pathogen invasion. The innate immune system immediately reacts to invading microbes in an invariant manner and with mechanisms that are pre-existent to the infection. The epithelial barrier, phagocytic cells such as macrophages and neutrophils, innate lymphoid cells (ILCs), natural killer (NK) cells and the complement system are part of innate immunity. The adaptive immune system is divided into humoral and cellular mediated immunity. Humoral immunity is mediated by B cell-produced antibodies, whereas cellular-mediated immunity is mediated by T lymphocytes.

2.1 Epithelial cells as first line of immune defense

Epithelial cells are the first line defense against pathogens (e.g. bacteria, fungi) and injuries. They prevent microbes and dangerous molecules to penetrate into the body. Besides this physical protection, epithelial cells are equipped of innate immune sensors such as Toll like receptors (TLR) that sense pathogen associated molecules and allow a first immune response\(^1\). When sensing pathogens, epithelial cells release anti-microbial molecules such as defensins and chatelicidins that damage invading pathogens and limit the infection\(^3\). Moreover, danger signals trigger the release of chemokines in epithelial cells, which in turn enhance the recruitment of non-resident immune cells to the site of inflammation\(^4\). Epithelial cells are also target of cytokines released by T cells during the inflammation. Upon effects of cytokines, epithelial cells secrete further pro-inflammatory molecules that amplify the inflammation\(^5\). Immune cells recruited to the site of inflammation mediate tissue damage\(^6,7\). The inflammatory phase terminates with a repair phase, characterized by remodeling of the tissue and wound healing\(^8\).
2.2 Effector cells of adaptive immunity - T cells

T lymphocytes or T cells belong to the adaptive immune system and are responsible for cellular immunity. They can be divided in functional distinct populations, among them CD4+ T helper (h) cells and CD8+ cytotoxic T cells. T cells recognize peptides bound to autologous proteins codified by genes present in the major histocompatibility complex (MHC) and expressed by the membranes of other cells. MHC-I molecules mount antigens derived from intracellular, while MHC-II molecules from extracellular pathogens. Two distinct T cell lineages recognize MHC molecules depending from the expression of CD8 or CD4 on the surface, which bind to MHC-I or MHC-II, respectively. The expression of CD4 or CD8 on mature T cell lineages is mutually exclusive and reflects the MHC restricted antigen recognition by T cells.

After antigen recognition on MHC-I, CD8+ cells mature in cytotoxic T cells (Tc) and are committed to kill cells infected by intracellular pathogens, such as viruses and intracellular microbes.

After antigen recognition on MHC-II, CD4+ cells release cytokines and directly modulate proliferation and differentiation of lymphocytes as well as other cell types, such as B cells, macrophages, other leukocytes and resident tissue cells.

2.2.1 CD4+ T helper (Th) cells

CD4+ cells influence the function of other immune cells by either helping or suppressing their function.

CD4+ T cells were discovered to help B cells in immunoglobulin production and were therefore named T helper (Th) cells. According to the release of their signature cytokines Interferon gamma (IFN-γ) and Interleukin (IL)-4 after antigen recognition Th cells were subdivided into Th1 and Th2, respectively. To develop into Th1 or Th2, naïve CD4+ T cells have to recognize their cognate antigen exposed by professional antigen presenting cells (APC) on MHC-II molecules. For the differentiation process three signals are essential: 1) specific antigen recognition through the T cell receptor (TCR), 2) presence of co-stimulatory factors such as B7 molecules and growth factors, 3) cytokines that
direct the phenotype into a certain subtype. The cytokines IL-12 and IL-4 determine if a naïve T cell will develop in Th1 or Th2, respectively\textsuperscript{12} (Figure 1).

In the following years, further T cell subsets such as T regulatory (Treg) and Th17 cells have been discovered.

Treg are key players in the maintenance of tolerance against self and harmless antigens. Naturally occurring CD4\textsuperscript{+}CD25\textsuperscript{+}FOXp3\textsuperscript{+} T cells (nTreg), IL-10 producing T cells (Tr1)\textsuperscript{15}, and TGF-b producing T cells (Th3)\textsuperscript{16,17} belong to the regulatory T cell compartment. While nTreg exert their inhibitory function by inhibiting T cell proliferation in a contact-dependent manner, Tr1 and Th3\textsuperscript{16} secrete inhibitory cytokines such as IL-10 and TGF-beta\textsuperscript{18}.

In the last decade much attention has been focused on Th17 cells because of their association with autoimmune diseases\textsuperscript{19}. Th17 cells are characterized by production of the signature cytokine IL-17A and other associated cytokines such as IL-17F, IL-21, IL-26 and IL-22 \textsuperscript{19,20}. Moreover, they express the transcription factor ROR-gamma and the chemokine receptors CCR6 and CCR4\textsuperscript{21,22}. TGF-b and IL-6 have been shown to be essential to promote Th17 cell polarization from the naïve compartment\textsuperscript{23} (Figure 1). However, the generation of Th17 cells co-producing IL-22 requires additional IL-23\textsuperscript{19}. IL-17 and IL-22 have been shown to synergize in the induction of innate molecules in epithelial cells\textsuperscript{20}.

More recently a new T cell subset named “Th22 cells” has been identified. This T cell subset is characterized by the production of IL-22 in absence of IFN-\textgamma, IL-4 and IL-17. Th22 cells are preferentially recruited in the skin according to expression of the chemokine receptors CCR10, CCR6, CCR4 and the cutaneous lymphocyte antigen (CLA)\textsuperscript{24,25}. TNF-\alpha and IL-6 have been shown to promote Th22 cell polarization from the naïve compartment while the aryl hydrocarbon receptor (AHR) and the transcription factor t-bet have been suggested to control the generation of Th22 cells on the transcriptional level\textsuperscript{26,27} (Figure 1).

Besides these well-defined T cells subsets, some less characterized T cells exist that co-express more than one signature cytokine\textsuperscript{28,29}. This phenomenon is called plasticity and raises the debate about the origin of these cells. Until today it is not clear if plastic T cells develop from the naïve compartment or result from the instability of some T cell subsets\textsuperscript{30,31}. 

Figure 1. Naïve T cell differentiation into Th subsets.
Naïve T cells are directed towards Th1, Th2, Th17 and Th22 depending from the cytokines encountered. T cell subsets are characterized by master transcription factors and by signature cytokines.
2.2.2 Functions of T helper cells

By releasing cytokines, Th cells promote immunoglobulin production in B cells, enhance the activity of other immune cells and activate epithelial cells\(^{32}\). Th1 cells release IFN-\(\gamma\) after the encounter of their cognate antigen. IFN-\(\gamma\) is strongly supporting the activity of innate immune cells such as NK cells and macrophages\(^{32}\). It is inducing the isotype switch in B cells towards the production of IgG\(^{32}\). Besides its effect on immune cells, IFN-\(\gamma\) plays an important role in epithelial cell responses. On IFN-\(\gamma\) exposure, epithelial cells release pro-inflammatory chemokines and cytokines that in turn amplify the recruitment of other immune cells to the site of inflammation\(^{33,34}\). Additionally, IFN-\(\gamma\) induces the expression of adhesion molecules such as MHC-I, MHC-II, and ICAM-1 on epithelial cells, enabling Th1 and Tc mediated cytotoxicity\(^6,33\) (Figure 2). Moreover, it has been reported to ameliorate fibrosis by inhibiting collagen synthesis, cell cycle arrest and apoptosis, as well as enhancing NK cell cytotoxicity\(^{35}\).

Th1 cells play an important role also in tissue damage. They induce epithelial cell apoptosis by two distinct mechanisms. The first mechanism is cytokine dependent. Here, IFN-\(\gamma\) induces epithelial cell apoptosis via down-regulation of anti-apoptotic factors in epithelial cells\(^7\). The second mechanism is contact dependent. By recognizing the antigen on MHC-II expressed by epithelial cells, Th1 cells adhere to the epithelium and via FAS-FAS-L engagement induce apoptosis. This second mechanism requires the adhesion molecule ICAM-1 (Figure 3)\(^6\).

Th2 cells are characterized by secretion of IL-4, IL-5 and IL-13. IL-4 further enhances the differentiation of Th2 cells and mediates the IgE class switch in B cells\(^{12}\). IgE is involved in defense against parasites by triggering mast cell and basophil degranulation\(^{12}\). However, by recognizing harmless allergens, IgE is a central molecule in allergic disorders\(^{12}\). By secretion of IL-5, Th2 cells interact with another granulocyte, the eosinophil. IL-5 in turn induces the maturation, enhances the survival, serves as chemoattractant and activates the degranulation of eosinophils\(^{12}\). By releasing IL-4 and IL13, Th2 also affect resident tissue cells. In fact, IL-4 has been shown to act synergistically with IFN-\(\gamma\) in the induction of
ICAM-1 on epithelial cells\textsuperscript{4,5}. Moreover, both IL-4 and IL-13, amplify the recruitment of eosinophils by enhancing chemokine release such as eotaxin-3/CCL26 by epithelial cells. Th2 cytokines also affect innate immune responses of epithelial cells by reducing beta-defensin-2 and S100/A11 expression in keratinocytes\textsuperscript{36}. Finally, Th2 cytokines mediate tissue remodeling by inducing collagen synthesis in dermal fibroblasts and are therefore important mediators of fibrotic processes (Figure 2)\textsuperscript{37}.

Th17 cells are involved in autoimmune diseases such as experimental autoimmune encephalitis (EAE), rheumatoid arthritis and inflammatory bowel disease\textsuperscript{38-40}. IL-22 reinforces the IL-17 mediated induction of many pro-inflammatory mediators such as IL-8, IL-6, CCL20 and GM-CSF. This synergism has been suggested to be involved in the pathogenesis of diseases such as psoriasis and cystic fibrosis\textsuperscript{41,42}. Moreover, Th17 cells play a key role in triggering innate immune responses in epithelial cells. IL-17 and IL-22 synergistically induce beta-defensin-2 in epithelial cells, a key factor for the clearance of candida infection (Figure 2)\textsuperscript{20}. An impaired Th17 functionality is associated with autosomal-dominant hyper-IgE syndrome and chronic mucocutaneous candidiasis\textsuperscript{43,44}. In both diseases, patients are affected by recurrent infections of the barrier by \textit{Candida albicans (C. albicans)} highlighting the relevance of Th17 cytokines in innate immune responses.

In contrast to the well-known T cell subsets, very little is known about the function and disease involvement of Th22 cells.
**Figure 2:** Th cells differentially instruct epithelial cells for distinct tissue responses.
2.3 Atopic Eczema

Atopic Eczema (AE) is a chronic inflammatory skin disease. AE patients suffer from an impaired quality of life and therapy causes heavy health care cost\textsuperscript{45,46}. The pathogenesis of AE is the result of a complex interaction between environment, genetic susceptibility, altered skin barrier function and the immune system\textsuperscript{47}. This interaction leads to the characteristics of the disease that are epidermal barrier dysfunction, trans-epithelial water loss and dry skin, pruritus and often sensitization to environmental allergens. Hereby, the skin on the flexural surface of the joints (knee and elbow) is mainly affected. The genetic background influences the barrier dysfunction of atopic eczema patients predisposing their skin to be very sensitive to external insults\textsuperscript{48}. Subjects carrying the null mutation in the filaggrin gene have a lower expression of filaggrin and are more susceptible to develop AE\textsuperscript{49,50}. Filaggrin plays a key role in epidermal barrier functions and its association with atopic eczema emphasizes the importance of barrier dysfunction in eczema pathogenesis. About two third of AE patients develop multi-allergen sensitizations and react to allergen with eczematous reactions and chronic eczematous inflammation. It is discussed if the sensitization can take place via the disrupted epidermal barrier and/or if it is the result of an immune deviation.

Innate immune responses also influence the outcome of AE. In fact, an impaired beta-defensin-2 expression has been shown in the skin of AE patients\textsuperscript{51}. Consistently, \textit{Staphylococcus aureus} (\textit{S. aureus}) colonizes more than 85% of patients and is associated with increased disease severity\textsuperscript{52}. It has been speculated that exotoxins produced by \textit{S. aureus} stimulate T cells infiltrating the skin during the eczematous reaction independently from their specificity\textsuperscript{52,53}. This mechanism may aggravate atopic eczema by boosting a non-specific T cell immune reaction.

Concerning the contribution of T cells, AE is considered a Th2-mediated disease\textsuperscript{54,55} (Figure3). Th2 cells support the generation of allergen-specific IgE that in turn mediates mast-cell activation and tissue damage. Upon allergen exposure, patients affected by AE react with a biphasic immunologic response
characterized by an initial Th2 pattern that switches towards a Th1 response in later phases of the disease\textsuperscript{54,55}. Besides Th2, increasing numbers of reports indicate that new T cell subsets such as Th17 cells may be also involved in the pathogenesis of AE. IL-17 has been shown to be expressed in the skin of AE patients and to be more expressed in the severe cases\textsuperscript{56}. However, the number of studies evaluating the contribution of IL-17 producing T cell subsets in the context of a Th2 dominated disease is very limited. IL-22 producing cells have been also described to infiltrate the skin of AE patients and suggested to play a role in the control of pathogen colonization.

\textbf{2.4 Allergic Contact Dermatitis}

Allergic contact dermatitis (ACD) is a type IV hypersensitivity reaction resulting from an imbalance in the regulatory and specific effector T cell compartment\textsuperscript{57,58}. Contact allergy affects about 20\% of the European population and is characterized by an erythematous reaction at the side of allergen contact\textsuperscript{59}. Sensitized patients react to small molecular weight allergens that are called haptens and penetrate the skin to induce an exaggerated T cell response\textsuperscript{60}. The most common contact allergens are nickel, fragrances and preservatives. Contact allergy is one of the most important reasons for work-related incapacity to work and therefore of high socioeconomic interest\textsuperscript{59}.

In murine models of ACD, CD8\textsuperscript{+} and CD4\textsuperscript{+} cells play two distinct roles. CD8 cells are main mediators of tissue damage while CD4\textsuperscript{+} cells are predominantly regulatory cells\textsuperscript{61}.

In humans, CD4\textsuperscript{+} cells play an important role in amplification of the eczematous reaction\textsuperscript{5}. Dendritic cells located in the tissue present the contact allergen to CD4\textsuperscript{+} cells. After antigen recognition T cells produce large amounts of cytokines such as IFN-\textgamma, IL-17, IL-22 and IL-2 that affect epithelial responses and amplify the extent of the immune reaction\textsuperscript{5} (Figure 3). Only a minor fraction of T cells infiltrating the ACD reaction is specific to the causative allergen and the role of non-antigen specific T cells during the effector phase of immune responses has been little investigated\textsuperscript{62}. 
Besides their amplificatory role, Th1 cells directly contribute to the epithelial damage during ACD. Upon IFN-γ exposure, epithelial cells up-regulate MHC-II and therefore become susceptible to Th1 mediated cytotoxicity\textsuperscript{33}. IL-17 has been shown to play an important role in mouse models of ACD\textsuperscript{63}. Mice lacking IL-17 expression have an impaired sensitization to haptens as well as a reduced elicitation of the effector phase of contact hypersensitivity. The reduced magnitude of the effector phase has been shown to be dependent from a diminished infiltration of granulocytes\textsuperscript{64}. Since neutrophilic infiltration in human ACD is a rare event, the contribution of Th17 cells to human ACD has to be determined in future studies.

### 2.5 Asthma

Asthma is a chronic inflammatory disease characterized by airflow obstruction and tissue remodeling\textsuperscript{65}. It is classified according to the allergic status and severity of the disease\textsuperscript{66}. Allergic asthma (extrinsic) affects the vast majority of asthmatic patients during childhood and about 30% of adult asthmatic patients. In allergic asthma allergen-specific IgE induces mast cell degranulation and the consequent release of factors mediating bronchial constriction and remodeling (Figure3). Non-allergic asthma (intrinsic) is mainly affecting adult asthmatic patients\textsuperscript{65}. To this group belongs the majority of patients affected by severe asthma, corticoid-resistance and severe asthmatic attacks\textsuperscript{67}. Severe asthma is characterized by strong remodeling with tendencies to fibrosis and tissue damage. T cells mediate remodeling by secretion of pro-fibrotic cytokines such as IL-13 and IL-17. By inducing molecules such as G-CSF, IL-6 and IL-8, IL-17 induces recruitment of immune cells that in turn further drives severe fibrosis\textsuperscript{42,68}. Moreover, IL-17 has also been shown to induce airway smooth muscle cell contraction leading to bronchiolar constriction\textsuperscript{69}. The role of IL-22 in lung diseases is matter of debate. Together with IL-17, IL-22 has been shown to induce recruitment of granulocytes aggravating the outcome of allergic asthma in mice. However, lung inflammation of mice lacking IL-17 expression is ameliorated by IL-22 injection\textsuperscript{70}. Besides animal studies, the role of IL-22 producing T cells in human asthma has not been investigated.
Figure 3. Role of T cells in the pathogenesis of Psoriasis, Allergic Contact Dermatitis, Atopic Eczema and Asthma.
3. Aim of the study

The aim of this study is to identify IL-17 and IL-22-producing T cell subsets infiltrating skin and lung tissue during inflammatory disorders and to investigate the functional consequences of IL-17 and IL-22 in the context of a Th1 and Th2 dominated tissue environment.
4. Material and methods

4.1 Patients
In the present study, patients affected by ACD, AE, psoriasis and asthma have been included. Human samples were obtained after informed consent and the local ethical committee approved all studies. Further details are provided in the material and method section of publications 1-5.

4.2 Immunohistochemistry
Biopsies were embedded in paraffin. Five-micrometer sections were firstly dewaxed in xylene, secondly in ethanol and next boiled in Tris-EDTA buffer. After quenching endogenous peroxidase, slides were incubated with polyclonal rabbit anti-human IL-22 (Novus Biologicals), goat anti-human IL-17 (R&D Systems) and/or rabbit anti-human CD3 (Dako). Further details are provided in the material and method section of publications 2 and 5.

4.3 Isolation of tissue infiltrating T cells
Lung and skin biopsies were cultured in complete RPMI 1640 medium supplemented with human serum and interleukin-2 (IL-2). Emigrating cells were expanded by means of IL-2 alone or in combination with anti-CD3/anti-CD28. Depending from the experiment, emigrating T-cells were collected between day 2 and day 13. Further details are provided in the material and method section of publications 1-5.

4.4 T cell cloning
T cell clones were obtained by plating T cells at limiting dilution (0.6 cells/well) in RPMI medium supplemented with 5% human serum, 10% fetal bovine serum (FBS) and IL-2 ranging between 20-60 U/ml. Further details are provided in the material and method section of publications 1-4.

4.5 Flow cytometry analysis
Staining of surface markers and intracellular cytokines were performed using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's
instructions. Acquisition and analysis were performed with the FACS-Calibur or FACS-Canto II (both BD Biosciences). Further details are provided in the material and method section of publications 1-5.

4.6 T cell proliferation assays
T cell antigen specific proliferation was measured by [³H]thymidine deoxyribose (Tdr) incorporation (Amersham Biosciences) or CFSE staining. Further details are provided in the material and method section of publications 1-3.

4.7 Keratinocyte and bronchial epithelial cell culture
Primary keratinocytes were derived from skin biopsies of patients and healthy donors. Cells of the first, second, and third passage were used in experiments. Primary human bronchial epithelial cells from healthy (NHBE) or asthmatic (DHBE) subjects were purchased and cultured in bronchial epithelial cell medium (Lonza). Epithelial cells were stimulated with recombinant cytokines or T cell clone supernatants in growth factor–free basal medium (BEBM; Lonza). Further details are provided in the material and method section of publications 1-5.

4.8 Dendritic cell generation and co-culture with T cells
Immature dendritic cells (iDCs) were generated from CD14+ monocytes. Briefly, CD14+ monocytes were isolated by MACS sorting (Miltenyi Biotech) and cultured in complete RPMI medium supplemented with human rIL-4, and rGM-CSF for 5 days. Mature DCs (mDCs) were generated stimulating iDCs with 50 μg/mL LPS (Invitrogen) for 24 hours. Further details are provided in the material and method section of publications 1 and 2.

4.9 ELISA
Measurement of TNF-α, IFN-γ, IL-4, IL-17, IL-22, IL-10, IL-13, (all R&D systems), and HBD-2 (Phoenix Pharmaceuticals) was performed in cell-free culture supernatants by using commercially available ELISA kits and following the manufacturer's instructions of the respective companies. Further details are provided in the material and method section of publications 1-5.
4.10 Real-time PCR analysis
Total RNA was isolated from human primary cells (Qiagen) and reversely transcribed with random oligo (dT) primers according to the manufacturer’s protocols of the respective kits (Roche, Applied Biosystems). The obtained cDNA was amplified using SYBR Green Mastermix (Applied Biosystems) and gene specific primers. Further details are provided in the material and method section of publications 1-5.

4.11 Whole-genome microarray analysis
Microarray analysis was performed by using “SurePrint G3 Human Gene Expression 8x60K kit” or “Agilent Whole Human Genome Oligo Microarrays 4x44K kit” (both Agilent Technologies). Differential gene expression was analyzed with the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware) or Genespring Software GX 11.0 (Agilent Technologies). Further details are provided in the material and method section of publications 3 and 5.

4.12 T cell-epithelial cell cytotoxicity
The T cell mediated cytotoxicity of EBV-transformed B cell lines (B-LCL) and primary autologous keratinocytes was measured by means of [3H]TdR release assay. The T cell mediated cytotoxicity on NHBE cells was measured by means of LDH cytotoxicity detection kit (Roche). Further details are provided in the material and method section of publications 2 and 5.

4.13 Keratinocyte-T cell adhesion
Keratinocytes were seeded in culture slides (BD Biosciences) and stimulated with recombinant cytokines. After stimulation, autologous nickel-specific T cell clones were stained with CFSE and incubated with autologous keratinocytes. After 6 hours, co-cultures were extensively washed in PBS, and adherent CFSE positive T cells were measured. Further details are provided in the material and method section of publication 2.
4.14 Wound repair assay
Confluent monolayers of primary epithelial cells were scratched with a tip. Wounded monolayers were then incubated with recombinant cytokines, T cell supernatants and/or blocking antibodies. The residual gap between migrating cells was measured with a computer-assisted image analysis system (AxioVision 4.5, Zeiss). Further details are provided in the material and method section of publications 3 and 5.

4.16 In vitro Candida infection model
Human oral keratinocytes were cultured in 96 well plates for the two dimensional infection model or on polycarbonate filter for the three dimensional infection model and next infected with C. albicans wild type strain SC5314. Recombinant cytokines and Th22 cell supernatants were incubated between 12 and 20 hours. Candida infection was measured either by LDH release or by immunohistochemistry studies. Further details are provided in the material and method section of publication 4.

4.17 In vivo experiments
Definite areas on the forearm of a patient with AE were challenged with commercially available D. pteronyssinus (Stallergenes). 36 hours later, 50 mg/cm² SEB were added onto one areal, additionally. Epidermis and blister fluids were obtained by suction blister 60 hours after allergen application. Further details are provided in the material and method section of publication 1.
5. Summary of results

5.1 Publication 1: IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response

Patients affected by AE often suffer of *S. aureus* skin colonization that aggravates the outcome of the disease. IL-17 is a key cytokine mediating epithelial innate immune responses against extracellular pathogens.

In the present study, I sought to investigate the contribution of IL-17 producing T cells to the antimicrobial immune responses of AE patients.

About 10% of T cells infiltrating the skin of AE patients were producing IL-17 upon PMA/ionomycin stimulation. I identified antigen specific Th0/IL-17 and Th2/IL-17 producing T cells infiltrating the skin of AE patients. The cognate antigen was effectively inducing the release of IFN-γ and IL-4 by specific Th2/IL-17 and Th0/IL-17 T cell clones, whereas was not sufficient for an efficient induction of IL-17. IL-17 secretion by Th2/IL-17 T cell clones was boosted by the *S. aureus*-derived super-antigen staphylococcus enterotoxin B (SEB), but not by tissue environmental cytokines such as IL-23, IL-1β, or IL-6. IL-17 induced beta-defensin-2 (HBD-2) release in keratinocytes in both, healthy and AE subjects. However, the Th2 cytokines IL-4 and IL-13 reduced the IL-17 mediated induction of HBD-2. In vivo, additional application of SEB to a house dust mite atopy-patch-test reaction boosted the secretion of IL-17 and HBD-2 in the skin, but only marginally the one of IL-4, IFN-γ, and IL-10.

In conclusion, IL-17 producing T cells infiltrate the skin of patients affected by AE. The cognate antigen is not sufficient for the full induction of IL-17 by specific T cells, while bacterial-derived super-antigens trigger IL-17 production. A Th2-dominated skin microenvironment impairs the IL-17-dependent up-regulation of HBD-2 contributing to the susceptibility of AE patients to colonization of *S. aureus*.

5.2 Publication 2: IL-17 amplifies human contact hypersensitivity by licensing hapten nonspecific Th1 cells to kill autologous keratinocytes

Allergic contact dermatitis is a self-limited skin immune disorder characterized by robust epithelial damage. Recently, a new T cell subset named Th17 has been
shown to be involved in autoimmune disorders and immune responses to pathogens. In this study, I aimed to investigate the contribution of IL-17-producing T cells to the pathogenesis of ACD.

IL-17 was expressed in skin of patients affected by ACD by heterogeneous IL-17 producing T cell subsets. Although pure Th17 infiltrated ACD skin, hapten specificity was restricted to Th1/IL-17, Th0/IL-17 and Th2/IL-17 T cell subsets. Compared to Th1 cells, Th1/IL-17 induced similar amounts of CXCL8, IL-6, and HBD-2 in keratinocytes. However, Th1/IL-17 T cells were inducing more ICAM-1 expression on keratinocytes compared to Th1 cells. The increased ICAM-1 expression by keratinocytes in turn increased the adhesiveness of T cells to keratinocytes and promoted the ICAM-1-dependent antigen non-specific T cell killing.

In conclusion, Th1/IL-17 is the most common specific IL-17 producing T cell subset, among the one infiltrating the skin of patients affected by ACD. In presence of IFN-γ, IL-17 enables non-specific Th1 cells to damage the epithelium amplifying the extent of T cell mediated epithelial damage.

5.3 Publication 3: Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling

Interleukin-22 is a cytokine involved in inflammatory and wound healing processes. Interleukin-22 has been shown to be expressed by Th1 cells and more recently by Th17 as well as a subpopulation of NK cells. In this work, I identified a T cell subset characterized by the production of IL-22 and TNF-α, but that lacks expression of IFN-γ, IL-4 and IL-17. These so called “Th22” cells infiltrate the skin of patients affected by psoriasis, AE and ACD. Th22 T cell clones derived from T cells expanded from psoriatic patients were stable when cultured with cytokines involved in T cell polarization. Th22 clones showed distinct transcriptome profiles compared to Th1, Th2, and Th17 clones. The Th22 cytokines TNF-α and IL-22 were synergistically promoting innate immune responses in primary keratinocytes. Moreover, Th22 supernatant promoted in vitro wound healing, which was exclusively dependent on IL-22.

In conclusion, our study identifies a new T cell subset lineage named Th22 that is modulating epithelial cell functions towards innate immune responses and
remodeling.

5.4 Publication 4: *IL-22 and TNF-α represent a key cytokine combination for epidermal integrity during infection with Candida albicans*

The newly identified Th22 cell subset has been described to produce IL-22 in combination with TNF-α and to be involved in epidermal immune responses. Aim of the present study was to evaluate the contribution of Th22 cells to innate immune responses against *C. albicans*. I show that IL-22 reinforces the TNF-α-mediated activation of primary human keratinocytes. This synergism of IL-22 and TNF-α induces several molecules involved in innate immunity such as complement factors, chemokines and antimicrobial peptides. Intracellularly, the synergism is mediated by MAP kinases and AP-1 family transcription factors. In an *in vitro* Candida infection model, the combination of IL-22 and TNF-α as well as supernatant derived from Th22 clones, effectively reduced growth of *C. albicans* in the same strength like IL-17. However, only the combination of IL-22 and TNF-α or Th22 supernatant was able to protect the integrity of the epidermal barrier in a 3D skin infection model compared to IFN-γ, IL-17, IL-22 or TNF-α alone. In conclusion, I showed that IL-22 and TNF-α act synergistically to protect the epithelium from *C. albicans* infection.

5.5 Publication 5: *IL-22 suppresses IFN-γ-mediated lung inflammation in asthmatic patients*

Asthma is a chronic inflammatory lung disease characterized by an excessive tissue remodeling. The role of IL-22 in lung inflammation is controversial. On the one hand, IL-22 amplifies the pro-inflammatory effects of IL-17 in mouse models of asthma. On the other hand, in absence of IL-17, IL-22 ameliorates lung inflammation. In the present study, I aimed to explore the anti-inflammatory properties of IL-22 in human asthma. The data highlight a functional tissue-restricted mutual antagonism of IL-22 and IFN-γ. Induction of the antimicrobial peptide S100A7 and bronchial epithelial cell migration mediated by IL-22 were antagonized by
IFN-γ. Conversely, IL-22 reduced T cell-mediated damage of the epithelium by reducing the MHC-I, MHC-II and CD54/ICAM-1 expression induced by IFN-γ. Moreover, IL-22 decreased IFN-γ mediated induction of pro-inflammatory chemokines such as CCL5/RANTES and CXCL10/IP-10 \textit{in vitro}. In line with these results, expression of IL-22 in bronchoalveolar lavage (BAL) fluids collected from asthmatic patients inversely correlated with CCL5/RANTES and CXCL10/IP-10 expression.

In conclusion, IL-22 has the potential to control the extent of IFN-γ-driven and epithelial cell-mediated lung inflammation.
6. Discussion

In this thesis I demonstrated that functions of IL-17 and IL-22 differ depending from the inflammatory context considered\(^{71}\). I give four examples of a local tissue inflammatory environment influencing IL-17 and IL-22 effects and I describe how these conditions shape the outcome of human diseases. Moreover, I demonstrated that the co-production of more than one signature cytokine determine distinct functions of “non-canonic” compared to the “canonic” T cell subsets.

6.1 Tissue infiltrating T cell subsets, specificity and plasticity

Since the discovery of Th1 and Th2 cells by Mosmann et al, the T cell family was initially extended to T regulatory and Th17 cells. Paralleling the improvement of technologies, new T cell subsets such as Th22\(^{25,26,24}\) and Th9\(^{30}\) cells joined the family, recently. However, the classical grouping scheme of Mosmann et al has its limits in explaining T cells that co-express more than one signature cytokine.

I identified a distinct IL-22 producing T cell subset that is lacking the production of the other signature cytokines IL-17, IFN-\(\gamma\) and IL-4\(^{24}\). This T cell subset was stable in culture and expressed little or none RORC, GATA-3 or T-bet. The idea that this so-called Th22 cells are an independent T cell subset is further supported by other studies showing that Th22 cells develop from the naïve compartment by a distinct polarization condition and that this polarization is driven by plasmacytoid dendritic cells (pDC) rather than conventional DC (cDC)\(^{25}\).

Consistently with previous evidences suggesting that Th22 cells express skin homing receptors such as CCR10, CCR4 and CLA, I found that Th22 cells were enriched in the epidermis of patients affected by psoriasis, atopic eczema and allergic contact dermatitis\(^{24}\). Together with the evidence that tissue cells selectively express the IL-22 receptor\(^{72}\), Th22 cells appear to play their main role in the instruction of tissue cells rather than helping B cells in immunoglobulin production.
It is increasingly appreciated that tissue infiltrating T cells are heterogeneous in terms of cytokine production. In fact, besides Th22 other T cell subsets are a source of IL-22 in the skin. I found T cells co-producing IFN-γ, IL-4 and/or IL17 together with IL-22. This heterogeneity may be the result of the instability of some T cells. While Th22 cells are stable in vitro, Th17 cells are sensitive to the effects of other molecules. It has been shown that agonists of the aryl hydrocarbon receptor (AHR) inhibit IL-17 while promoting IL-22 expression in Th17 cells. Ligands include environmental toxins, and endogenous ligands, such as heme metabolites, indigoids, dietary components, and tryptophan metabolites. Therefore, factors released during tissue damages may influence the Th17/Th22 balance. This observation indicates that Th17 might terminally differentiate into Th22 cells in the tissue upon AHR activation.

The highly plastic phenotype of Th17 cells is in line with our finding that heterogenic IL-17 producing T cells infiltrate the skin of patients affected by AE and ACD. Besides Th17, I found IL-17 being co-produced with IFN-γ (Th1/IL-17) and IL-4 (Th2/IL-17). It is in debate if Th1/IL-17- and Th2/IL-17-producing cells are generated by the naïve compartment upon special conditions or if they originate from Th17 plasticity.

Th1/IL-17 cells have been observed in healthy as well as patients affected by Crohn’s disease. The chemokine receptor repertoire of these cells is slightly different compared to Th17 cells. However, it has been shown that stimulation with IL-12 induces T-bet and IFN-γ expression in Th17 clones while IL-23 reduces their expression, indicating that Th1/IL-17 may generate from memory Th17 cells.

In contrast, Th2/IL-17 cells have been shown to generate from the naïve compartment when IL-4 is added to the Th17 polarization condition. These cells co-express RORC and GATA3, the master transcription factors of both Th17 and Th2 cells. Thus, the heterogeneity of T cell subsets infiltrating the tissue maybe a consequence of the balance between T cell polarization of naïve cells and T cell plasticity of effector cells.
The specificity of pure Th17 and Th22 cells is still a matter of debate. Pure Th17 cells have been found to be specific for *C. albicans* and *S. aureus* while the specificity of Th22 cells is still unknown. It has been shown recently that a subset of IL-22-“only producing” CD4+ cells were activated after encounter of CD1a expressed by autologous tissue cells. But it is still unclear if these auto-reactive CD4+ cells are Th22 cells or “NK-like” immune cells.

Although pure Th17 and Th22 cells are infiltrating the tissue during skin hypersensitivity disorders, they did not show allergen reactivity. By cloning T cells out of allergen-induced eczema lesions, Nickel and Der p 1-specific Th1/IL-17, Th2/IL-17, Th0/IL-17 clones but not “pure Th17 or Th22” clones could have been identified in ACD and AE, respectively.

Thus, while Th1/IL-17 and Th2/IL-17 are responsive to the cognate allergen and play a main role in allergies, the role of pure Th17 and Th22 remains unclear.

In this scenario, Th1/IL-17 and Th2/IL-17 could be the main IL-17-producing cells during allergic reactions, while pure Th17 cells are mainly involved in chronic and/or pathogen related diseases. This hypothesis would be in line with the effectiveness of anti-IL-23 therapy in Psoriasis but not in ACD.

### 6.2 IL-17 in Atopic Eczema

Th2 cells and cytokines dominate the initial phase of an AE reaction. In this context, I identified a previous unknown Th17 subset that is co-producing IL-4 and IL-17 and named “Th2/IL-17 cells”. The existence of this subset has been further confirmed by other authors in Th2 diseases and it has been suggested that these cells derive from Th17 cells.

Although Th2/IL-17 cells were producing both IL-4 and IL-17 upon a strong stimulation such as PMA/Ionomycin, they were releasing IL-4 but not IL-17 when exposed to the cognate antigen Der p 1. This observation indicates firstly that Th2/IL-17 cells behave as Th2 cells when exposed to the cognate antigen contributing to the establishment of a Th2 dominated environment in AE. Secondly, recognition of the antigen presented by DC is not sufficient for full activation of a multi-potent T cell, and that secretion of some T cell cytokines may require additional signals.
I investigated if local environmental factors may influence the Th2/IL-17 functionality\textsuperscript{28}. I exposed Th2/IL-17 clones to the Th17 related cytokines IL-23, IL-1b and IL-12\textsuperscript{20,82}. Moreover, since the skin of AE patients is often colonized by \textit{S. aureus}, I further stimulated Th2/IL-17 clones with \textit{S. aureus} enterotoxin B (SEB). When Th2/IL-17 clones were exposed to SEB, but not to cytokines such as IL-23, IL-1b and IL-12, the production of IL-17 was boosted. Similar to Th17 cells, Th2/IL-17 are committed to \textit{S. aureus} responses but show a double threshold of activation. In response to the cognate antigen they behave as common Th2 cells but in presence of \textit{S. aureus} they respond with additional secretion of IL-17\textsuperscript{28}. Thus, \textit{S. aureus} colonization differentially directs a Th2 dominated reaction towards an IL-4/IL-17 response by affecting the single T cell behavior. This finding underlines the role of a local tissue inflammatory environment in modulating the function of infiltrating T cells.

The influence of an inflammatory microenvironment on the disease outcome is not restricted to effects of inflammatory factors on T cells but extended to effects on epithelial cells. In fact, a Th2-dominated environment impaired the IL-17 induced beta-defensin-2 (HBD-2) release by keratinocytes\textsuperscript{28}. This evidence is in line with previous studies showing an impaired beta-defensin-2 expression in AE patients\textsuperscript{51}. Thus, by reducing the efficiency of the IL-17/HBD-2 axis a Th2 dominated environment contributes to the continuous \textit{S. aureus} skin colonization in AE patients.

\textbf{6.3 IL-17 promotes tissue damage in Allergic Contact Dermatitis}

While IL-17 effects are inhibited by IL-4\textsuperscript{28}, IL-17 amplifies the epithelial cell damage in a Th1 dominated environment\textsuperscript{77}. In presence of IFN-\gamma, IL-17 intensifies the epithelial damage by enabling non-antigen-specific T cells to induce epithelial cell apoptosis.

IL-17 is known to contribute to the pathogenesis of several autoimmune diseases via recruitment of granulocytes to the site of inflammation\textsuperscript{63}. Similar to autoimmune diseases, blocking of IL-17 in mouse models of contact hypersensitivity (CHS) has been shown to ameliorate CHS by reducing numbers of infiltrating granulocytes\textsuperscript{64}. Although IL-17 induces production of the
neutrophil chemoattractant CXCL8 in human keratinocytes, neutrophilic infiltration in ACD is a rare event implying that the role of IL-17 is not limited to neutrophil recruitment. This discrepancy highlights how mouse models differ in some features from human diseases. Cytokines expressed in ACD but not in CHS may be specifically inhibiting the IL-17 dependent neutrophilic recruitment.

In ACD, IL-17 is produced in presence or together with IFN-γ. In fact, the ACD skin environment is dominated by IFN-γ. Moreover, Th1/IL-17 is the hapten-specific and most abundant IL-17 producing T cell subset infiltrating the skin during ACD.

The co-production of IL-17 and IFN-γ established a unique tissue signature profile of Th1/IL-17 cells. On the one hand Th1/IL-17 cells were inducing similar mediators in human keratinocytes compared to Th17 cells such as IL-6 and HBD-2. On the other hand, Th1/IL-17 were inducing less CCL-5 but more ICAM-1 expression in keratinocytes when compared to Th1 cells. Indeed, IL-17 synergistically up-regulated IFN-γ mediated ICAM-1 expression that in turn stabilized the T-cell-keratinocyte contact allowing non-specific Th1 cells to induce keratinocyte apoptosis. This mechanism virtually involves the totality of non-antigen-specific Th1 cells infiltrating the skin. Therefore, non-specific infiltrating cells might turn from spectators to direct actors in tissue damage in presence of an IFN-γ/IL-17 dominated environment.

Further studies are needed to evaluate the role of non-specific T cell mediated cytotoxicity caused by Th1/IL-17 in autoimmune/auto-inflammatory diseases.

6.4 IL-22 maintains epidermal integrity upon Candida albicans infection

The role of IL-22 in inflammatory skin diseases is unclear. The effects of IL-22 on epithelial cells are limited to the modulation of a small amount of genes. Alone, IL-22 is inducing genes involved in epithelial cell migration and differentiation. Besides anti-microbial peptides, IL-22 is not inducing pro-inflammatory chemokines suggesting a major role in innate and tissue protective responses.

In contrast to other T cell subsets, Th22 cells promote epithelial cell migration and modulate chemokines and innate related molecules released by keratinocytes. Although it has not been proven that Th22 cells mediate tissue...
remodeling in vivo, IL-22 has been shown to be a key player in epithelial cell proliferation and differentiation. Mice lacking of IL-22 production are impaired in regeneration of the lung epithelium and thymus suggesting an important role in wound healing. Moreover, Th22 cells release factors such as FGFs that are also involved in tissue regeneration. Together these evidences indicate that Th22 cells may be key players in epithelial remodeling.

While the Th22-induced epithelial migration is mediated by IL-22, the induction of chemokines and cytokines in epithelial cells is the result of a synergism between IL-22 and TNF-α. This cytokine combination allows Th22 cells to effectively activate epithelial cells. Indeed, TNF-α enhances parts of the IL-22-mediated effects for instance the IL-22 induced release of antimicrobial peptides and the consequent clearance of C. albicans. In addition, the combination of TNF-α and IL-22 is as powerful as IL-17 in the induction of HBD-2 indicating that Th22 and Th17 cells have some redundant functions. However, a depletion of both subsets caused by gain-of function STAT1 mutation can lead to the rare disease chronic mucocutaneous candidiasis (CMC), where infections with C. albicans cannot be controlled by the immune system.

The synergism of IL-22 and TNF-α is not mediated by the classical IL-22 and TNF-α intracellular pathways but by the activation of AP-1 family transcription factors indicating a unique tissue signature of this cytokine combination. These results are in line with the role of IL-22 in CMC as well as with side effects of anti-TNF-α therapy such as skin and respiratory infections.

Similarly, Th22 cells efficiently enhance epithelial cell migration and C. albicans clearance suggesting that by releasing both TNF-α and IL-22, Th22 play a key role in protecting the integrity of the tissues from damage and C. albicans infection.

6.5 Regulatory role of IL-22 in asthma
Studies in mouse models of fibrosis and asthma show that the pro-and anti-inflammatory role of IL-22 is controlled by co-expression of IL-17. Another
study shows the potential of IL-22 to inhibit chemokine release and recruitment of inflammatory cells to the lung\textsuperscript{90}. Paralleling the finding in mice, IL-22 reduces the chemokine release by human bronchial epithelial cells\textsuperscript{73}. This reduction is depending from the mutual antagonistic effects of IL-22 and IFN-\(\gamma\) on epithelial cells. In fact in presence of IL-22, chemokine release such as CCL-5, CXCL-10 and T cell mediated cytotoxicity induced by IFN-\(\gamma\) are reduced. The antagonism between IL-22 and IFN-\(\gamma\) highlights an anti-inflammatory role of IL-22 in mice and human. Notably, IL-22 is inhibiting the expression of IFN-\(\gamma\) mediated MHC-I on epithelial cells\textsuperscript{73}. This is consistent with the role of IL-22 in the clearance of extracellular but not intracellular pathogens\textsuperscript{42,91}. In line with its tissue protective role, IL-22 reduces the recruitment of immune cells into the lung, limits an excessive T cell mediated tissue damage\textsuperscript{92}, promotes wound repair and induction of molecules involved in tissue regeneration\textsuperscript{94}. By releasing IL-22 in absence of IL-17 or other pro-inflammatory cytokines, Th22 cells might therefore mediate tissue protection rather than pro-inflammatory effects.
7. Synthesis

T cell cytokines affect the tissues according to the local inflammatory environment. In ACD IL-17 is expressed together with IFN-\(\gamma\) and induces overexpression of ICAM-1 on epithelial cells increasing the adhesion and cytotoxicity of Th1 cells and promoting tissue damage\(^{77}\). On the contrary, in AE IL-17 is expressed in a Th2-dominated environment and thus in presence of IL-4. In this context, the antibacterial effects of IL-17 are inhibited leading to an impaired response to *S. aureus* in patients affected by AE\(^{28}\). IL-22 reinforces the innate immune responses mediated by TNF-\(\alpha\) reinforcing the response against *C. albicans* and promoting innate related functions of Th1 cells\(^{24,85}\). In contrast to IL-17, IL-22 inhibits the IFN-\(\gamma\) mediated pro-inflammatory effects protecting the tissues from T cell-mediated damage\(^{73}\). Thus in presence of Th1 mediators IL-22 ameliorates tissue damage but enhances Th1-mediated innate immunity.

Therefore, the tissue local environment strongly affects the overall function of cytokines and the disease outcome. Therapies aiming to block selectively one cytokine may change the balance between synergistic and antagonistic effects of T cell cytokines and boost the effects of non-targeted cytokines. This homeostasis explains why therapeutical effects of blocking antibodies targeting single cytokines are often below the expectation. Thus, effects of single inflammatory mediators should be considered in the inflammatory context. Therefore, modulating the local tissue microenvironment rather targeting single T cell cytokines could yield the development of novel therapeutical approaches.
8. References


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9. Declaration

With this I declare that the presented work, title “Interleukin-17 and Interleuki-22: key cytokines in tissue inflammation” was written by myself and that I did not use other resources than those indicated. I did not undertake an unsuccessful graduation attempt and this thesis was not submitted to another institution before.

I contributed to every publication included in the present thesis as follows:

**Publication 1:** “IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response”

Within this manuscript, I contributed in designing and performing experiments, interpreting data and writing the paper.

Figure 1: Isolation of T cells from biopsies of AE patients and characterization by means of flow cytometry
Analysis of the data, graph generation and figure layout

Table I: Generation and stimulation of T cell clones

Table II: Generation of T cell clones and characterization by means of flow cytometry
Analysis of the data, table generation

Figure 2: Generation of T cell clones
Generation of dendritic cells
Performing co-culture experiments
Analysis of the data, graph generation and figure layout

Figure 5: Experiment design
Analysis of the data, graph generation and figure layout

Figure E1: Generation and characterization of T cell clones

Figure E2: Generation of T cell clones
Generation of dendritic cells
Performing co-culture experiments
Analysis of the data, graph generation and figure layout

Figure E3: Generation of T cell clones
Isolation of CD14+ cells
Performing co-culture experiments
Analysis of the data, graph generation and figure layout

Figure E4: Generation of T cell clones
Generation of dendritic cells
Performing co-culture experiments
Analysis of the data, graph generation and figure layout
**Publication 2:** “*IL-17 amplify human contact hypersensitivity by licensing hapten non-specific Th1 cells to kill autologous keratinocytes*”

Within this manuscript, I contributed in designing and performing experiments, interpreting data and writing the paper.

- **Figure 1:** Analysis of immunohistochemistry, quantification, generation of graphs and layout of the figure
- **Figure 2:** Isolation of T cells from biopsies of ACD patient.
  Characterization of skin derived T cell lines by means of flow cytometry
  Analysis of the data, graph generation and figure layout
- **Figure 3:** Generation of T cell clones from blood and skin derived T cells
  Characterization of T cell clones by means of flow cytometry and ELISAs (A-D)
  Proliferation measurement of skin derived T cell lines to antigen exposed to autologous DCs (E)
  Analysis of the data, graph generation and figure layout
- **Figure 4:** Characterization of nickel specific IL-17 producing cells by flow cytometry (A-F)
  Analysis of the data, graph generation and figure layout
- **Figure 5:** Flow cytometry analysis of keratinocytes (C,D)
  Generation of T cell clone supernatants for keratinocyte stimulation
  Analysis of the data, graph generation and figure layout
- **Figure 6:** Generation of EBV lines
  Generation of nickel specific T cell clones
  Co-culture experiments
  Analysis of the data, graph generation and figure layout
- **Figure 7:** Culture of primary keratinocytes
  Adhesion experiments
  Generation of nickel specific T cell clones
  Analysis of the data, graph generation and figure layout
Publication 3: “Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling”

Within this manuscript, I contributed in designing and performing experiments as well as interpreting data.

Figure 1: Characterization and generation of skin derived T cell lines (A-F)

Table 1: Generation of T cell clones

Figure 2: Generation of Th22 clones and characterization by means of flow cytometry

Figure 4: Generation of Th22 cell clones needed for microarray analysis

Figure 5: Isolation and characterization of T cells from dermis and epidermis (A,B)

Figure 6: Generation of Th22 supernatants for keratinocyte stimulation

Figure 7: Generation of Th22 supernatants for keratinocyte stimulation

Figure 8: Generation of Th22 supernatants for keratinocyte stimulation

Designing the wound healing experiment

Supplemental Figure 1: Characterization and generation of skin derived T cell lines (A,B)

Supplemental Figure 2: Characterization of Th22 T cell clones (A,B,C)

Supplemental Figure 3: Culture of Th22 T cell clones upon deforming conditions and flow cytometry characterization

Supplemental Table 1: Characterization of skin derived T cell lines

Publication 4: “IL-22 and TNF-α represent a key cytokine combination for epidermal integrity during infection with Candida Albicans”

Within this manuscript, I contributed in designing and performing experiments, as well as interpreting data.

Figure 4: Generation of T cell clone supernatant (A,B)

Figure 5: Generation of T cell clone supernatant
Publication 5: “IL-22 suppresses IFN-γ mediated lung inflammation in asthma”

Within this manuscript, I contributed in designing and performing experiments, interpreting data, writing the paper and coordinating the project.

Figure 1: Generation and characterization of lung derived T cell lines (A-H) Analysis of the data, graphs generation and figure layout

Table 1: Measurement of chemokines in BAL fluids Analysis of the data, table generation and layout

Figure 2: Analysis of the data, graphs generation and figure layout (A-E)

Figure 3: Designing and performing the wound healing experiments (A,B) Analysis of the data, graphs generation and figure layout

Figure 4: Bronchial epithelial cells culture and stimulation ELISAs and flow cytometry measurements (A-D) Cytotoxicity experiments (E,F) Analysis of the data, graphs generation and figure layout

Figure 5: Measurement of chemokines in BAL fluids Analysis of the data, graphs generation and figure layout

Figure E1: Generation and characterization of lung derived T cell lines (A,B) Analysis of the data, graphs generation and figure layout

Figure E2: Analysis of the data, graphs generation and figure layout

Figure E3: Analysis of the data, graphs generation and figure layout

Figure E4: Bronchial epithelial cells culture and stimulation ELISAs and flow cytometry measurements (A-D) Designing and performing the wound healing experiments (E) Analysis of the data, graphs generation and figure layout

Figure E5: Designing and performing the wound healing experiments Analysis of the data, graphs generation and figure layout

Figure E6: Bronchial epithelial cells culture and stimulation ELISAs and flow cytometry measurements (A-D) Analysis of the data, graphs generation and figure layout
10. Publications arising from this thesis

IL-17 in atopic eczema: Linking allergen-specific adaptive and microbial-triggered innate immune response

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Background: Patients with atopic eczema (AE) regularly experience colonization with Staphylococcus aureus that is directly correlated with the severity of eczema. Recent studies show that an impaired IL-17 immune response results in diseases associated with chronic skin infections.

Objective: We sought to elucidate the effect of IL-17 on antimicrobial immune responses in AE skin.

Methods: T cells infiltrating atopy patch test (APT) reactions were characterized for IL-17 secretion to varying stimuli. IL-17–dependent induction of the antimicrobial peptide human β-defensin 2 (HBD-2) in keratinocytes was investigated.

Results: Approximately 10% of APT-infiltrating T cells secreted IL-17 after phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation. Among these, 33% belonged to the newly characterized subtype T_{reg}/IL-17. Despite the capacity to secrete IL-17, specific T-cell clones released only low amounts of IL-17 on cognate allergen stimulation, whereas IL-4, IFN-γ, or both were efficiently induced. IL-17 secretion was not enhanced by IL-23, IL-1β, or IL-6 but was enhanced by the S aureus–derived superantigen staphylococcal enterotoxin B. Both healthy and AE keratinocytes upregulated HBD-2 in response to IL-17, but coexpressed IL-4/IL-13 partially inhibited this effect. In vitro, additional application of staphylococcal enterotoxin B induced IL-17 in APT reactions, whereas IL-4, IFN-γ, and IL-10 were marginally regulated. Induced IL-17 upregulated HBD-2 in human keratinocytes in vitro.

Conclusion: IL-17–capable T cells, in particular T_{reg}/IL-17 cells, infiltrate acute AE reactions. Although IL-17 secretion by specific T cells is tightly regulated, it can be triggered by bacteria-derived superantigens. The ineffective IL-17–dependent upregulation of HBD-2 in patients with AE is due to a partial inhibition by the type 2 microenvironment, which could partially explain why patients with AE do not clear S aureus. (J Allergy Clin Immunol 2009;123:59-66.)

Key words: Atopic eczema, T_{reg}17, IL-17, superantigen, defensin

Atopic eczema (AE) is a chronic relapsing–remitting inflammatory skin disorder beginning mostly in early childhood as a first step of the atopic march. It is highly pruritic and severely affects the quality of life of the individual and his or her environment. The incidence of AE is continuously increasing, which implies a high socioeconomic effect. The underlying pathogenesis of AE is a complex interaction of genetic predisposition and environmental factors. Recently, a strong association with loss-of-function mutations in the filaggrin gene was reported, which results in epidermal barrier dysfunction. Moreover, AE is often associated with type 1 (T_{reg}2-dominated) immune hyperreactivities mediated by allergen-specific IgE to common environmental or food allergens. The atopic patch test (APT) has been widely accepted as a model for allergen-specific induction of acute AE by type 1 allergy-inducing proteins, such as pollen- or house dust mite–derived allergens. AE and APT reactions share histologic similarities with delayed-type hypersensitivity responses, with the exception that in acute AE and APT lesions T_{reg}2 cytokines, such as IL-4, IL-5, and IL-13, are abundantly present. Beyond deregulations of the adaptive branch of the immune system, innate immune responses are critical for the outcome of AE. More than 85% of patients with AE are affected by skin colonization with facultative pathogenic microbes, such as Staphylococcus aureus, without imminent clinical signs of infection. However, colonization of eczematous lesions with S aureus is strongly associated with increased disease severity. A suspected underlying mechanism is stimulation of skin-infiltrating T cells by exotoxins that are frequently produced by S aureus species colonizing AE skin. These exotoxins stimulate T cells bearing particular T-cell receptor (TCR) VB chains regardless of their specificity and are therefore called superantigens. Recently, the superantigen staphylococcal enterotoxin B (SEB) was shown to enhance house dust mite–induced patch test reactions in patients with AE. Although interactions between adaptive and innate immunity in AE in terms of a facilitated T_{reg}2 response by superantigens has been suggested, the underlying mechanisms are not well understood.

The recently described T-cell subtype T_{reg}17 is characterized by the production of IL-17 and IL-22 and the expression of CCR6. In addition to a putative role in autoimmune diseases, recent studies show that T_{reg}17 cells are essential for the first-line defense of the human organism. An impaired or absent IL-17 immune response underlies the orphan syndromes autosomal-dominant hyper-IgE syndrome and chronic mucocutaneous candidiasis, both of which are associated with recurrent and persistent infection of skin and mucosal membranes. A possible mechanism for host defense against microorganisms by IL-17 and IL-22 in the skin is the upregulation of antimicrobial peptides, the so-called defensins, in...
human keratinocytes.\(^7\) Defensins are critical for killing \textit{S. aureus},\(^5\) and they induce migration of CCR6\(^1\) cells into the skin.\(^6\) In patients with AE, however, evidence exists that defensin levels are reduced compared with those seen in other immune-mediated skin diseases, such as psoriasis, and this might be responsible for the high \textit{S. aureus} skin colonization.\(^23\)

Therefore the aim of this study was to elucidate the effect of IL-17 on antimicrobial immune responses in AE skin.

**METHODS**

**Patients**

Patients with AE according to the criteria of Hanifin and Rajka (n = 3) and a positive skin prick test response to \textit{Dermatophagoides pteronyssinus}, RAST class of 3 or greater to Der p 1, and positive APT reaction to \textit{D. pteronyssinus} were included in the study. Before blood or skin samples were obtained, each participant provided informed consent. The study was approved by the ethics committee of the Istituto Dermatomatologico Dell’Immacolata.

**Cytokines and antibodies**

The following antibodies were used in flow cytometric analysis: CD4\(^+\) fluorescein isothiocyanate (FITC, SK3), CD14\(^+\)FITC (MoP9), CD4\(^+\)phycoerythrin (PE; clone SK3), CD69-FITC (NCAM16.2; all BD Biosciences Mississauga, Ontario, Canada), CD63-FTC (HB15e), CD86-FTC (2331 FUN1), CD1a-FITC (H149), CD88-FITC (RPA-T8), CCR6-PE (clone 11A9), IL-10-PE (JES5-19F1), IL-4-IFN (MP4-25D2), IFN-CD86 (B27), IFN-γ-allophycocyanin (B27), TNF-α-FITC (Mab1; all BD PharMingen), San Jose, Calif), CCR4-PE (Clone 20510), CCR5-PE (clone 4900.111A), IL-22-PE (142928), IL-4-PE (500.11; all R&D Systems, Minneapolis, Minn), and IL-17A-PE (clone SK3; ebscience, San Diego, Calif).

For cell culture and stimulation, the following cytokines were used: purified natural Der p 1, recombinant Der p 1 (both Indoor Biotechnologies, Charlottesville, Va), anti-CD3, anti-CD28 (both BD Biosciences), IL-2 (Novartis), IL-1β, IL-6, IL-13, IL-17, IL-23 (all R&D Systems), GM-CSF (Schering-Plough, Deerfield, Ill), IFN-γ (BD PharMingen), LPS, SEB (both Sigma, St Louis, Mo), anti-HLA-DR, anti-HLA-DQ, anti-HLA-DR, anti-IL-4 receptor, and anti-IL-17 (AF-317; all R&D systems).

**Isolation and expansion of T cells, antigen-presenting cells, and keratinocytes**

T-cell clones derived from 48-hour positive APT reactions to \textit{D. pteronyssinus} (Stallergenes, Antony, France) were isolated as previously described.\(^24\) Briefly, biopsy specimens were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 0.05 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 mg/mL streptomycin (all Invitrogen, Carlsbad, Calif; RPMI complete) and 5% human serum and 10% heat-inactivated FBS on a feeder layer of irradiated PBMCs, 30 U/mL IL-2, and 1% PHA (Sigma). Fresh medium containing IL-2 was added 3 times a week, and clones were restimulated with irradiated feeder PBMCs all 3 weeks. Der p 1 reactivity of both T-cell lines and clones was determined by using the titrated thymidine assay.

Dendritic cells (DCs) were generated from CD14\(^+\) monocytes, as described previously.\(^25\) Briefly, CD14\(^+\) monocytes were cultured in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 0.5 mmol/L 2-mercaptoethanol, 20 μg/mL gentamicin (all from Invitrogen), 10% FBS, 50 U/mL human rGM-CSF, and 200 U/mL human rIL-4 (complete DC medium), with a complete change of medium after 3 days. At day 5, a part of the cells was stimulated with 50 μg/mL LPS (Invitrogen) for 24 hours. Immediately before coculture experiments, cells were harvested and characterized for maturation markers (CD83, CD86, and HLA-DR) and CD1a by using flow cytometry.

Antigenic keratinocytes were isolated by using the suction blister method, as described previously.\(^26\) Briefly, blisters were induced by generating a vacuum on normal skin of the forearm. Epidermal sheets were obtained from blister roofs, treated with 0.05% trypsin (Invitrogen) to obtain single-cell suspensions, and seeded on a feeder layer of irradiated J774 cells in modified Green’s medium. At 70% to 80% confluence, keratinocytes were detached with 0.05% trypsin, placed in aliquots, and cryopreserved in liquid nitrogen. Keratinocytes of second and third passages were used in experiments.

**Coculture experiments**

T cells (10\(^6\)) and between 100 and 10,000 immature or mature DCs were cocultured in flat-bottom 96-well plates (Falcon; BD Biosciences) in RPMI complete 5% human serum for 36 hours with 5 μg/mL Der p 1, 10 μg/mL phorbol 12-myristate 13-acetate (PMA)/1 μg/mL ionomycin, 5 μg/mL SEB, or 5% medium as a control, respectively. The cell-free supernatant was obtained, and T-cell proliferation was read by using the titrated thymidine method.

Substantial expression of IL-17 was defined as a cytokine expression of at least 10% of the total releasing capacity measured by means of ELISA after 48 hours of PMA/ionomycin stimulation and a biologic effect of IL-17 in the supernatant. Biologic effect was defined by inducing detectable amounts of human β-defensin 2 (HBD-2) in human keratinocytes measured by means of ELISA.

**Flow cytometric analysis**

Intracellular cytokine staining was performed with a kit (BD Biosciences), according to the manufacturer’s instructions. Briefly, cells were stimulated with PMA and ionomycin for 6 hours in the presence of monensin. After 2 hours, brefeldin A was added. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and incubated with antibodies. Acquisition and analysis was done with a FACSCalibur (BD Biosciences).

**ELISA**

Concentrations of IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-22, TNF-α (all R&D Systems), and HBD-2 (Phoenix Pharmaceuticals, Inc, Burlingame, Calif) in cell-free culture supernatants were measured by using commercially available sandwich ELISA kits from indicated companies.

**RNA isolation and real-time PCR**

Total cellular RNA was extracted with the TRIzol (Invitrogen) method, according to the manufacturer’s instructions. RNA was reverse transcribed with oligo(dT) primers. PCR reactions were performed with the following synthetic oligonucleotides for HBD-2: 5'-TTCTCCTCGTCTGCTTCATATT-3' and 5'-TATGACCGTTACAGGACGTCG-3'.

**In vivo experiments**

Commercially available \textit{D. pteronyssinus} (Stallergenes) was applied at the 2 forearms of a patient with AE. Thirty-six hours after allergen application, 50 μg/mL SEB was added on 1 forearm. Sixty hours after allergen application, epidermal sheets and blister fluid of induced eczematous reactions were
FIG 1. Distinct populations of IL-17-producing T cells in the skin infiltrate of a positive APT reaction. A-C, Intracellular cytokine staining of skin-derived lymphocytes with IL-4/IFN-γ (Fig 1, A), IL-4/IL-17 (Fig 1, B), and IL-17/IFN-γ (Fig 1, C). D, Gating on IL-17+ cells after triple staining with IL-4, IFN-γ, and IL-17. Percentage of cells is indicated for each quadrant. Shown is 1 representative experiment. APC, Allophycocyanin.

TABLE I. Functional characterization of APT-derived IL-17+ T-cell clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sl to Der p 1</th>
<th>Sl to SEB</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-10</th>
<th>IL-13</th>
<th>IL-17</th>
<th>IL-22</th>
<th>TNF-α</th>
<th>Subtype</th>
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<tr>
<td>3</td>
<td>75</td>
<td>45</td>
<td>496</td>
<td>4,285</td>
<td>13,484</td>
<td>13,738</td>
<td>12,494</td>
<td>2,031</td>
<td>7,779</td>
<td>T2/IL-17</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
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<td>1,578</td>
<td>947</td>
<td>13,788</td>
<td>2,505</td>
<td>1,596</td>
<td>T2/IL-17</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>30</td>
<td>5,385</td>
<td>2,922</td>
<td>10,623</td>
<td>13,074</td>
<td>11,000</td>
<td>1,126</td>
<td>5,348</td>
<td>T2/IL-17</td>
</tr>
<tr>
<td>91</td>
<td>20</td>
<td>15</td>
<td>0</td>
<td>3,252</td>
<td>16,260</td>
<td>18,317</td>
<td>3,519</td>
<td>7,000</td>
<td>4,620</td>
<td>T2/IL-17</td>
</tr>
<tr>
<td>96</td>
<td>40</td>
<td>45</td>
<td>9,946</td>
<td>3,477</td>
<td>16,159</td>
<td>14,302</td>
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<td>T2/IL-17</td>
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<tr>
<td>141</td>
<td>40</td>
<td>1</td>
<td>0</td>
<td>4,710</td>
<td>1,149</td>
<td>20,472</td>
<td>5,500</td>
<td>219</td>
<td>861</td>
<td>T2/IL-17</td>
</tr>
</tbody>
</table>

Cytokine levels are measured by means of ELISA after PMA/ionomycin stimulation. Sl, Stimulation index to Der p 1 and SEB.

TABLE II. Surface markers of APT-derived IL-17+ T-cell clones: Flow cytometric analysis of resting T-cell clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>TCR Vβ chain</th>
<th>CD4</th>
<th>CD8</th>
<th>CCRI</th>
<th>CCRII</th>
<th>CCRII</th>
<th>CCRIII</th>
<th>CXCR3</th>
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<tbody>
<tr>
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<td>94%</td>
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<td>73%</td>
<td>85%</td>
<td>14%</td>
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<td></td>
</tr>
<tr>
<td>27</td>
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<td>98%</td>
<td>0%</td>
<td>95%</td>
<td>99%</td>
<td>1%</td>
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<tr>
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<td>3</td>
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<td>0%</td>
<td>85%</td>
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<td>14</td>
<td>98%</td>
<td>0%</td>
<td>89%</td>
<td>90%</td>
<td>3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>ND</td>
<td>90%</td>
<td>0%</td>
<td>1%</td>
<td>54%</td>
<td>45%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>ND</td>
<td>99%</td>
<td>0%</td>
<td>45%</td>
<td>55%</td>
<td>1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shown is the percentage of positive cells compared with the isotype control.
ND, Clone expresses a TCR Vβ chain that is not detectable by the Jost Beta Mark Repertoire Kit (Beckman Coulter, Fullerton, Calif).

obtained by using the suction blister method. RNA was prepared from epidermal roofs, as described above; suction blister fluid was analyzed by using the Luminex method (Fiterokine MAP multiplex human cytokine panel A, R&D systems) and by using HBOD-2 ELISA, as described above.

Statistical analysis
Statistical analysis was done with the Student t test.

RESULTS
IL-17–producing T lymphocytes infiltrate the skin during APT reactions: Newly characterized T42/IL-17 subset

Three patients with AE with a documented hypersensitivity to D. pteronyssinus were challenged with APTs to D. pteronyssinus. Biopsy specimens were taken from the resulting eczematous reactions, and infiltrating T cells were isolated and characterized by means of intracellular cytokine staining with flow cytometric techniques. In line with the hypothesis of a type 2 domination in early AE, the majority of skin-derived T cells activated in vitro by PMA plus ionomycin expressed high levels of IL-4 (Fig 1, A). Moreover, about 9% (9% ± 3%) of all infiltrated T cells were capable of producing IL-17 (Fig 1, B). IL-17 and IL-22 were not necessarily coexpressed (Fig 1, C). Interestingly, about one third of IL-17–releasing T cells coexpressed IL-4 (T42/IL-17 T cells) or IL-4 plus IFN-γ (T40/IL-17 T cells). Fifty percent of IL-17–producing T cells were pure T42/IL-17 T cells, and a minor proportion coproduced IL-17 and IFN-γ (T41/IL-17; Fig 1, D).
**A subpopulation of Der p 1-specific T cells has the capacity to produce IL-17**

T-cell lines isolated from biopsy specimens with positive APT reactions to *D. pteronyssinus* were cloned by means of limiting dilution to further characterize skin-derived IL-17-producing T lymphocytes. Expanded T-cell clones (in total 142 T-cell clones obtained from 3 patients with AE) were characterized for Der p 1 specificity, chemokine receptor expression, and the release of IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-22, and TNF-α. Consistent with the observations obtained from the APT-derived T-cell line, a high number of APT-derived T cells (24% ± 1.9%) were capable of producing IL-17 after activation with PMA plus ionomycin. The
newly identified Tq2/IL-17 subset was identified also on the clonal level. The relative distribution of IL-17–producing subpopulations was comparable with the results obtained from T-cell lines, with a more than 40% pure Tq17 phenotype (41% ± 4.1%), one third Tq2/IL-17 cells (32% ± 3.6%), and one fourth Tq1/IL-17 cells (26% ± 4.5%). Pure Tq17 cells were not specific for Derp 1. A correlation of IL-17 with IL-22 was not obvious on the clonal level. Sixty-nine percent (± 4.8%) of skin-infiltrating T-cell clones were capable of producing IL-22. A characterization of IL-17–producing T-cell clones is shown in Tables I and II (see also Fig E1 in this article’s Online Repository at www.jacionline.org).

**Stimulation with cognate antigen induces IL-4 release, IFN-γ release, or both but no or very low amounts of IL-17**

We then analyzed the physiologic reaction pattern of specific skin-derived Tq2/IL-17 and Tq0/IL-17 T-cell clones stimulated with native or recombinant Der p 1 in the presence of different antigen-presenting cells (APCs). Surprisingly, IL-17 was not or only marginally secreted by specific Tq2/IL-17 and Tq0/IL-17 cells when the allergen was presented by varying numbers of immature (Fig 2) or mature (see Fig E2 in this article’s Online Repository at www.jacionline.org) DCs or CD14+ monocytes (see Fig E3 in this article’s Online Repository at www.jacionline.org). Incrementing TCR stimulation intensity by increasing allergen concentration (see Fig E4 in this article’s Online Repository at www.jacionline.org) or the number of APCs (up to a DC/cell ratio of 1:10) only marginally upregulated IL-17 secretion (Fig 2, A and B). In contrast, even at low levels of stimulation intensity (DC/cell ratio of 1:1000 and 5 μg/mL Der p 1), high amounts of IL-4 and a strong induction of proliferation were detected, without substantial differences between different APC populations. Thus in contrast to the maximal stimulation induced by PMA and ionomycin, physiologic TCR triggering failed to upregulate the release of IL-17, although it was capable of inducing Tq2 cytokines, which predominate in the early phase of AE.

**SEB induces high secretion of IL-17 by Der p 1–specific T cells**

Because a defect in IL-17 secretion results in recurrent infections of the skin and mucosal membranes,19,20 we investigated whether microbial-derived products could induce substantial production of IL-17. We stimulated skin-derived Der p 1–specific T-cell clones (n = 5) with the proinflammatory bacterial substances LPS and SEB, which is commonly present on AE skin. Although 50 μg/mL LPS did not alter the cytokine secretion of T-cell clones (data not shown), addition of 5 μg/mL SEB to DC/T-cell cocultures strongly promoted IL-17 release by T cells expressing SEB-sensitive TCR VB chains (4/5; Fig 2, A and B; see also Figs E2 and E3). Secretion of IL-10 and IFN-γ, but not that of IL-4, was also affected in these clones. However, the increase in IL-17 secretion was by far most prominent, resulting in an increased percentage relative to other T-cell cytokines in all clones examined (Fig 2, C and D). A predominating VB chain was not detected in IL-17–producing T-cell clones (Table II). In line with the literature, blocking TCRs by adding neutralizing antibodies against MHC class II molecules abrogated SEB stimulation almost completely (Fig 3). This finding indicates that secretion of inflammatory IL-17 by T lymphocytes is tightly regulated and requires additional stimulation beyond cognate antigen
T cell–derived IL-17 potently induces HBD-2 production in AE keratinocytes, whereas secreted IL-4 partially inhibits IL-17–dependent HBD-2 upregulation

IL-17 strongly induces HBD-2, but this antimicrobial peptide is diminished in patients with AE. To clarify whether the ineffective upregulation in AE is due to intrinsic defects in AE keratinocytes or due to inhibitory effects of the microenvironment, we stimulated primary keratinocytes from patients with AE (n = 3) and healthy donors (n = 3) with different T-cell cytokines. We found that IL-17 strongly induced HBD-2 release in both AE and healthy keratinocytes in vitro (Fig 4). However, IL-17–induced HBD-2 upregulation was partially inhibited by the TGFβ cytokines IL-4 and IL-13 (Fig 4, A and B). Accordingly, experiments performed with supernatants from APT-derived T-cell clones demonstrated that neither TGFβ nor IL-10 could induce HBD-2 release by AE keratinocytes. The TGFβ1/IL-17–derived supernatant was the most effective in HBD-2 induction, whereas the coexpression of IL-4 in the supernatant of TGFβ2/IL-17 cells partially, but not completely, blocked the induction of HBD-2 release. Finally, preincubation of TGFβ1 and TGFβ2/IL-17 supernatant with a neutralizing antibody against IL-17 abrogated HBD-2 induction almost completely (Fig 4, C).

SEB strongly upregulates HBD-2 mRNA and protein release in D pteronyssinus–induced AE

To confirm the role of superantigens in triggering the IL-17/HBD-2 axis in vivo, we applied D pteronyssinus on both forearms of a patient with AE. Thirty-six hours after allergen application, we added 50 μg/cm² SEB on 1 forearm. In concordance with previous reports,15 the clinical reaction was severely aggravated (classified as “+++” vs “+-” according to the European Task Force on Atopic Dermatitis 2000 reading key16) and maintained for substantially longer (10 days vs 4 days) in the SEB-exposed lesion. Sixty hours after allergen application, epidermal sheets of induced eczematous reactions were obtained by using the suction blister method, and suction blister fluid was investigated for cytokine content. In line with our in vitro results, IL-17 was induced more than 2-fold in the SEB-challenged patch test site, whereas IFN-γ induction was unchanged. IL-4 level were less than the detection level, and IL-10 levels were marginally increased (Fig 5, A-D). Consequently, HBD-2 mRNA was 2-fold increased in keratinocytes from SEB-exposed epidermal sheets (Fig 5, E). An increased HBD-2 concentration in the SEB-treated APT reactions was confirmed at the protein level by using ELISA assays performed on blister fluids (Fig 5, F).

DISCUSSION

Increasing evidence suggests a central role of IL-17 in encompassing host defense against microorganisms at barrier surfaces. In patients with AE, disease severity positively correlates with skin colonization of S aureus. This observation led us to investigate IL-17 in the pathogenesis of AE.

In this study we demonstrate that distinct subpopulations of IL-17–secreting T cells infiltrate acute skin lesions, where they trigger keratinocytes to produce the antimicrobial peptide HBD-2. However, this induction is substantially impaired in the presence of the type 2 cytokines abundantly present in the AE skin microenvironment.

By isolating and characterizing the lymphocytic infiltrate in APT reactions, we directly demonstrated IL-17-releasing T cells in acute AE lesions. Hereby we confirm and extend a previous report describing the detection of IL-17 mRNA by means of PCR in AE skin.26 When we further characterized skin-infiltrating T cells, we identified distinct subpopulations of IL-17-producing T-cell clones in addition to the previously described pure TGFβ1 and TGFβ3/IL-17 cells that coexpress IFN-γ,25 a newly described population coproducing IL-17 and type 2 cytokines was classified as TGFβ2/IL-17 cells. Although in the APT lesion no Der p 1–specific TGFβ1 cells were found on the clonal level, we were surprised to observe that stimulation of Der p 1–specific TGFβ2/IL-17 and TGFβ3/IL-17 cells with their cognate antigen resulted in a strong induction of proliferation and IL-4 secretion, but IL-17 was poorly secreted. Neither the TGFβ1–differentiating cytokines IL-12 and IL-23 nor IL-26, described to maintain survival and cytokine secretion of the mouse,17 and, to a lesser degree, also of human TGFβ1 cells, strongly increased secretion of
IL-17 in our differentiated effector T cells stimulated with DCs and their cognate antigen. To find an in vivo stimulus for substantial IL-17 secretion, we investigated whether microbial-derived products could be adequate stimuli as evidence increases that IL-17 secretion is critical for clearing infections selectively at skin and mucosal membranes. Consensus exists that *S. aureus* colonization of AE skin significantly aggravates the intensity and accounts for the persistence of eczematous reactions; however, the mechanisms remain unclear. Under natural exposure conditions, *S. aureus*-derived superantigens, such as SEB, could contribute to the amplification of the inflammatory reaction by stimulating infiltrating T cells bearing particular TCR V β chains. Indeed, when we stimulated SEB-sensitive T β T cells bearing particular TCR V β chains, IL-17 was strongly enhanced compared with that seen with cognate TCR triggering alone both in vitro and in vivo. Thus our data underline a role of the environment in triggering full effector functions of tissue-infiltrating T cells.

However, despite the availability of SEB-triggered IL-17, which is a very efficient stimulus for HBD-2, expression of HBD-2 in patients with AE was reported to be diminished in comparison with that seen in T β T cells bearing particular TCR V β chains, such as psoriasis. We therefore investigated whether AE keratinocytes show an intrinsic defect in responding to IL-17 or whether coexpressed type II cytokines could account for the diminished HBD-2 induction, as reported for the IFN-γ- and TNF-α-induced expression of antimicrobial peptides.

We found that AE keratinocytes are not hyporesponsive to IL-17 in vitro, but rather the AE skin microenvironment containing abundant IL-4 and IL-13 partially inhibits the IL-17/HBD-2 axis. This could, at least in part, explain the persistent colonization of AE skin with *S. aureus* that represents a continuous trigger of cutaneous inflammation.

**Key messages**

- IL-17–producing T cells, in particular T β T cells, infiltrate lesional skin in patients with acute AE.
- Secretion of IL-17 by Der p 1–specific T cells is tightly regulated and requires microenvironmental stimuli, such as SEB.
- IL-17 is involved in linking adaptive and innate immunity through the IL-17/HBD-2 axis, which is partially impaired by T β cytokines in patients with AE.

**REFERENCES**

**FIG E1.** Cytokine pattern of IL-17–secreting T-cell clones confirms the existence of T_{h}17, T_{h}1/IL-17, and T_{h}2/IL-17 subpopulations in APT reactions. Cytokine secretion after 48-hour PMA/ionomycin stimulation of all 34 identified IL-17–producing T-cell clones is shown.

**FIG E3.** Stimulation of CD14^{+} monocytes with IL-17 secreting T-cell clones results in high IL-17 secretion on SEB but not on allergen stimulation. A and B, Dose-dependent secretion of IL-17 and IL-4 (left panel) and addition of cytokines and SEB (right panel) are shown. Type of APC: CD14^{+} monocytes (monos). Error bars indicate SDs of 1 representative experiment performed in triplicate.
**FIG E4.** Varying concentrations of Der p 1 have little effect on IL-17 secretion by T_{H}2/IL-17 clones: **A.** 1 μg/mL Der p 1; **B.** 5 μg/mL Der p 1. iDC, Immature DCs. Error bars indicate SEMs from 3 independent experiments with clone 3.
IL-17 Amplifies Human Contact Hypersensitivity by Licensing Hapten Nonspecific Th1 Cells to Kill Autologous Keratinocytes

Davide Pennino,* Kilian Eyerich,* Claudia Scarponi,* Teresa Carbone,* Stefanie Eyerich, Francesca Nasorri,* Simone Garcovich, Claudia Traidl-Hoffmann, Cristina Albanesi,* and Andrea Cavani*

Th17 is a newly identified lineage of effector T cells involved in autoimmune and immune responses to pathogens. We demonstrate in this study the pathogenic role of IL-17–producing CD4+ T lymphocytes in allergic contact dermatitis (ACD) to skin-applied chemicals. IL-17+ T cells infiltrate ACD reactions and predominantly distribute at the site of heavy spongiosis. Skin IL-17+ T cells were functionally and phenotypically heterogeneous: although pure Th17 prevailed in ACD skin, hapten responsiveness was restricted to Th1/IL-17 (IFN-γ−IL-17+) and Th0/IL-17 (IFN-γ−IL-17−IL-4+) fractions, and to lesser extent Th2/IL-17 cells. In the IFN-γ-dominated ACD environment, IL-17–releasing T cells affect immune function of keratinocytes by promoting CXCL18, IL-6, and HB-2 production. In addition, compared with Th1, supernatants from Th1/IL-17 T cells were much more efficient in inducing ICAM-1 expression on keratinocytes and keratinocyte–T-cell adhesiveness in vitro. As a consequence, exposure to combined IFN-γ and IL-17 rendered keratinocytes susceptible to ICAM-1–dependent Ag nonspecific T cell killing. Thus, IL-17 efficiently amplifies the allergic reaction by rendering virtually all of the T lymphocytes recruited at the site of skin inflammation capable to directly contribute to tissue damage. *The Journal of Immunology, 2010, 184: 4880–4888.

Allergic contact dermatitis (ACD) is a worldwide prevalent disease that results from an unbalanced immune response to small-molecular, highly reactive chemicals—the hapten (1, 2). These substances contacting the skin induce in susceptible individuals a specific immune response. The ensuing effector phase—clinically apparent as acute eczema—depends on the expansion and rapid migration of chemical-reactive CD8+ T cytotoxic I and effector CD4+ Th1 cells, possibly as a consequence of an impaired function of CD25+CD4+ regulatory T cells and IL-10–releasing T regulatory 1 lymphocytes (3–5). It has been demonstrated in murine models of contact hypersensitivity (CHS) that CD8+ and CD4+ T cells have distinct functions, the former being pathogenic and the latter being predominantly regulatory (6). However, the relative contribution of these subsets is much less defined in human beings. Expansion of nickel-specific cytotoxic CD8+ T cells appears to be critical for the development of nickel contact allergy. However, the intensity of the inflammatory reaction is mostly controlled by effector CD4+ T cells, which outnumber CD8+ T lymphocytes in CHS skin and release proinflammatory lymphokines affecting the immune function of resident cells. In particular, IFN-γ and TNF-α promote the release of cytokines and chemokines and the induction of MHC class II and ICAM-1 expression in keratinocytes (7). Thus, T cell–keratinocyte interactions are critical for the full expression of the inflammatory reaction.

Alongside IFN-γ, other cytokines, such as IL-4 and IL-17, are actively released by subsets of skin-infiltrating chemical-reactive T lymphocytes and may modulate ACD responses (8). IL-17A (IL-17) and IL-17F are coreleased by a well-defined subpopulation of effector CD4+ T lymphocytes, named Th17 cells (9–11). Th17 cells have been linked to a variety of autoimmune diseases, including murine experimental encephalomyelitis and collagen-induced arthritis (12–15). In addition, Th17 appears to be involved in protective immune responses to several pathogens, such as *Candida albicans* (16–18). More recently, evidence has been provided by us and others that IL-17 is also involved in the regulation of allergic skin diseases, such as atopic dermatitis, and in the pathogenesis of skin immunemediated skin conditions, such as psoriasis (19–21). In mice, naive T cell maturation toward the Th1 or Th17 phenotype appears mutually exclusive and under the reciprocal control of dendritic cell–derived IL-12 or IL-23. However, evidence exists that IL-17 and IFN-γ are coexpressed by a relevant number of human T lymphocytes isolated from both peripheral blood and from inflamed tissues, such as gut and skin (8, 22–24).

In this study, we investigated the expression of IL-17 in ACD, and we functionally characterized IL-17–releasing T cell subsets involved in the immune reaction. We show that Th1/IL-17 T cells infiltrating ACD amplify the immune responses to hapten by inducing chemokine and cytokine release from keratinocytes and by intensifying the ICAM-1–dependent keratinocyte–T-cell interaction, thus promoting nonspecific T cell–induced keratinocyte apoptosis.
Materials and Methods

Patients
Peripheral blood or 4-mm skin biopsies, or both, were obtained from seven ACD patients. Keratinocyte primary cultures from suction blisters were obtained from three ACD patients. Diagnosis was confirmed by clinical history and by positive patch tests to NIS0 (n ≥ 3), fragrance mix (n = 1), thiamin (n = 1), and cobalt (n = 1). Blood and skin samples were obtained after informed written consent according to the Declaration of Helsinki with regard to scientific use and upon approval of the ethical committee of the Istituto Dermopatico dell’Immacolata, Rome, Italy.

Culture medium, reagents, and Abs
T cell lines were cultured in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Lonza, Basel, Switzerland) (complete RPMI) plus 5% human serum (HS) (Sigma-Aldrich, St. Louis, MO). T cell clones (Tecs) in complete RPMI plus 5% HS and 10% FBS (HyClone, Logan, UT). Keratinocytes were grown in keratinocyte modified medium (Lonza) or in supplemented Ham’s F12 and DMEM (Biochrem, Berlin, Germany) medium, as previously described.

For cell culture, the following cytokines were used: recombinant human IFN-γ, TNF-α, and IL-17 were from R&D Systems (Minneapolis, MN), and IL-2 was from Novartis Pharmaceuticals (East Hanover, NJ). Mouse PEC, allogeneic, or FITC-conjugated mAb anti-human CD4 (SK3, IgG1), CD8 (SK1, IgG3), HLA-DR (L234, IgG3), HLA A, B, C (G46-2.6, IgG1), CD69 (L78, IgG1), IFN-γ (B27, IgG1), and TNF-α (Mab11 IgG1) were from BD Biosciences (San Diego, CA), and mouse anti–IL-22 (142928, IgG1), IL-4 (300711, IgG1), CD3 (UCHT1, IgG1), and CD54 (BDH57, IgG1) were from R&D Systems. Mouse Alexa Fluor 647- and PE-conjugated anti-human IL17A (EBio564DEC17, IgG1) or AS.B3, IgG1 was from Biolegend (San Diego, CA). Mouse IgG isotype controls were purchased from BD Biosciences.

Immunohistochemistry
Noninvolved skin and positive patch tests to 5% NIS0 applied on the backs of two sensitized patients punch biopsied at 24, 48, 72, and 96 h were paraffin-embedded. Five-micrometer sections, pretreated with hydrogen peroxide in PBS, were incubated in a pH 6 epitope retrieval solution (Dako, Carpinteria, CA) and subsequently incubated with goat anti-human IL-17 (R&D Systems) or rabbit anti-human CD3 (Dako), or both, followed by a biotynylated secondary Ab. Streptavidin peroxidase was added for 10 min to the samples. In single staining immunohistochemistry, IL-17 was detected by using a 3-amino-9-ethyl-carbazole (red: Dako) as a substrate. In double staining, IL-17 was detected by using alkaline phosphates (blue; Vector Laboratories, Burlingame, CA), whereas CD3 was detected by using 3-amino-9-ethyl carbazole complex substrate solutions. Slides were counterstained with hematoxylin. Immunohistochemistry results were quantified by two independent analyzers. Positive cells were counted in 20 total photographic fields for each condition considered.

Isolation of skin-infiltrating T cells and T cell cloning
Skin biopsies were minced with a scalpel and placed in culture in complete medium 60 U/ml IL-2. After 2–5 d, T cells emigrated from tissue samples were collected for phenotypic and functional characterization and for T cell cloning by limiting dilution (0.6 cells per well) in the presence of irradiated allogeneic feeder cells plus 1% PHA.

Flow cytometry analysis
Skin-derived lymphocytes were stimulated with PMA and ionomycin (Sigma-Aldrich) for 6 h in the presence of monensin and brefeldin (BD Bioscience), permeabilized with BD Cytofix/CytoPerm (BD Biosciences), and incubated with Abs toward surface markers and cytokines. Acquisition and analysis was done using a FACSaria (BD Biosciences).

T cell proliferation and activation assays
Tecs were cocultured with autologous monocytes in the presence or absence of 20 µg/ml NIS0 (Sigma-Aldrich) for 48 h. A total of 1 µCi/ml [3H] thymidine deoxyribose (TRK/Amherst Biosciences, Little Charston, UK) was added to the cultures for the last 8 h. Radioactivity incorporation was measured in a β counter. Results are given as mean cpm ± SD of triplicate cultures.

To assess the proliferation to nickel of T cell lines, skin-derived lymphocytes or peripheral blood CD4+CD25− T cells, purified with immuno magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), were incubated with CFSE (Molecular Probes, Eugene, OR) prior to culture with autologous monocytes in the presence or absence of nickel. At day 3, the proliferating fraction was measured by flow cytometry. To evaluate the activation induced by nickel in Th17 subsets, PBMCs of nickel-allergic patients were isolated and cultured in RPMI plus 5% HS for 3 d in the absence or presence of 20 µg/ml NIS0. After immunomagnetic separation, the CD4+ fraction was permeabilized with BD Cytofix/CytoPerm (BD Biosciences) and incubated with Abs toward surface markers and cytokines.

Keratinocyte cultures
Primary keratinocyte cultures were obtained from enzymatic digestions of the roofs of suction blisters by treatment with 0.05% trypsin/0.02% EDTA (Biochrem) for 30 min at 37°C. Keratinocytes were then expanded on mitomycin C-treated mouse fibroblast ST3 cells. Experiments were performed using secondary passage keratinocytes cultured in keratinocyte modified medium and pulsed for 3 h in the absence of hydrocortisone with a combination of recombinant human IFN-γ, TNF-α, and IL-17 or with supernatants of Th1/IL-17 or Th1 Tecs activated for 48 h with plate-coated anti-CD3 plus soluble anti-CD28 Abs. Total RNA was extracted after 18 h by using TRIZol reagent (Invitrogen Italia, San Giuliano Milanese, Italy), whereas keratinocyte supernatant was collected at 48 h for cytokine and chemokine content determination.

ELISA
Cell culture supernatants were collected, filtered, and measured for their content of IFN-γ, IL-4, IL-17, IL-22, TNF-α, IL-6, and CCL5 by using ELISA DuoSet kits from R&D Systems; GM-CSF, CXCL10, CXCL8, and CCL2 were measured by using BD OptEIA kits, whereas CCL25 release was measured by using the Ab pair MAB-360 and BAF-360 (all from BD Biosciences).

Real-time PCR analysis
Keratinocyte total RNA was reverse-transcribed into cDNA by using oligo (dT) primers and analyzed by real-time RT-PCR. Real-time PCR was performed using the SYBR Green PCR master mix or Taqman PCR master mix (Applied Biosystems, Branchburg, NJ). The forward and reverse primers used for PCR were as follows: for Bel-2 5′-ATGGAATCTGGCC TTATCG-3′ and 5′-TACTACCTCTTCGTGATGTTATTTT-3′; for Bel-15 5′-GGATCTTTTGTGAAGCTATG-3′ and 5′-CAGTTGAA GGCCTTCGTGG-3′; for CCL5 5′-CTACTGGCTCTCGTCCTC-3′ and 5′-TTGCTTGGGAGAAATATGGG-3′; for β-actin 5′-CATCGACGAC CCCATGGTCA-3′ and 5′-TAAGGAGCTGCGTGAGA-3′ as an endogenous control. HBBD-3 and IL-37 mRNA were analyzed by using specific PCR primers and probes (25) and normalized to 18S rDNA expression (Taqman Gene Expression Assay HS99999901 from Applied Biosystems). Quantification of mRNA expression was performed using the method described by Schmittgen et al. (26). Real-time PCR was performed in duplicate for each sample, and the procedure was performed in three independent experiments.

Keratinocyte apoptosis and T cell-mediated cytotoxicity
Keratinocyte cultures were treated for 48 h with recombinant cytokines (200 U IFN-γ; 50 ng/ml TNF-α; 50 ng/ml IL-17) or Tc supernatants. Apoptosis was evaluated by determining the caspase 3 and 7 content (Caspases 3 & 7 FLICA kit, ImmunoChemistry Technologies, Bloomington, MN) and analyzed by flow cytometry.

T cell-mediated killing of autologous keratinocytes or EBV-transformed B cell lines (B-LCL) were evaluated by measuring DNA fragmentation by the 1HTdR release assay, as previously described (27). Briefly, target cells were preincubated with 2 µg/ml [3H] thymidine 12–14 h prior to coculture with effector T cells. Th1- and Th17/L7–mediated killing were compared by incubating 105 T cells with 105 B-LCL cells or 48 h cytokine-stimulated keratinocytes for 6 h in the presence or absence of 20 µg/ml nickel. Blocking experiments were performed by incubating keratinocytes with 10 µg/ml anti-CD54 prior to coculture with effector T cells.

Keratinocyte–T cell adhesion assays
Subconfluent keratinocytes seeded in culture slides (BD Biosciences) were stimulated with IFN-γ and/or in combination with IL-17 for 48 h before addition of 5 × 105 CFSE-stained nickel-specific autologous T cells clones. Cocultures were performed in the presence or absence of 20 µg/ml nickel. In blocking experiments, keratinocytes were incubated 1 h with 10 µg/ml anti-CD54 prior to coculture with effector T cells. After 6 h of incubation, cocultures were extensively washed in PBS, fixed in 4%
paraformaldehyde, and counterstained with hematoxylin. T cells that adhered to keratinocytes were counted in 20 casual fields for each condition, as fluorescent dots using a fluorescent microscope (Zeiss, Oberkochen, Germany), and average T cell number per square millimeter ± SD was calculated.

**Statistical analysis**

Statistical analysis was done using Student t test. Statistically significant differences were defined as p < 0.05.

**Results**

**CD3⁺/IL-17⁺ in ACD skin correlates with the extent of the inflammatory reaction**

Epicutaneous application of 5% NiSO₄ in petrolatum onto the backs of nickel-allergic individuals (patch test) induces an ACD reaction. To investigate the expression of IL-17 and the distribution of IL-17⁺ T cells during ongoing reactions, we performed immunohistochemical studies on skin biopsies of noninvolved skin and 24, 48, 72, and 96 h NiSO₄ positive patch tests performed on two allergic donors. Sparse IL-17⁺ cells were already detected in normal noninflamed skin (average number of positive cells per area = 9 ± 1.4) (Fig. 1A). At 24 h, IL-17 did not significantly increase (average number of positive cells per area = 20 ± 10) (Fig. 1B, 1H) whether a noticeable increment was detected up to 48 (average number of positive cells per area = 40 ± 7) and 72 h (average number of positive cells per area = 46 ± 12), when positive cells could be also observed in the papillary dermis (Fig. 1C, 1D, 1H). Interestingly, at 96 h, we observed numerous intraepidermal IL-17⁺ cells, mostly distributed at the site of epidermal spongiosis and microvesiculation (average number of T cells per area = 64 ± 27) (Fig. 1E, 1H). CD3⁺/IL-17⁺ cells include almost the totality of IL-17⁺-producing cells and represent ~14% of the total CD3⁺ cells (average number of CD3⁺ cells per area = 241 ± 13; number of IL-17⁺ cells per area = 42 ± 11; number of CD3⁺/IL-17⁺ cells per area = 35 ± 12) (Fig. 1F, 1G). In general, the presence of IL-17⁺ cells reflected the course of the inflammatory reaction. In the epidermis, IL-17⁺ cells were particularly located at the site of intense tissue damage (Fig. 1E and inset).

**Skin T lymphocytes infiltrating ACD reactions contain distinct IL-17⁺ T cell subpopulations**

T cells lines were prepared from skin biopsies from five allergic donors (one patch test to nickel and four cases of acute spontaneous ACD to nickel, cobalt, thurium, and fragrances, respectively) and characterized phenotypically and functionally. As previously described, the microenvironment in ACD, to happen, is dominated by IFN-γ. Herein, we confirm this finding ex vivo showing that IFN-γ-releasing T cells are the most frequent infiltrating T cell population (22–42% of the total number of infiltrating T cells, as determined by intracellular staining upon activation with PMA/ionomycin (average ± SD = 35 ± 9%) (Fig. 2A, 2E). IL-17-releasing T cells ranged from 12 to 21% (average ± SD = 15 ± 3%) of total skin-infiltrating lymphocytes (Fig. 2A, 2E). The great majority of human skin IL-17⁺ T cells belonged to the CD4⁺ subset, whereas CD8⁺IL-17⁺ cells represented ~1% of skin T cells (Fig. 2C, 2D). The percentage of IL-4⁺ T cells was substantially lower compared with that of IFN-γ⁺ lymphocytes, ranging from 11 to 16% (average ± SD = 13 ± 3%). Among skin IL-17⁺ T cells, four subsets could be identified on the basis of their cytokine profile: pure Th17, defined as IL-17⁺/IFN-γ− IL-4⁺ T lymphocytes, which represent >50% (average ± SD = 56 ± 5.5%) of the IL-17⁺ T cells infiltrating ACD; IFN-γ⁺IL-4⁺ T cells (Th1/IL-17), which ranged from 42 to 18% (average ± SD = 25 ± 9.8%) of the total IL-17⁺ T lymphocytes, and finally two minor subpopulations of IL-4⁺ IL-17⁺ T cells (Th2/IL-17, average ± SD = 8.2 ± 1.4%) and IL-17⁺/IFN-γ−IL-4⁺ T lymphocytes (Th0/IL-17, average ± SD = 7.8 ± 1.9%) (Fig. 2B, 2F). Interestingly, IL-17 release was highly correlated with TNF-α production but not with that of IL-22 (Fig. 2A, 2B). Thus, skin IL-17⁺ T cells are heterogeneous in terms of cytokine release and may differentially affect ACD expression. Additionally, although IL-17⁺ CD8⁺ T cells have been described to play a role in murine CHS, human skin-derived IL-17⁺ T cells mostly belong to the CD4⁺ T cell subset (Fig. 2C, 2D).

**Figure 1.** CD3⁺ cells produce IL-17 during ACD reactions. Four-millimeter skin biopsies were fixed in formalin and stained with anti–IL-17 only or double stained with anti–IL-17 and anti–CD3 as described in Materials and Methods. Representative IL-17 staining in (A) uninvolved skin from allergic donor is compared with ACD reaction to nickel at (B) 24 h, (C) 48 h, (D) 72 h, and (E and inset) 96 h. Single staining with 3-amin0-9-ethylcarbazole, counterstained with hematoxylin. Original magnification ×100; inset ×200. Representative double staining of CD3 (3-amin0-9-ethylcarbazole, red) and IL-17 (alkaline phosphatase substrate kit III, blue) in a 48 h patch test. Original magnification ×100. (F). Quantification of (G) CD3⁺/IL-17⁺ cells in 48 h patch tests and (H) IL-17⁺ cells during time courses of ACD reactions was performed measuring positive stained cells on randomly acquired photographic fields obtained from two distinct patients.**

**Th1/IL-17, Th0/IL-17, and Th2/IL-17 but not Th17 cells are hapten-reactive**

Skin-derived T cell lines from two nickel-allergic donors (one isolated from a positive patch test and one isolated from spontaneous nickel skin hypersensitivity) were cloned by limiting dilution, assayed for cytokine release by ELISA, and further confirmed by flow cytometry. A total of 320 CD4⁺ Tcells were obtained and investigated for cytokine release (Fig. 3A); 71 Tcells released IL-17 upon PMA plus ionomycin activation. Among these, 25 Tcells displayed an IL-17⁺IFN-γ−IL-4⁺ phenotype, 30 Tcells were IL-17⁺IFN-γ⁺IL-4⁺, 11 Tcells were IL-17⁺IFN-γ−IL-4⁺, and, finally, a minor fraction was characterized by a IL-17⁺IFN-γ−IL-4⁻ cytokine asset (Fig. 3B). Proliferation to Ag was assayed in the presence of autologous monocytes and 20 μg/ml NiSO₄. Seventy-two out of 320 Tcells displayed a proliferation index ≥5 in the presence of nickel (data not shown and Fig. 3C); of these, none belonged to the Th17 subset, 11 Tcells were Th1/IL-17, 4 Tcells were Th0/IL-17, and 2 Tcells displayed a Th2/IL-17 phenotype (Fig. 3D).
FIGURE 2. Distinct CD4⁺ IL-17-producing T cell subpopulations are involved in ACD reactions. T lymphocytes were isolated from acute ACD reactions to fragrances (1), thiuram (1), cobalt (1), and nickel (1) and from 48 h positive patch test to NiSO₄ (1). T cell lines isolated from acute ACD reaction to fragrances were characterized for (A, B) cytokine release by four-color FACS analysis upon PMA/ionomycin stimulation and (C) CD4/CD8 expression. The percentages of IL-17⁺ T lymphocytes among CD4⁺ and CD8⁺ T cell subsets (D), the percentages of IFN-γ⁺ and IL-17⁺ T cells (E), and the relative distributions of the distinct IL-17-producing T cell subsets (F) as determined in five ACD T cell lines are shown.

The observation that only 22% of the Tccs isolated from skin biopsies were reactive to the causative Ag prompted us to investigate the frequency of specific T cells that infiltrate nickel positive patch tests. CFSE-labeled skin T cell lines were incubated with autologous monocytes and 20 μg/ml nickel, and the dividing cell population was evaluated as CFSE⁺ cells with flow cytometry (Fig. 3E). Nickel dividing cells represented a minor fraction of skin-infiltrating T cells (16.4 and 17.2%, respectively). In aggregate, these findings indicate that despite Th17 being the major IL-17-relasing T cell subpopulation in ACD, only Th1/IL-17 and Th0/IL-17 T cells show a productive response to the metal. Secondly, the great majority of the T cells infiltrating the skin during the acute phase of ACD reactions are not responsive to the causative Ag.

FIGURE 3. In a nickel-induced ACD reaction, only a minority of the infiltrating T cells, comprising Th1/IL-17, Th0/IL-17, and Th2/IL-17, but not Th17 cells, are nickel-specific. Tccs were obtained by limiting dilution from skin T cell lines from two nickel-allergic donors and characterized for cytokine release by ELISA upon PMA/ionomycin stimulation. Each dot represents an individual Tcc. IFN-γ and IL-17 production (A) and IFN-γ and IL-4 (B) release by IL-17⁺ skin Tccs pooled from two nickel-allergic individuals. IFN-γ and IL-17 (C) and IFN-γ and IL-4 (D) release of IL-17 nickel-specific Tccs. Nickel specificity was measured as cpm in a β counter after overnight incubation with [³H]TDR in the presence and absence of Ag. E. Skin T cell lines from skin biopsies of acute ACD reactions of nickel-allergic donors were incubated with CFSE and cocultured 5 d with autologous monocytes in the presence of nickel and examined by flow cytometry. The CFSE⁺ fraction represents the T cell population that undergoes cellular division in the presence of the Ag. Data of one representative T cell line out of two experiments performed is shown.
IL-17–producing nickel-reactive T cells from peripheral blood of nickel-allergic donors belong to the Th0–Th1/IL-17 subsets

The absence of proliferative in vitro responses to nickel in our skin-derived Th17 T cells moved us to investigate the cytokine profile of nickel-reactive T cells isolated from peripheral blood of nickel-allergic donors. Purified CD4⁺CD25⁻ T cells were labeled with CFSE and cocultured with adherent monocytes for 5 d in the presence or absence of nickel. T cell lines were restimulated with PMA and ionomycin, and cytokine release in proliferating and nonproliferating T cells was assessed by flow cytometry. Average percentages of IFN-γ— and IL-17—releasing cells were 0.9 ± 0.37% and 0.33 ± 0.11%, respectively (negative control 0.15 ± 0.01% and 0.05 ± 0.03%, respectively) (Fig. 4A–D). The dividing, CFSE⁺ fraction of IL-17⁺ T cells correlated with IFN-γ plus IL-4 (Fig. 4E), thus belonging to the Th1/IL-17 and Th0/IL-17 subsets. In contrast, the nonproliferating fraction of IL-17⁺ T cells consisted mainly of pure Th1 T cells. To confirm the unresponsiveness of pure Th17 to nickel, PBMCs from allergic donors were cocultured 3 d with 20 μg/ml NISO₄, and the purified CD4⁺ fraction was evaluated for expression of the activation marker CD69 and expression of IL-17, IFN-γ, and IL-4 in a four-color cytofluorimetric analysis (Fig. 4G–M). Results showed that ~1.5% of circulating CD4⁺ lymphocytes express the CD69 activation marker and release IL-17. Interestingly, the majority of the region IL-17 gated subpopulation coreleased IFN-γ (Fig. 4M), thus confirming that most IL-17–producing nickel-reactive CD4⁺ T cells belong to the Th1/IL-17 subset. This finding confirms the hypothesis that IL-17 is coreleased with IFN-γ in most of the hapten-reactive T cells isolated both from peripheral blood and inflamed tissues.

Supernatants of Th1/IL-17 clones modulate innate immune function of keratinocytes

The intensity of ACD reactions depends on the cytotoxic potential of infiltrating T cells, which target hapten-loaded keratinocytes, resulting in spongiosis and microvesiculation (27, 28). In addition, keratinocytes activated by T cell-derived mediators release a plethora of cytokines and chemokines, which promote the accumulation of leukocytes and mediate the inflammatory response. To disclose how IL-17–releasing T cells could affect keratinocyte immune function, we compared the effects of supernatant of skin-derived activated Th1/IL-17 cells to those of Th1 lymphocytes on primary human keratinocytes. As a control, cocktails of recombinant cytokines were used. Th1/IL-17 supernatant was 4-fold more efficient than supernatant of Th1 T cells (both releasing comparable amounts of IFN-γ and TNF-α) to induce CXCL8 production in human keratinocytes. Th1/IL-17 supernatant also increased keratinocyte release of IL-6, as compared with Th1 supernatant. Both effects were IL-17–dependent and could be reversed by anti–IL-17–neutralizing Ab. Conversely, natural IL-17 inhibited the IFN-γ–induced CCL5 production by human keratinocytes, confirming previous reports on the effects of recombinant IL-17 on cultured keratinocytes (8). Other chemokines involved in T cell recruitment at the ACD site, such as CXCL10, CCL2, and CCL20, were not affected by IL-17 (Fig. 5A). In addition to the modulation of chemokines, Th1/IL-17 strongly induced mRNA of HBD-2 in an IL-17–dependent manner, without affecting HBD-3 or LL-37 (Fig. 5B). In line with previous reports indicating a synergistic effect of IL-17 on the IFN-γ–dependent induction of ICAM-1 expression on keratinocytes, we could demonstrate that Th1/IL-17 was much more effective than Th1 supernatant in inducing keratinocyte expression of ICAM-1 but not MHC molecules (Fig. 5C).

Finally, to disclose whether IL-17 could directly affect survival or susceptibility to apoptosis of human keratinocytes, we investigated the effects of IL-17 exposure on keratinocytes treated with IFN-γ plus TNF-α, two potent inducers of apoptosis (Fig. 5D). A 48-h treatment with IFN-γ plus TNF-α doubled the percentage of caspase 3/7 keratinocytes (Fig. 5D) and strongly reduced the expression of the antiapoptotic molecule Bcl-2 but not that of Bcl-xl. Addition of IL-17 did not affect the proapoptotic
FIGURE 5. Th1/IL-17 T cells modulate the immune function of human keratinocytes by affecting their chemokine, cytokine, and defensin release and by increasing the expression of ICAM-1. Primary cultures of human keratinocytes were treated 48 h with recombinant cytokines or with supernatants of activated Th1 and Th1/IL-17 T cell clones, releasing comparable amounts of IFN-γ and TNF-α. Chemokine and cytokines released by keratinocytes were detected by ELISA in the culture supernatants (A). mRNA of HBD-2, HBD-3, and LL-37 was measured by RT-PCR (B), and expression of surface molecules was determined by flow cytometry and shown as mean fluorescence intensity (C). Keratinocyte spontaneous apoptosis was measured as the percentage of caspase III/VII⁺ cells by flow cytometry, whereas mRNA expression of Bcl-2 and Bcl-xl were determined by RT-PCR (D).

effect of IFN-γ and TNF-α or the mRNA levels of Bcl-2 and Bcl-xl, indicating that IL-17 does not prevent apoptosis in a proinflammatory environment. In contrast to the results obtained with recombinant cytokines, keratinocyte exposure to Th1- or Th1/IL-17–derived supernatants did not significantly affect keratinocyte apoptosis, as measured by the percentage of caspase III/VII⁺ cells. We may speculate that additional soluble factors released by T lymphocytes could exert a protective role in keratinocyte survival, thus preventing or counteracting the apoptosis induced by IFN-γ and TNF-α.

Overall, these results suggest that IL-17 strongly affects the innate immune function of human keratinocytes, by inducing HBD-2, CXCL8, and IL-6, and has a prominent role in increasing ICAM-1, an adhesion molecule involved in keratinocyte–T cell adhesiveness.

Th1/IL-17 T cells are cytotoxic toward Ag-loaded target cells

The observation of a high number of IL-17⁺ cells in close proximity to spiongiotic areas in ACD skin prompted us to investigate the cytotoxic properties of Th1/IL-17 cells compared with those of Th1 cells. For that purpose, nickel-specific Th1 and Th1/IL-17 T cell clones were incubated for 6 h with nickel-loaded EBV-transformed autologous B cell lines, and cytotoxicity was measured as DNA fragmentation by the [³H]Tdr release assay. Although Th1 cells were generally more efficient in killing target cells, all Th1/IL-17 T cells showed a significant cytotoxic activity, thus suggesting that they could directly contribute to the expression of ACD (Fig. 6).

T cell-derived IL-17 augments the ICAM-1–dependent T cell–keratinocyte adhesiveness and induces T cell-mediated keratinocyte killing in an Ag-independent mechanism: the inducer–amplifier model of ACD

To investigate the functional consequences of increased ICAM-1 expression on keratinocytes upon exposure to IL-17, we investigated T cell–keratinocyte adhesiveness in an in vitro cell-contact model. A monolayer of human keratinocytes was treated for 48 h with IFN-γ alone or IFN-γ plus IL-17, labeled with nickel, and cocultured for 5 h with autologous CFSE-labeled nickel-specific T cell clones (Fig. 7A–C). After extensive washing,
FIGURE 6. Th1/IL-17 T cells are cytotoxic toward Ag-loaded target cells. Nickel-specific Th1 (n = 6) and Th1/IL-17 (n = 6) Tccs were cocultured with autologous [3H]thymidine-pulsed EBV-transformed B cell lines in the presence or absence of nickel for 6 h. Specific cytotoxicity was measured in triplicate as the difference between the [3H]Tdr release in the presence of the AG and that in the absence of the Ag. Statistical analysis was determined using unpaired Student t test. *p > 0.3

the number of adherent T cells was determined by counting CFSE<sup>+</sup> cells with a fluorescence microscope. T cells barely adhered to resting keratinocytes, independently of the presence of the relevant Ag (average numbers of T cells per square millimeter = 36 ± 13 and 16 ± 7 in the presence or absence of nickel, respectively). In contrast, numerous T lymphocytes adhere to IFN-γ-treated keratinocytes (average number of T cells per square millimeter = 176 ± 20), and their number doubled in the presence of the cognate Ag (average number of T cells per square millimeter = 374 ± 14). Interestingly, exposure of keratinocytes to IFN-γ plus IL-17 strongly increased adhesiveness of T cells in the absence of nickel (average number of T cells per square millimeter = 387 ± 47) compared with that of IFN-γ-treated keratinocytes and matched that observed in the presence of nickel (average number of T cells per square millimeter = 379 ± 17). Moreover, T cell–keratinocyte adhesiveness was strongly decreased by blocking anti-ICAM-1 Ab in both IFN-γ (average number of T cells per square millimeter in the presence of nickel = 35 ± 20; in the absence of nickel = 42 ± 19) and IFN-γ/IL-17<sup>+</sup> (average number of T cells per square millimeter in the presence of nickel = 33 ± 10; in the absence of nickel = 40 ± 13) treated keratinocytes (Fig. 7B, 7C). This finding confirms that the increased expression of ICAM-1 induced by IFN-γ/IL-17 cotreatment is relevant for T cell–keratinocyte adhesiveness, in particular in the absence of the relevant Ag.

Because ICAM-1 is a critical mediator of CD4<sup>+</sup> T cell killing, we further investigated the susceptibility of autologous keratinocytes activated via IFN-γ or IFN-γ plus IL-17 to Th1-mediated killing. As expected, IFN-γ-pretreated keratinocytes were only efficiently killed by T cells when optimal concentrations of cognate Ag were added (Fig. 7D). Surprisingly, upon IL-17 pretreatment, keratinocytes became susceptible to T cell-mediated killing independently of Ag recognition. The role of ICAM-1 in the T cell-mediated keratinocyte killing was confirmed by the strongly reduced keratinocyte apoptosis in the presence of anti-ICAM-1 blocking Abs. Thus, in the IFN-γ-dominated ACD

FIGURE 7. IL-17, acting synergistically with IFN-γ, increases keratinocyte–T cell adhesiveness and licenses Th1 lymphocytes to kill autologous keratinocytes in an Ag-independent manner. Nickel-specific Th1 Tccs were stained with CFSE and then cocultured 6 h with untreated, IFN-γ–, or IFN-γ plus IL-17–treated adherent keratinocytes in the presence or absence of nickel. After extensive washing, the number of FITC<sup>+</sup> adherent T cells were counted as described in Materials and Methods. A, Light and fluorescent microscopy of a representative picture of the monolayer of adherent IFN-γ–treated keratinocytes cocultured with CFSE-labeled Tccs. Original magnification ×20. Keratinocytes were counterstained with hematoxylin. B, Representative adherence experiment, showing CFSE<sup>+</sup> T cells that adhere to untreated, IFN-γ–, or IFN-γ plus IL-17–treated keratinocytes in the presence or absence of nickel. In blocking experiments, keratinocytes were incubated 1 h with anti-ICAM-1 blocking Ab prior to cocultures. C, Quantification of adherent T cells per square millimeter ± SD as measured as fluorescent dots in 20 casual fields for each experimental condition. Difference in T cell adherence to IFN-γ or IFN-γ plus IL-17–treated keratinocytes was analyzed using an unpaired Student t test. *p < 0.0001. Representative data of one experiment out of three performed is shown. D, IFN-γ– or IL-17/IFN-γ–treated autologous keratinocytes obtained from suction blisters were used as target cells in cytolysis assays using nickel-specific Tccs as effector cells. Percentage of apoptotic keratinocytes was measured in triplicate in [3H]Tdr release assays. Anti-ICAM-1 Ab was added to selected triplicates at a concentration of 10 μg/ml. The figure shows the result obtained with one representative Tcc out of five Tccs investigated. Statistical analysis was determined using an unpaired Student t test. *p > 0.05; **p < 0.05.
environment, the additional release of IL-17 enables non-Ag-specific T lymphocytes, which represent the major part of the infiltrating T cells, to directly attack ICAM-1+ keratinocytes, thus serving as an extremely efficient amplification mechanism of the inflammatory response.

Discussion
In this study, we characterized distinct IL-17-producing T cell populations infiltrating the skin during acute ACD, showing their multiple roles in the amplification of the inflammatory response. We demonstrate that IL-17 secreted by skin-derived T cells modulates innate immunity by keratinocytes, increases T cell–keratinocyte adhesiveness, and thereby promotes ICAM-1-dependent non-Ag-specific keratinocyte killing by T lymphocytes.

Previous reports support a strong impact of IL-17 in murine CHS. IL-17+ mice show reduced hapten-specific CD4+ T cell responses, a decreased secretion of chemokines and cytokines, and lower expression of ICAM-1 on keratinocytes at the site of hapten challenge (29). Thus, IL-17 may influence multiple steps of the immune response to hapten. In line with these findings, it has been shown that neutralization of IL-17 suppresses the elicitation of murine CHS and that IL-17–producing CD8+ T lymphocytes may be relevant in the efferent phase of murine CHS (30, 31).

In humans, nickel-specific IL-17+CD4+ T lymphocytes isolated from sensitized donors have already been described by our and other groups (8, 32); however, their role in ACD expression remained obscure. Interestingly, a low number of IL-17+ cells is already detectable in normal uninvolved skin, and their number gradually increases at 48–96 h, thus paralleling clinical symptoms of the allergic reaction. Interestingly, in fully expressed eczematous reactions, IL-17+ lymphocytes are greatly enriched at the site of heavy spongiosis and vesiculation. Reduced susceptibility of IL-17–producing T cells to activation-induced cell death, as recently observed, may explain this finding (33).

The major sources of IL-17 in ACD skin are infiltrating CD4+ T lymphocytes, whereas IL-17+CD8+ T cells represent <1% of the total number of T cells obtained from ACD lesions. Whether this limited number of IL-17+CD8+ T cells can affect the magnitude of ACD remains to be determined. Interestingly, skin-infiltrating CD4+ T lymphocytes that release IL-17 upon stimulation are highly heterogeneous. Although in the mouse system the differentiation of Th17 and Th1 appears to be mutually exclusive, a striking T cell plasticity is observed in the human system. Besides Th17, T lymphocytes coreleasing IFN-γ and IL-17 (Th1/IL-17) have been described in many immune-mediated human diseases, including Crohn’s disease, CHS, and in healthy subjects (8, 22–24). Additionally, we have recently demonstrated that atop eczema is enriched in IL-4+IL-17 (Th2/IL-17) T cells (19). Overall, these findings clearly demonstrate that besides the well-defined Th17 population, IL-17 can be coreleased by a variety of Th1 as well as Th2-polarized T lymphocytes.

Interestingly, although abundantly present in ACD skin, Th17 Tccs are not responsive to nickel in vitro. This finding confirms and extends our previous observation in Dermatophagoides-induced atopy patch tests, where we demonstrated Dermatophagoides specificity in Th2/IL-17 and Th1/IL-17 but not in Th17 Tccs. Accordingly, all nickel-reactive IL-17+ T cells obtained from skin and peripheral blood of nickel-allergic individuals corelease IFN-γ or IL-4, or both. Experiments are needed to disclose whether unresponsiveness of Th17 is the consequence of an anergic state or a consequence of the plasticity of IL-17+ T cells, which may convert to a Th1/IL-17 phenotype upon terminal differentiation.

In line with the suspected primary function of Th17 cells, T cell–derived IL-17 modulates innate immune responses in the skin. IL-17 directly induces the release of CXCL8, IL-6, and HBD-2 by keratinocytes, while inhibiting CCL5. In contrast to previous reports, we were not able to confirm the effects of IL-17 on CCL20 release by keratinocytes (34). Thus, IL-17 contributes to the recruitment of neutrophils and establishes a protective immune response against extracellular pathogens.

Beyond modulation of innate immunity, this study reveals a second central function of IL-17 in inflammatory skin reactions. Induction of keratinocyte apoptosis by T cells is a key element in the pathogenesis of eczematous disorders. Previous studies demonstrated two pathways eliciting apoptosis in keratinocytes: one being Ag-dependent and mediated via both Fas/Fas ligand and perforin/granzyme B and a second pathway that is Ag-independent and involves IFN-γ and Fas/Fas ligand (27, 35, 36). Our study clearly demonstrates that IL-17 links these two processes in a kind of inducer–amplifier model. IL-17 is coreleased with IFN-γ by Ag-specific Th1/IL-17 cells and synergistically enhances the non-specific pathway by increasing ICAM-1 expression on keratinocytes, which in turn strongly enhances T cell–keratinocyte adhesiveness and consequently renders keratinocytes susceptible to non-Ag-specific T cell attack. Given the fact that Ag-specific T cells represent the minor fraction of skin-infiltrating T cells, nonspecific T cell killing represents an extremely efficient amplification mechanism of the initially Ag-specific allergic reaction, rendering virtually all T lymphocytes recruited at the site of skin inflammation capable to directly contribute to tissue damage.

In conclusion, our data demonstrate that IL-17 is a central proinflammatory mediator in the skin microenvironment through modulating keratinocyte immune responses and amplifying a non-specific cytotoxic cascade that results in a severe and sustained cutaneous inflammatory reaction.

Disclosures
The authors have no financial conflicts of interest.

References


Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling

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Th subsets are defined according to their production of lineage-indicating cytokines and chemokines. In this study, we have identified a subset of human Th cells that infiltrates the epidermis in individuals with inflammatory skin disorders and is characterized by the secretion of IL-22 and TNF-α, but not IFN-γ, IL-4, or IL-17. In analogy to the Th17 subset, cells with this cytokine profile have been named the Th22 subset. Th22 clones derived from patients with psoriasis were stable in culture and exhibited a transcriptome profile clearly separate from those of Th1, Th2, and Th17 cells; it included genes encoding proteins involved in tissue remodeling, such as FGFs, and chemokines involved in angiogenesis and fibrosis. Primary human keratinocytes exposed to Th22 supernatants expressed a transcriptome response profile that included genes involved in innate immune pathways and the induction and modulation of adaptive immunity. These proinflammatory Th22 responses were synergistically dependent on IL-22 and TNF-α. Furthermore, Th22 supernatants enhanced wound healing in an in vitro injury model, which was exclusively dependent on IL-22. In conclusion, the human Th22 subset may represent a separate T cell subset with a distinct identity with respect to gene expression and function, present within the epidermal layer in inflammatory skin diseases. Future strategies directed against the Th22 subset may be of value in chronic inflammatory skin disorders.

Introduction

Th subsets play a discriminative role in translating antigen-specific immune responses into tissue functions or immunopathology. The identification of novel T cell subsets, such as Th17 cells, is important in order to define the role of the specific immune response in human disease. Across the board of different pathologies, distinct T cell subsets secrete cytokines that not only function on other immune cells, but also instruct target cells (1–3). Each T cell subset secretes tissue-instructing cytokines such as IFN-γ (Th1), IL-4 (Th2), and IL-17 (Th17), which induce MHC-expression in nonlymphoid cells, mucus secretion in epithelial cells, and IL-6 expression in keratinocytes and/or epithelial cells, respectively (4).

A less well-defined tissue-instructing cytokine is IL-22, which is expressed by Th17 cells (5, 6), but also by NK cells (7). Recent studies have determined that some T cells express IL-22 independently of IL-17, particularly CCR10+ T cells (2, 8–10). IL-22 belongs to the IL-10 cytokine family and binds to a heterodimeric receptor consisting of the IL10 receptor (IL-10R) β chain and the IL-22R. Despite this receptor sharing, IL-22 signaling acts independently of IL-10 (11) and, in contrast to IL-10, also triggers 3 MAPK pathways (12). The IL-22R is expressed almost exclusively on nonimmune cells, such that IL-22, like IL-17, acts primarily on tissue cells (13).

The function of IL-22 is difficult to generalize: It is not anti-inflammatory, nor is it necessarily proinflammatory. However, it is consistently described as having epithelial innate immune responses, which can be detrimental or protective. An example of a detrimental effect is epithelial hyperplasia in psoriasis (PS), which also can be induced by IL-22 in human artificial skin cultures (14). Furthermore, IL-22 synergizes with IL-17 in the induction of proinflammatory cytokines in human bronchial epithelial cells (3) and colonic myofibroblasts (15).

Examples of protective effects of IL-22 were reported in Gram-negative bacterial pneumonia, in which IL-22 induces the secretion of antimicrobial substances in lung epithelial cells (3). In addition, IL-22, but not IL-17, protects from hepatitis-induced liver damage by preventing hepatocyte apoptosis (16, 17). In the skin, IL-22 induces antimicrobial peptides, promotes keratinocyte proliferation, and inhibits differentiation, which suggests a role in remodeling wound healing and in innate defense mechanisms (14). The role of IL-22 in common skin disorders such as atopic eczema (AE) and allergic contact dermatitis (ACD) is currently not known.

Here we describe a Th22 subset of T cells, which was clearly separate from Th17 and other known T cell subsets. Th22 cells expressed several FGFs, were associated with epidermal repair responses, and synergized with TNF-α to induce a characteristic Th22 signature in keratinocytes.
Figure 1
Th22 cells represent a distinct T cell subset enriched in inflammatory skin diseases. (A–D) Representative 4-color intracellular cytokine stainings of primary human T lymphocytes derived from PBMCs (A), PS (B), AE (C), and AC (D) for IL-22, IL-17, IFN-γ, and IL-4. Shown are dot plots for IL-22 and IL-17 and of IL-17-IL-22- gated cells (red outline) for IFN-γ and IL-4. Th22 cells are shown by red shading. Numbers indicate relative percentages per quadrant. (E) Frequency of IL-22- in PBMCs (n = 4) and in PS (n = 3), AE (n = 4), and AC (n = 3). Symbols denote individual determinations, and horizontal bars denote mean. *p < 0.05; **p < 0.01. (F) Plasticity in T cell phenotypes, as shown by coexpression of IL-22 with other cytokines in PS (n = 3), AE (n = 4), and AC (n = 4). Gray oval denote scale, indicating 1% of total cells. (G) The majority of IL-22+ T cells belong to the CD4+ subpopulation, as shown for CD4/CD8 sorted cells from PBMCs and skin T cell lines of PS, AE, and AC. Numbers indicate relative percentages per quadrant.

Results
IL-22-expressing cells represent a tissue T cell subset. To investigate the cytokine profile of circulating and skin-homing T lymphocytes, we analyzed freshly isolated PBMCs (n = 4) and short-term cultures of skin biopsies obtained from patients with PS (n = 3), AE (n = 4), and AC (n = 4). T cells were permeabilized, and their cytokine profile was studied by flow cytometry and multicolor intracellular staining. As expected, obvious differences between the samples were observed. AE-derived T cells were characterized by a large proportion of IL-4-expressing T cells (30.5% ± 10.0%), whereas PS- and AC-derived T cells showed a prevalence of IFN-γ-expressing T cells (34.3% ± 3.7%), AC, 36.4% ± 4.8%; Figure 1F and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI40202DS1). The IL-17-expressing population was largest in PS (17.4 ± 4.2%), followed by AC (13.1% ± 3.1%) and AE (9.0% ± 1.2%; Figure 1). This IL-17-expressing population overlapped with IFN-γ-producing T cells and, to a lesser degree, with those expressing IL-4, whereas approximately 50% were pure Th17 cells (both IL-4 and IFN-γ negative). IL-22-producing T cells were observed in both PBMCs and inflammatory skin diseases. Although interindividual differences were high, the frequency of IL-22+ T cells was significantly higher in skin-derived T cell lines than in the circulation (PBMCs, 2.3% ± 0.4%; PS, 15.9% ± 2.3%; AE, 13.8% ± 4.0%; AC, 12.9% ± 2.4%; Figure 1E). Thus, IL-22+ cells were enriched in inflamed skin.

In addition to distinct IL-4, IFN-γ, and IL-17 populations, a subset of T cells secreting IL-22 alone was present as a robust single-positive entity. Depending on the disease, about one-third of total IL-22+ cells were clear-cut Th22 cells (Supplemental Figure 1), and another third were Th17 cells coproducing IL-22; thus, TH22 and TH17 cells were the major T cell sources of IL-22. The overlap of cytokine expression observed in T cells derived from inflammatory skin disorders is shown in Figure 1F. These data illustrate that the size and likely plasticity from TH1, TH2, TH17, and TH22 subsets toward neighboring phenotypes was distinct for the different diseases.

Because IL-22 is not released solely by T lymphocytes, we investigated IL-22+ cells from blood and skin for their expression of T cell markers. The majority of IL-22+ cells were found within the CD4+ subpopulation in circulating PBMCs and AE, whereas in both PS and AC, a minor, but substantial, fraction was IL-22+ CD4− (Figure 1G). Our data highlight that CD4+ Th cells represent the major source of IL-22 in the skin, although IL-22 was also secreted by other leukocyte subsets, such as NK cells and CD8+ T cells (data not shown).

Characteristics of Th22 clones. T cell subsets are defined by an epi-genetically imprinted cytokine profile that is assumed to be stable over multiple cell divisions, thereby allowing amplification of immune responses by clonal expansion. Starting from T cell lines, as analyzed in Figure 1, we generated 244 distinct T cell clones from 2 donors with AE (143 clones), 44 clones from a patient with AC, and 57 clones from a patient with PS (Table 1).

Consistently with the single-cell analysis from biopsies, multiple Th22 clones were characterized by solitary IL-22 expression, which did not show substantial protein secretion of IL-4, IFN-γ, or IL-17 (Figure 2, A–C, left). As observed for the large majority of T cell clones isolated from skin, Th22 cells were capable of secreting IL-10 and/or TNF-α. The cytokine secretion pattern of isolated Th22 clones was remarkably stable even after 4 restimulations over sequential 10-week cultures. Th22 clones released IL-22 within 6 hours, reaching a peak at 12 hours and persisting at this level for at least 48 hours (Figure 2, A–C, right). Surface expression profiling of Th22 clones confirmed the CD3 CD4+ phenotype of Th22 cells, whereas the CD8 and NK cell markers CD56, Nkp44, and Nkp46 were negative (Supplemental Figure 2).

Th22 cells represent an independent and stable lineage of Tb cells. Because Th cell are increasingly understood to differ in plasticity and differentiation status, we investigated the phenotype stability of Th22 cells under different polarizing conditions. Skin-derived memory Th22 clones did not lose the capacity to secrete IL-22 under Th1, Th2, Th17, and Treg conditions, nor did they gain the capacity to produce substantial amounts of an additional subset-defining cytokine, although IFN-γ was slightly induced under different conditions (Supplemental Figure 3). These findings were confirmed in freshly isolated CD45RA+CCR10+ T cell lines from PBMCs of 3 independent donors (Figure 3). CCR10+ sorting enriched IL-22-producing, antigen-experienced T cells (Supplemental Figure 4 and ref. 9). The frequency of IL-22+ T cells and the secretion of IL-22 remained at high levels under all polarizing conditions (Figure 3, B–F, and Supplemental Figure 4), being slightly diminished only under Treg conditions. Importantly, induction of IFN-γ under Th1 conditions and IL-17 under Th17 conditions was not caused by a conversion of Th22 cells into Th1 or Th17 cells, as the number of Th22 cells within the CCR10+ T cell lines was not diminished under different polarizing conditions (Supplemental Figure 4). In contrast, both Th2 and Th22 conditions tended to increase the number of Th22 cells, whereas IL-13 and IL-4 were induced only marginally or not at all (Supplemental Figure 4). Thus, Th22 cells represent a stable T cell lineage, and memory Th22 cells do not convert into another subset.

Transcriptome of Th22 cells. Th22 clones were subjected to full transcriptome analysis to validate the phenotype compared with known T cell subsets, specifically Th17 cells. We compared 3 Th22 cell clones originating from different AE and PS patients with Th1, Th17, and Th22 clones (n = 5; Table 1). To exclude the possibility that Th22 clones derive from Th17 cells, we chose IL-22-producing Th17 clones for gene chip comparison (Table 1). Despite the genetic distance, the Th22 clones showed surprisingly low variance (Figure 4A). The transcriptome for Th22 cells demonstrated similar numbers of up- and downregulated transcripts, whereas the profile for Th22 cells was distinct and not closely related to those of other known T cell subsets (Figure 4B). The gene chip analysis also confirmed the selectivity of IL-22 expression and
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Supernatants were measured 48 hours after anti-CD3/CD28 stimulation. *Clone was included in full transcriptome analysis. †Clone was used for in vitro wounding assay.

The IL-23R. Transcription factors also underlined the individual expression pattern of Th22 cells, given the reduced RO92C, GATA3, and T-bet expression and the presence of BNC-2 and FOXO4 (Figure 4C). It remains to be demonstrated that these factors are also involved in the polarization process upon Th22 differentiation. The expression profile clearly identified Th22 cells as a separate subset, including subset-specific surface receptors and transcription factors (Figure 4D).

Epidermal location of Th22 cells. Epidermis and dermis of skin biopsies were separated by disperse treatment, and infiltrated lymphocytes of each fraction were analyzed for the expression of lead cytokines. Figure 5A shows a representative 3-color FACS analysis, which revealed a higher proportion of IL-22+ T cells and Th22 cells in the epidermal compartment. On average, a 2.2-fold enrichment of IL-22+ cells was observed in epidermal compared with dermal isolations of inflamed skin of AE (n = 4) and ACD (n = 4) patients (median of 8 total dermis, 10.98; median epidermis, 24.05; P < 0.05; Figure 5B; see Supplemental Figure 1B for disease-separated values).

The increased number of IL-22-producing T cells in the epidermis was the result of an increase in Th22 cells and in IFN-y/IL-22-coproducing T cells. The distribution of IL-22+ T cell subpopulations shifted toward IFN-y/IL-22-coproducing T cells (dermis, 30.5%; epidermis, 42.0%), whereas Th22 cells were comparably distributed in the dermal (35.3%) and epidermal (31.0%) compartments. Accordingly, IL-4/IL-22-coproducing T cells were diminished in the epidermal compartment (dermis, 4.6%; epidermis, 2.8%).

Th22 cells orchestrate potentially novel innate immune responses by keratinocytes. To investigate the potential activities of the Th22 resident within the epidermis on epidermal cells, human primary keratinocytes of healthy donors were exposed for 12 hours to medium or Th22 supernatants of 3 independent and activated Th22 clones. Whole transcriptome analysis of keratinocytes revealed alterations of multiple genes (Figure 6A), including genes involved in the intracellular transport of proteins, genes for chemokines regulating different T cell subsets and cytokines, and genes belonging to the innate immune system (Figure 6B and Supplemental Table 4). The Th1-activating and antimicrobial chemokines CXCL9, CXCL10, and CXCL11 were indeed up to 400-fold. A minor, 10-fold induction was also observed for monocyte-activating CCL2, the antiviral and antibacterial Th1 chemokine CCL5, the CCR6+ cell-activating CCL20, and the fibroblast- and eosinophil-attracting CCL26. Furthermore, Th22 supernatants induced T cell growth factors IL-7 and IL-15 and the macrophage differentiation factor IL-32. Surprisingly, Th22 supernatants also substantially induced C15 and C1r, inductible factors of the classi...
Figure 2
Th22 clones show a stable phenotype. Skin T cell lines were cloned by limiting dilution and analyzed by intracellular cytokine staining and ELISA. Th22 clones 1 (A), 2 (B), and 3 (C; see Table 1) produced exclusively IL-22, as determined by multicolor flow cytometry. Stability of the Th22 phenotype is also shown in a time course after TCR stimulation and over a culture time of 10 weeks with subsequent TCR restimulations. Cytokine content of cell-free supernatant is shown for 1 representative experiment.

Discussion
We have shown that Th22 cells represent a distinct human T cell subset characterized by a unique expression profile, a functional spectrum that we believe to be novel, and prominence within inflammatory skin diseases. Similar to Th17 cells, Th22 cells were rarely found in PBMCs, while they were clearly detected in T cell populations isolated from the skin of patients with Ps, AE, and ACD. This is consistent with the previously suggested role of
Figure 3

Th22 cells are stable and cannot be deformed into other T cell phenotypes. Freshly isolated CCR10+T cells did not convert into another Th subset. Shown are intracellular stainings for CCR10+CD45RA+ cells after 5 days under nonpolarizing (A), Th1 (B), Th2 (C), Th17 (D), Th22 (E), and Treg (F) conditions and subsequent stimulation with PMA/ionomycin. Shown are dot plots for IL-17 and IFN-γ, for IL-22 and IL-17, and of IL-17-IL-22-gated cells (red outline) for IFN-γ and IL-4. Th22 cells are shown by red shading. Numbers indicate relative percentages per quadrant. Data are representative for 3 independent donors. Mean values and corresponding IL-22, TNF-α, IL-13, and IL-4 secretion are shown in Supplemental Figure 3.

Th17-related IL-22 in Ps (18, 19), atopic dermatitis (20), and contact dermatitis (21, 22).

Although we demonstrated a pure Th22 population, we observed a substantial proportion of Th22 cells that also secreted IFN-γ or IL-17. This plasticity is also observed for other T cell phenotypes, such as Th17 cells expressing IFN-γ or Tregs expressing IL-17 (23, 24). It is of interest that the plasticity expressed by Th22 cells in AE was also apparent in a small percentage of IL-4-expressing cells, but not within Ps or ACD. Our present data demonstrated that the extent and composition of this plasticity varied among different inflammatory disease entities even within the same organ. We anticipate that Th22 plasticity is also related to disease severity, as severity-associated factors such as IL-12 (25) are known to modulate T cell plasticity (23, 26).

Although T cell plasticity is now an accepted component of the specific immune system, the stability of a T cell phenotype over multiple cell divisions remains a hallmark of Th subsets, conferring immunologic memory, as we have demonstrated for Th22 cell clones in the current study. Th22 clones responded within 12 hours with maximal IL-22 secretion and were also able to produce moderate amounts of TNF-α and IL-10. Although NK cells show a similar narrow cytokine profile (27–29), the Th22 cells described herein were clearly defined as non-NK cells, demonstrated by CD4 and TCR expression and the absence of NK cell markers. Recent studies indicate that IL-6 and TNF-α, along with the help of plasmacytoid DCs, can promote the Th22 phenotype (9); however, precise culture requirements lacking serum and APCs remain to be defined.

Th22 cells showed distinct differences in the profile of altered genes compared with Th1, Th2, and Th17 cells, confirming an individual signature for the Th22 subset. In contrast to Th17, expression of CCL20 and IL-23R (30) was absent in Th22 clones. Furthermore, RORC, the transcription factor essential for Th17 differentiation (31), was strongly diminished in Th22 cells. This confirms recent reports (9, 10) that highlight the aryl hydrocarbon receptor in the differential regulation of IL-22 over
Th22 cells represent a terminally differentiated and independent T cell subtype. The Th22 expression profile revealed several FGFs: FGF1 is a powerful mitogen exhibiting strong action on different cell types, including endothelial cells (32, 33), and FGF5 also acts on neuronal differentiation (34) and is associated with inhibition of hair growth (35, 36). These cytokines play a role in various stages of development and morphogenesis as well as in angiogenesis and wound healing processes. Several members of the FGF family have previously been shown to be involved in Ps (37, 38). Consistent with this upregulation of fibrogenic factors, the SPRY7 gene, an antagonist of FGFs, was significantly reduced in Th22 cells. A potential role for Th22 in remodeling or repair of the epithelial barrier is further supported by the chemokine expression pattern characterized by CCL7, involved in tissue fibrosis (39), and CCL15 and possibly CCL23 splice variant 2, both implicated in angiogenesis (40, 41). We note that results with CCL23 splice variant 1 contradicted this finding (Figure 4C), but no publications did not clarify which isoform was detected.

Th22 cells, like Th17 cells, show an epidermal homing characteristic reflected by a remarkable transcriptome response of primary human keratinocytes in response to Th22 supernatants: namely, the chemokines CXCL9, CXCL10, and CXCL11 are likely to recruit further T cells and also show antimicrobial activity. However, the transcriptome profile also revealed characteristics of keratinocytes that we believe to be previously unknown, including the ability to contribute to expression of complement factors such as C1s, C1r, and CFB and expression of TLR3 and TLR6, in addition to the previously described ability of keratinocytes to express S100 genes and defensins in response to IL-22 (14). Th22 cells also have potential to promote adaptive and innate immunity.
by virtue of the induction of T cell and NK promoting factors, such as IL-15 and IL-7, in keratinocytes. Of particular interest is the expression of the TNF-α-enhancing cytokine IL-32 by keratinocytes, which corresponds to the ability of Th22 cells to also express TNF-α. We hypothesize that IL-22 and TNF-α represent the key combination of cytokines expressed by Th22 cells that unlocks the activation of keratinocytes, as in vitro cultures using either Th22 supernatants or a combination of IL-22 and TNF-α maximally induced the Th22 signature in keratinocytes, whereas anti-IL-22 did not fully block — and recombinant IL-22 alone was not sufficient to induce — expression of these genes. This dual key is a recurring scheme observed in the lymphoid regulation of tissue cells in inflammatory context, such as the regulation of epithelial cells by IL-17, which is only maximal in synergism with IFN-γ, IL-1, or TNF-α (42, 43). In contrast to major effects on the innate immune response by keratinocytes in a proinflammatory context, IL-22 and TNF-α did not synergize in the enhanced wound healing clearly induced by Th22 cells. This effect was only IL-22 dependent and reflected the induction of promigratory genes in keratinocytes by IL-22 (8, 14, 44, 45). These data indicate a double function of IL-22 in cutaneous immunity: on the one hand, being protective and regenerative, and on the other hand, amplifying TNF-α-induced signals to contribute to a proinflammatory microenvironment during skin immune reactions.

In conclusion, our present results demonstrate that Th22 cells represent a T cell subset uniquely able to regulate epidermal responses in inflammatory skin diseases. The subset is not only defined by a stable and distinct expression profile, but also characterized by what we believe to be a novel functional profile. The identification of Th22 cells provides a cellular target for therapeutic intervention and may shed light on thus far unknown pathways in the control of tissue immunity and remodeling.

Methods

Patients. Adult Ps patients (n = 3) suffered from stable, moderate to severe plaque Ps, defined by a Ps area and severity index (PASI) score of at least 12, a minimum involvement by Ps of 10% body surface area, and a history of Ps of at least 6 months before sample collection. AE patients (n = 4) were diagnosed according to the criteria of Hanifin and Rajka (46). ACD patients (n = 4) displayed positive patch tests following criteria of the International Contact Dermatitis Research Group (47). Exclusion criteria were local treatment with corticosteroids, phototherapy, or systemic corticosteroid therapy within 4 weeks of sample collection. Before blood or skin samples were taken, each participant gave his informed consent. The study was approved by the ethical committee of the Instituto Dermatológico del Iberg Espanol. Isolation and expansion of T cells and primary human keratinocytes. T cell clones derived from inflammatory skin conditions were isolated as previously described (48). Briefly, whole biopsies, or biopsy material treated with 0.5% dispase (Roche) for 1 hour at 37°C in order to separate the epidermis and dermis, were cultured in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-mercaptoethanol, 1% penicillin/streptomycin (all InVitrogen; RPMI complete), and 5% human serum (Sigma-Aldrich) supplemented with 60 U/ml IL-2. Migrated cells were collected after 2–4 days and cloned after 6 days by limiting dilution (0.6 cells/well in 96-well U-bottom microtiter plates) in RPMI complete 5% human serum and 10% heat-inactivated FBS (HyClone) on a feeder layer of irradiated PBMCs, 30 U/ml IL-2, and 1% PHA (Sigma-Aldrich). Fresh medium containing IL-2 (Novartis) was added 3 times per week, and clones were restimulated with irradiated feeder PBMCs every 3 weeks. Allergen or hapten reactivity of both T cells and clones was determined using the 3H-thymidine incorporation assay. Cytoxic content of clone supernatants was analyzed using ELISA in order to identify Th subset identity (see Table 1).

Aplastic keratinocytes were isolated using the previously described method of suction blister (43). 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 (inviGenot) to obtain single-cell suspension, and seeded on a feeder layer of irradiated 3T3/12 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 secondary and third passage were used in experiments. Isolation of CCR10+ T cells and phenotype stability. CCR10+ effector T cells were magnetically isolated from CD45RA depleted PBMC with a combination of a PE-labeled anti-CCR10 antibody (R&D Systems) and anti-PE microbeads (Miltenyi Biotech). CCR10+ cells were restimulated for 5 days in AIMV medium with anti-CD3 and anti-CD28 antibodies and the following cytokines and blocking antibodies: 25 ng/ml IL-12 and 5 μg/ml anti-IL-4 (Th1 condition); 25 ng/ml IL-4, 5 μg/ml anti-IL-12, and anti-IFN-γ (Th2 condition);
20 ng/ml IL-1β, 20 ng/ml IL-6, 20 ng/ml IL-23, 5 ng/ml TGF-β, 5 μg/ml anti-IL-12, and 5 μg/ml anti-IL-4 (Th17 condition); 5 ng/ml TGF-β, 5 μg/ml anti-IL-12, and 5 μg/ml anti-IL-4 (Treg condition); 50 ng/ml TNF-α, 20 ng/ml IL-6, 5 μg/ml anti-IL-12, and 5 μg/ml anti-IL-4 (Th22 condition).

**Stimulation of keratinocytes.** A total of 10^4 keratinocytes were seeded in 6-well microtiter plates in keratinocyte basal medium (KBM) with supplements (Invitrogen) for 5 days. At day of stimulation, medium was replaced by KBM and KBM plus 50 ng/ml recombinant TNF-α and/or 50 ng/ml IL-22. Alternatively, supernatant was obtained from Th22 clones stimulated for 48 hours with anti-CD3 and anti-CD28 (both BD Biosciences) and centrifuged to avoid cellular contamination. Cytokine content was measured by ELISA. Natural IL-22 was neutralized by incubation with 10 μg/ml anti-IL-22 (AF-782; R&D systems) 3 hours prior to keratinocyte stimulation. Full medium cells served as negative control. After 12 hours of stimulation, period cells were washed, detached with 0.056 trypsin, shock-frozen in liquid nitrogen, and conserved at -80°C until further analysis.

**Flow cytometry analysis.** The following antibodies were used in flow cytometry analysis: FITC-conjugated CD4 (clone SK3), PE-conjugated CD4 (clone SK5), PE-conjugated CD8 (clone SK1), and FITC-conjugated CD56 (clone NCAM16.2), all from BD Biosciences; FITC-conjugated IL-4 (clone MP4-25D2), FITC-conjugated IFN-γ (clone B27), and allophycocyanin-conjugated IFN-γ (clone B27), all from BD Biosciences – PharMingen; PE-conjugated IL-22 (clone 142928) and PE-conjugated IL-4 (clone 3007.11), all from R&D Systems; and PE-conjugated IL-17A (clone SK3) from eBioscience.

Intracellular cytokine staining was performed using a kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were stimulated with PMA and ionomycin for 6 hours in the presence of monensin. After 2 hours, brefeldin A was added. Cells were fixed and permeabilized with Cytofix/CytoPerm (BD Biosciences) and incubated with antibodies. Cells were analyzed using FACS Aria (BD Biosciences), and data were illustrated by FlowJo software (Tree Star Inc.).
Figure 7

Th22 cells influence keratinocyte functions by a combination of IL-22 and TNF-α. Human primary keratinocytes were stimulated with Th22 supernatants in the presence or absence of neutralizing anti–IL-22 antibodies (left) or with recombinant cytokines (right). mRNA was extracted after 12 hours, and gene expression was analyzed by real-time PCR. (A) Chemokine expression. (B) Innate immune response. (C) Cytokine expression pattern. Data are representative for at least 3 independent experiments. Error bars indicate SD.
Figure 8
Th22 supernatant enhances wound healing in a functional in vitro injury model. Microscopic photography after wound induction on a confluent monolayer of primary human keratinocytes in a time course from 4 to 24 hours revealed that wound healing was efficiently enhanced by Th22 supernatant and by IL-22, but less so by other T cell subset supernatants. Neutralization of IL-22 in Th22 supernatant reverted this effect. Brackets show initial wound size for comparison. Graphs below show relative wound closure over time, based on the wound gap compared with initial wound size. Error bars indicate SD.
ELISA. Concentrations of IFN-γ, IL-4, IL-10, IL-17, IL-22, and TNF-α in cell-free culture supernatant were measured using commercially available sandwich ELISA kits (all from R&D systems).

RNA extraction, cDNA generation, and real-time PCR analysis. Total RNA was isolated from frozen cell pellets with a RNeasy Mini Kit (Qiagen) and reversely transcribed with random hexamers using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s protocols. Primers for real-time PCR, designed with Primer Express software (version 1.2; Applied Biosystems), are listed in Supplemental Table 2. PCR was performed in 384-well plates using EP Motion 5075 (Eppendorf) for pipetting all reagents. The cDNA was amplified with SYBR Green Mastermix (Applied Biosystems) in an ABI Prism 7000HT Sequence Detection System (Applied Biosystems). The comparative ΔΔct method was used to calculate the relative quantification and the range of confidence.

Whole genome microarray analysis. Quality and integrity of RNA samples was checked with the Agilent 2100 Bioanalyzer platform (Agilent Technologies). 0.5 µg of each total RNA sample was amplified and Cy3 labeled using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) following the hybridization protocol. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit and Agilent Whole Human Genome Oligo Microarrays 4×44K (Agilent Technologies). Fluorescence signals of the hybridized arrays were detected using Agilent’s Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software was used to read out and process the microarray image files, and differential gene expression Feature Extraction Software-derived output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware).

Keratinocytes in vitro injury model. Confluent monolayers of cultured primary human keratinocytes were scratched with the tip of a P-200 pipette to create a uniform cell-free zone in each well. Cellular debris was removed by PBS washing. wounded monolayers were then incubated in Th1-, Th2-, and Th17-conditioned RPMI media. Reconstituent human IL-22 (10 ng/mL; R&D Systems), alone or in combination with reconstituent human TNF-α (10 ng/mL; R&D Systems), and Th2 supernatant preincubated with neutralizing anti-IL-22 antibody (AF-782; R&D Systems) served as controls. KBM plus RPMI represented the negative control. Microscopy pictures were taken at different time points after injury with a digital camera. The residual gap between migrating keratinocytes was measured with a computer-assisted image analysis system (Axiovision; Zeiss) and expressed as a percentage of the initial scratched area.

Statistical analysis was performed using 2-tailed Student’s t test. Statistically significant differences were defined as P < 0.05.

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Online supplemental material

Supplemental Figure 1: IL-22+ T cells comprise about one third “pure” Th22 cells. A) Algorithm to calculate the relative frequency of Th22 cells within the cellular infiltrate of inflammatory skin diseases. A representative 4-color intracellular staining of skin-infiltrating T cells shows the relative frequency of IL-22+IL-17- and of IL-22+IL-17+ T cells, in the example 15.94% (I). The relative frequency of Th22 within total T cellular infiltrate is calculated as the percentage of IL-22+IL-17- cells (dot plot I, 7.4%) that are also negative for IFN-g and IL-4 (lower left quadrant of dot plot II, 41.2% of IL-22+IL-17- cells). The relative frequency of Th22 within total IL-22+ cells is calculated as Th22/total IL-22+ cells, in the shown example 19.1% (III). B) Relative frequency of Th22 cells within IL-22+ T cells in peripheral blood (PBMC, n=4), Psoriasis (PS, n=3), atopic eczema (AE, n=4) and contact dermatitis (ACD, n=4). Shown are mean percentages +/- standard error of the mean.

Supplemental Figure 2: Surface phenotype of Th22 clones. The surface phenotype of the Th22 clones shown in Figure 2 was further analysed by flow cytometry. Shown are representative flow cytometric stainings of clone 1(a), clone 2 (b) and clone 3 (c) in resting state (red lines) as compared to isotype control (blue lines).

Supplemental Figure 3: Th22 clones were cultured under Th1, Th2, Th17 and Treg conditions. 4 color-intracellular staining after PMA/ionomycin stimulation after 4 and 7 days shows that Th22 conserved a high secretion of IL-22 (x-axis of dot plots), but did not gain the capacity to produce substantial amounts of another lineage-indicating cytokine (y-axes of dot plots) despite a slight increase in IFN-γ. Data shown are representative and show “clone 3” (Fig 2c).

Supplemental Figure 4: Th22 cells are stable and cannot be deformed into other T cell phenotypes. Freshly isolated Th22 cells are stable under different T helper subset-polarizing conditions. Th22 cells from peripheral blood of three different donors were enriched by isolating CD45RA-CCR10+ T cells and cultured under Th1, Th2, Th17,
Th22 and Treg conditions. After 5 days, they were stimulated with anti-CD3/anti-CD28 for 48 hours, and cytokine content was measured by ELISA (a) or stimulated with PMA/Ionomycine and stained intracellularly (b). Cytokine content of cell-free supernatant is given in the y-axes. As recombinant cytokines were added, TNF-α under Th22 and IL-4 under Th2 conditions is not shown (marked with “X”). Shown are three independent experiments. Error bars indicate Standard error of the mean.

**Supplemental Table 1:** Quantity of distinct T cell phenotypes and plasticity of T cell subsets varies among inflammatory skin disorders. Shown are relative percentages of primary human T lymphocytes derived from psoriasis (n=3), AE (n=4), and ACD (n=4) that stain positive for IL-22, IL-17, IFN-γ and IL-4 in 4 color-intracellular stainings, as shown in the Venn blot in figure 1f.

**Supplemental Table 2:** Primer sequences used for real time PCR analysis.

**Supplemental Table 3:** Bioinformatic extraction of genes up- or downregulated in Th22 clones (n = 3) in comparison to effector T cell clones (n = 5). Genes, which were at least two-fold up- or downregulated entered the analysis. The functional groups were filtered using the Biblioshere software (Genomatix). All tables were curated and corrected for double assignments.

**Supplemental Table 4:** Bioinformatic extraction of genes up- or downregulated in keratinocytes upon exposure to Th22 supernatants in comparison to medium. Genes that were at least two-fold up- or downregulated entered the analysis. The functional groups were filtered using the Biblioshere software (Genomatix). All tables were curated and corrected for double assignments.
Supplemental figure 1

A

I. Total IL-22+ cells

II. Fraction of Th22 cells on total T cells

III. Fraction of Th22 cells on IL-22+ cells

B

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% Th22 of total IL-22+ cells

n=4 n=3 n=4 n=4
Supplemental Figure 2

Suppl. Figure 3

**Th22 clones under deforming conditions**

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Suppl Fig. 4

a  Cytokine secretion (ELISA) of CCR10⁺ CD45RA⁻ T cells (n=3)

5 day culture (anti-CD3/CD28)
neutral conditions (medium)

\n
T₄¹ conditions (IL-12 +α-IL-4)
T₄² conditions (IL-4+α-IL-12)
T₄十七条 (IL-1β+IL-6+α-IFN-γ+α-IL-4)
T₄²条件 (TNFα+IL-6)
Treg conditions (TGF-β+α-IFN-γ+α-IL-4)

[Graphs showing cytokine levels (IL-22, TNF-α, IL-13, IL-4)]

b  Mean of Intracellular FACS analysis of CCR10⁺ CD45RA⁻ T cells (n=3)

5 day culture (anti-CD3/CD28)
neutral conditions (medium)

\n
T₄¹ conditions (IL-12 +α-IL-4)
T₄² conditions (IL-4+α-IL-12)
T₄十七条 (IL-1β+IL-6+α-IFN-γ+α-IL-4)
T₄²条件 (TNFα+IL-6)
Treg conditions (TGF-β+α-IFN-γ+α-IL-4)

[Graphs showing percentage of IL-22⁺ cells and Th22 cells (IL-22⁺, IFN-γ⁺, IL-17⁺)]
### Supplemental Table 1

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### Supplemental Table 2

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<td>TGAAGGTKAAAGATGGCAGCAA</td>
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<td>TGTTCCATCATGTTTCCAT</td>
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<td>CTG AAG GCC CGA ATG CAC CAG</td>
<td>GCA AAG GTG GTG TCA GTA TC</td>
</tr>
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</table>
IL-22 and TNF-α 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-α. Here, we demonstrate that IL-22 increases the TNF-α-dependent induction and secretion of several immune-modulatory molecules such as initial complement factors C1r and C1s, antimicrobial peptides S100A7 and HBD-2 (human β defensin 2), and antimicrobial chemokines CXCL-9/10/11 in primary human keratinocytes. The synergism of IL-22 and TNF-α 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-α effectively inhibit the growth of *Candida albicans* and maintain survival of epithelia. Accordingly, the combinatorial stimulation of keratinocytes with IL-22 and TNF-α most efficiently conserves the integrity of the epidermal barrier in a three-dimensional skin infection model as compared with IFN-γ, IL-17, IL-22 or TNF-α alone. In summary, we demonstrate that IL-22 and TNF-α represent a potent, synergistic cytokine combination for cutaneous immunity.

**Keywords:** *Candida albicans* • Epidermal integrity • IL-22 • TNF-α

**Introduction**

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

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(IL-10R) and the IL-22 receptor (IL-22R) [7]. While IL-10R 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 in 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 β 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-α and IL-22 [4].

The aim of this study was to investigate the molecular mechanisms underlying the synergism of TNF-α and IL-22 and the functional impact of this synergistic effect. It is demonstrated that IL-22 and TNF-α 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-α 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-α and IL-22 on primary human keratinocytes [4]. Table 1 confirms the synergism of TNF-α and IL-22 in the induction of some innate immunity genes in primary keratinocytes obtained from healthy individuals. At protein level, TNF-α induced CXCL-10 secretion in primary keratinocytes (n = 6) by ten-fold (Fig. 1A), CXCL-11 by six-fold (Fig. 1B) and HB-2 by 21-fold (Fig. 1C). In contrast, IL-22 only marginally induced CXCL-10, CXCL-11 and HB-2. Co-stimulation with IL-22 and TNF-α consistently and significantly enhanced the secretion over the level of an additive effect by 20-fold (p<0.001 versus IL-22/p<0.01 versus TNF-α) and 41-fold (p<0.001 versus IL-22/p<0.01 versus TNF-α), respectively. To estimate the biological relevance of this synergistic induction, we also stimulated keratinocytes with known inducers of these proteins. IL-22 and TNF-α stimulation lead to an upregulation of CXCL-10, CXCL-11 and HB-2 in the same dimension as IFN-γ and IL-17 respectively. This synergistic CXCL-10 induction and secretion becomes significant after 36 h (four-fold; p<0.05 versus IL-22/p<0.05 versus TNF-α) and is maintained over three days (17-fold after 48 h p<0.005 versus IL-22/p<0.05 versus TNF-α; 42-fold after 72 h; p<0.001 versus IL-22/p<0.01 versus TNF-α). (Fig. 1D). Similar results have been obtained for CXCL11 and HB-2 (data not shown).

Table 1. Synergistic induction of mRNA expression of genes belonging to the innate immune system in primary human keratinocytes by TNF-α and IL-22

<table>
<thead>
<tr>
<th>Gene</th>
<th>IL-22</th>
<th>TNF-α</th>
<th>IL-22 + TNF-α</th>
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<tr>
<td>CXCL-9</td>
<td>1±0.2</td>
<td>98±211</td>
<td>238±19</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>2±0.2</td>
<td>1848±778</td>
<td>7410±3232</td>
</tr>
<tr>
<td>CXCL-11</td>
<td>1±0.1</td>
<td>309±116</td>
<td>1757±741</td>
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<tr>
<td>CILs</td>
<td>3±0.4</td>
<td>40±9</td>
<td>399±151</td>
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<tr>
<td>CD40L</td>
<td>3±0.1</td>
<td>48±14</td>
<td>238±76</td>
</tr>
<tr>
<td>S100A7</td>
<td>3±0.1</td>
<td>3±0.4</td>
<td>8±0.7</td>
</tr>
<tr>
<td>HBD-2</td>
<td>6±0.8</td>
<td>359±74</td>
<td>826±99</td>
</tr>
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</table>

3) 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 and TNF-α 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-α 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-α or a combination of both. TNF-α 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-α promote activation of AP-1 family transcription factors, but not of STAT3 or NF-κB

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-α on these transcription factors in primary human keratinocytes. In line with our previous results, sole stimulation with IL-22 or TNF-α weakly induced AP-1 (1.30±0.08 relative luminescence or 1.33±0.1 relative luminescence), as measured by a dual luciferase system. In contrast, co-stimulation with IL-22 and TNF-α resulted in a significant activation of AP-1 (1.84±0.17 relative luminescence, Fig. 3A). To identify single members of the AP-1 family, we screened for activation of the AP-1 family transcription factors. These include c-Fos, c-Jun, Jun-B and AP-1. In line with our previous results, c-Fos and c-Jun were strongly induced by TNF-α and IL-22, respectively. Co-stimulation with IL-22 and TNF-α resulted in a significant activation of c-Fos and c-Jun.
Figure 1. IL-22 reinforces TNF-α-induced innate immunity in keratinocytes. Primary human keratinocytes (n = 6) were stimulated with TNF-α, IL-22, IL-17 (50 ng/mL each) and IFN-γ (300 U/mL) or a combination of TNF-α and IL-2. Content of (A) CXCL-10, (B) CXCL-11 and (C) HBD-2 after 72 h of treatment was measured 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<0.05, **p<0.01.

Figure 2. The synergism of TNF-α and IL-22 is mediated by the MAP kinase p38. Primary human keratinocytes stimulated with 50 ng/mL TNF-α, 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 (β-actin). The Western blot shown is representative of six independent experiments.

AP-1 family, TransAM ELISA systems were used to detect nuclear translocation. TransAM experiments demonstrated that c-fos (Fig. 3C) was synergistically induced by IL-22 and TNF-α (1.89±0.17 fold induction, p<0.001 versus IL-22/p<0.01 versus TNF-α). ATF-2, another AP-1 family member, showed a non-significant trend of induction by interaction of both cytokines (1.95±0.33 fold induction) (Fig. 3B). STAT3 (Fig. 3F) was only induced by IL-22 (1.23±0.06 fold induction), whereas c-jun (Fig. 3D) and NF-κB (Fig. 3E) were only activated by TNF-α (1.83±0.16 fold induction, p<0.001 versus control; 2.22±0.18 fold induction, p<0.001 versus control).

The synergism of TNF-α 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-α and IL-22 in an in vitro Candida infection model. Candida growth was inhibited by supernatant of keratinocytes stimulated with TNF-α plus IL-22 or Th2 supernatant respectively (Fig. 4A). In contrast, IL-22 alone had no effect and TNF-α only a weak inhibitory effect or Candida growth. Furthermore, both TNF-α plus IL-22 (Fig. 4B, upper graph) and Th2 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±6.16%, p<0.01 and 66.12±8.55%, p<0.01, respectively). Again, TNF-α and IL-22 alone had little or no protective effect (90.55±7.2%, and 104.79±5.31%). These results indicate that a Th22-like combination of cytokines synergistically induces an effective innate immune response of epithelial cells.
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 \(C.\) \textit{albicans}. Stimulation with TNF-\(\alpha\) or IL-22 in the absence of \(C.\) \textit{albicans} resulted in a mild hyper-proliferation of the three-dimensional skin models (Fig. 5, left pictures). While \(C.\) \textit{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 \textit{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 \(C.\) \textit{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
TNF-α-induced regulation of multiple innate defense genes such as complement cascade proteins, antimicrobial peptides and antimicrobial chemokines.

The interaction of IL-22 and TNF-α is mediated through the IL-22R heterodimer and tumor necrosis factor receptor 1 (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-α strongly induces 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-α (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-α strongly induces the NF-κB signaling cascade in keratinocytes (29). Since NF-κB is not synergistically activated by the combination of TNF-α and IL-22, the observed synergism does not cover the whole functional spectra of TNF-α and IL-22, but is rather limited to aspects such as innate immunity. This may explain functional diversity of TNF-α 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-α it becomes immune-stimulatory and arms epithelia for innate responses.

Figure 4. TNF-α 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-α, 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.

Figure 5. The combination of TNF-α 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 × 40).

The stimulation of the epithelial immune system by the IL-22/TNF-α 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-α therapy (Infliximab) are serious respiratory and skin infections (31), which could be explained by the missing interaction of IL-22 with TNF-α. Therefore, the IL-22/TNF-α 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-α 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 (Inovitrogen) 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-α, 50 ng/mL IL-22 (both R&D Systems) or a combination of both. For some experiments, 10^6 cells of human Th2 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-α, IFN-γ, 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'-GCTGACGATGACGAAAGGACAT-3', reverse 5'-GTAATTTGAGCCCTTTTTGGCACA-3'); HD2 (forward 5'-CTCCCTTCTCTGGGCTTCTTACAT-3', reverse 5'-AGGATGCTTACAACCACCAAAA-3'); CXCL-9 (forward 5'-TCAGCTCTGGTCAATCGG-3', reverse 5'-CCCTAAACAACTCCCAAGG-3'); CXCL-10 (forward 5'-GCTGATGCAAGTCAAGGCT-3', reverse 5'-CACATGAAACTAAATGAGGA-3'); CXCL-11 (forward 5'-ATGCAAAAGACACCGCTCT-3', reverse 5'-GAAAGCATGAGTGAAGACTG-3'), C1s (forward 5'-GAAAGTCTTCTCTGAGCT-3', reverse 5'-TGAGGAGATGACCCTGTGGC-3'), C1r (forward 5'-TCCCGAGGCTTTTTATATCCA-3', reverse 5'-GAAAGCTGCTCTCCAGGCT-3'). The comparative ΔΔCt 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× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL FMSF, 50 μg/mL aprotinin, 100 μM sodium orthovanadate and 10 μg/mL rosetting inhibitor cocktail (Sigma). Cell lysates were collected in a microfuge for 15 min at 15,000 × g. Supernant was collected and utilized for SDS-PAGE. After cell lysis, the supernatant was diluted 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-β-Actin (Sigma) (0.25 μg/mL), anti-JNK1/2 (1 μg/mL), anti-phospho-JNK1/2 (1 μg/mL), anti-P38 and anti-phospho-P38 (1 μg/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 μg/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-Dimethyl-1, 3- dimethyl-4, 5-dimethyl-2H-tetrazolium) and 1:1 HygroX – 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-α or a combination of both were measured using commercially available sandwich ELISA kit according to the manufacturer’s instructions (CXCL10, CXCL11: R&D Systems, HBD-2: Phoenix Pharmaceuticals).

In vitro Candida infection model

C. albicans wild-type strain SCS314 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% CO₂.

For two-dimensional skin infection models, 30,000 human oral keratinocytes (TR146) were plated per well in 96-well plates in antibiotic and antymycotic free culture medium. Twenty-four hours after plating, cells were treated with 50 ng/mL TNF-α 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 × 10⁶ human oral keratinocytes (TR146) were seeded on inert filter substrates (Nunc, polycarbonate filter, 0.4 μm pore size, 0.5 cm²) 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 substrate. Epithelium was treated with IFN-γ (300U/mL), IL-17, IL-22, TNF-α (50 ng/mL each), IL-22/TNF-α combination or Th22 supernatant directly before infection with 2 × 10⁶ Candida yeasts for 12 h. Light microscopic 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 < 0.05, **p < 0.01, ***p < 0.001.

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Abbreviation: HD-2: human 5-b Defensin 2 - IL-10RI: IL-10 receptor 
- IL-22R1: IL-22 receptor - LDH: lactate dehydrogenase

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IL-22 suppresses IFN-γ–mediated lung inflammation in asthmatic patients

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Background: IL-22 controls tissue homeostasis by both proinflammatory and anti-inflammatory effects. However, the anti-inflammatory mechanisms of IL-22 remain poorly investigated.

Objective: We sought to investigate the anti-inflammatory role for IL-22 in human asthma.

Methods: T-cell lines derived from lung biopsy specimens of asthmatic patients were characterized by means of flow cytometry. Human bronchial epithelial cells from healthy and asthmatic subjects were stimulated with IL-22, IFN-γ, or the combination of both cytokines. Effects of cytokine stimulation were investigated by using whole-genome analysis, ELISA, and flow cytometry. The functional consequence of cytokine stimulation was evaluated in an in vitro wound repair model and T cell–mediated cytotoxicity experiments. In vivo cytokine expression was measured by using immunohistochemistry and Luminex assays in bronchoalveolar lavage fluid of healthy and asthmatic patients.

Results: The current study identifies a tissue-restricted antagonist interplay of IL-22 and the proinflammatory cytokine IFN-γ. On the one hand, IFN-γ antagonized IL-22–mediated induction of the antimicrobial peptide S100A7 and epithelial cell migration in bronchial epithelial cells. On the other hand, IL-22 decreased epithelial susceptibility to T cell–mediated cytotoxicity by inhibiting the IFN-γ–induced expression of MHC-I, MHC-II, and CD54/intercellular adhesion molecule 1 molecules. Likewise, IL-22 inhibited IFN-γ–induced secretion of the proinflammatory chemokines CCL5/RANTES and CXCL10/interferon-γ-inducible protein 10 in vitro.

Consistently, the IL-22 expression in bronchoalveolar lavage fluid of asthmatic patients inversely correlated with the expression of CCL5/RANTES and CXCL10/interferon-γ-inducible protein 10 in vivo.

Conclusions: IL-22 might control the extent of IFN-γ–mediated lung inflammation and therefore play a tissue-restricted regulatory role.

Key words: T22 cells, IL-22, IFN-γ, asthma, human bronchial epithelial cells, epithelial regulation

Asthma is a chronic inflammatory disorder of the airways characterized by airway obstruction with characteristics of remodeling and evidence of ongoing epithelial injury and repair. 1 T cells contribute to chronic asthma by inducing direct tissue damage in epithelial airways, secreting proinflammatory cytokines, and releasing factors that contribute to epithelial remodeling. T22 cells are primary effector cells in asthmatic patients because they secrete IL-4, IL-5, and IL-13, leading to IgE production of B cells and eosinophil–mast cell–basophil-mediated inflammation. T22, T17, and cytokotoxic T cells (Tc) contribute to lung inflammation through the release of a large number of cytokines, such as IFN-γ, IL-17, and IL-22, and induction of apoptosis in lung epithelial cells. 2–5 IFN-γ is a key proinflammatory cytokine in lung inflammation. It stimulates epithelial cells to release chemokines 6 relevant for the recruitment of immune cells and therefore amplifies the ongoing immune reaction. 7 IFN-γ also promotes the induction of MHC class I (MHC-I), MHC class II (MHC-II), and intercellular adhesion molecule 1 (ICAM-1) expression on epithelial cells, thereby enhancing adhesion of T cells and induction of apoptosis by CD8+ and CD4+ T cells. 8–11 Although the role of IFN-γ in the inflammatory process has been extensively investigated, the contribution of IL-22 remains unclear. IL-22 is a member of the IL-10 cytokine family, which is produced by many immune cells, such as natural killer cells, CD11c+ myeloid cells, lymphoid tissue inducer–like cells, Tc cells, and the recently described T22 T-cell

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METHODS

Patients
Healthy subjects (n = 11) and asthmatic patients (n = 28) were included according to the American Thoracic Society Workshop on Refractory Asthma. 27 Patients affected by mild (n = 14) and severe (n = 14) asthma were enrolled in the study. Pulmonary function tests were performed and biopsy specimens and bronchoalveolar lavage fluid (BALF) were taken during baseline symptoms. The baseline symptoms correlated with pulmonary function test results. Bronchial biopsies and bronchial alveolar lavage were performed according to the local ethics committee. Each participant provided informed consent.

Cytokines and antibodies

The following antibodies were used for flow cytometric analysis: CD4–peridinin-chlorophyll-protein complex (SK3), IFN–γ–V450 (B27; both from BD Biosciences), CD8–phycoerythrin (RPA-T8), CD8–APC-Cy7 (SK1), IL–17A–Alexa Fluor 488 (N49-655; all from BD Pharmingen, San Jose, Calif), IL–4–phycoerythrin (5010.211, BD FastImmune), IL–22–allophycocyanin (142928), CD54–fluorescein isothiocyanate (BHIG-11; both from R&D Systems, Minneapolis, Minn), HLA-DR–allophycocyanin (LN5), and HLA A–B–C–flourescein isothiocyanate (W6/32; both from ebioScience, San Diego, Calif). For cell culture, stimulation, and blocking experiments, the following recombinant cytokines and antibodies were used: IL–2 (Novartis, Basel, Switzerland), IL–22 and IFN–γ (R&D Systems), purified anti–CD3 (UCHT1) and anti–CD28 (CD28.2; both from BD Biosciences), anti–IFN–γR1 (MAB0372), anti–IL–22Rα1 (AF2770), mouse IgG1 (MAB002), and polyclonal goat IgG (AB-108-C; all from R&D Systems).

Isolation and expansion of lung-derived T cells

Lung biopsy specimens of asthmatic patients were cultured in complete RPMI 1640 supplemented with 10 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL gentamycin, 5% human serum (Sigma, St Louis, Mo), and 20 U of IL–2/mL (Novartis). Emigrating cells were expanded by means of anti–CD3/anti–CD28 stimulation. After 10 to 13 days, T-cell lines were collected and characterized by using flow cytometry.

Flow cytometric analysis

Surface and intracellular cytokine staining were performed with the Cytotoxicity/Cytokine kit (BD Biosciences), according to the manufacturer’s instructions. Acquisition and analysis were performed with the FACSCanto II (BD Biosciences).

Human bronchial epithelial cell culture and stimulation

Primary human bronchial epithelial cells derived from 3 healthy subjects (NHBE cells) and 3 asthmatic patients (DHBE cells) were purchased from Lonza (Basel, Switzerland). Bronchial epithelial cells were cultured in complete human bronchial epithelial cell culture (BEGM, Lonza). Cells of the first, second, and third passages were used in experiments. Confluent and subconfluent epithelial cells were stimulated with recombinant 50 ng/mL IL–22 and 10 ng/mL IFN–γ in growth factor–free BEGM (Lonza). After 36 hours of stimulation, flow cytometric staining of MHC-I (HLA-A, B, C), MHC-II (HLA-DR), and CD54 (ICAM-1) were performed. Supernatants were collected after 48 hours of stimulation, and the content of CCL5/RANTES was analyzed by using ELISA (R&D Systems).

Whole-genome microarray analysis and real-time PCR

The total RNA sample was amplified and Cy3 labeled by using the 1-color Low Input Quick Amp Labeling Kit, according to the manufacturer’s protocol. Hybridization to SurePrint G3 Human Gene Expression 6x60K Microarrays was performed by using the Gene Expression Hybridization Kit. Differential gene expression was analyzed with the Genespring Software GX 11.0 (Agilent Technologies, Santa Clara, Calif). Genes regulated more than 2-fold change were further analyzed by using the paired Student’s t test and filtered for P value ≤ 0.05. The primers listed in Table E1 in this article’s Online Repository at www.jacionline.org were used in real-time PCR to validate the microarray data.

Wound repair assay

Confluent monolayers of NHBE cells and DHBE cells were scratched with the tip of a pipette to create a uniform cell-free zone in each well. Wounded monolayers were then incubated with IL–22 (50 ng/mL) alone or in combination with IFN–γ (10 ng/mL). BEBM basal medium represented the negative
control. In some experiments blocking anti-IFN-γ receptor 1 and the relative isotype control were added to the cultures. Closure of the wounded area was monitored microscopically at 6 hours and 16 hours after stimulation and recorded with a digital camera. The residual gap between migrating cells was measured with a computer-assisted image analysis system (AstoVision 4.5; Carl Zeiss, Oberkochen, Germany) and expressed as a percentage of the initial scratched area.

**T cell–mediated cytoktopy experiments**

The cytoktopy assay was performed with the cytoktopy detection kit LDH, according to the manufacturer’s instructions (Roche, Mannheim, Germany). Briefly, NHBE cells were seeded in flat-bottom 96-well plates in complete BGM. Confluent NHBE cells were washed with PBS and stimulated with recombinant cytokines (10 ng/mL IFN-γ and 50 ng/mL IL-22) in basal BIBM. After 56 hours, NHBE cells were extensively washed and collected for 6 hours with 20% fetal bovine serum (FBS) and CD4+ and CD8+ cells and isolated with the CD8+ and CD4+ T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), respectively.

**Immunohistochemistry**

Lung biopsy specimens of asthmatic (n = 6) and healthy (n = 3) donors were paraflin embedded. Five-micrometer sections were dewaxed in xylene and ethanol and boiled in Tris-EDTA buffer (pH 9.0) at 96°C. Lung biopsy specimens of asthmatic (n = 3) donors were paraffin embedded. Five-micrometer sections were dewaxed in xylene and ethanol and boiled in Tris-EDTA buffer (pH 9.0) at 96°C.

**BALF**

BALF collected from asthmatic (n = 22) and healthy (n = 8) donors was concentrated 50- to 70-fold by using the Amicon Centriprep-3K Centrifugal Filter Device (Millipore, Temecula, Calif). ELISA (R&D Systems) was performed to measure cytokine content. The results were corrected for concentration factor and thus represent the unconcentrated content of proteins in BALF (Table E1).

**Statistical analysis**

Mann-Whitney U analysis and 1-way ANOVA with the Bonferroni multiple comparison test were used to determine significant differences between groups. The Spearman test was used to evaluate correlations (Prism 5; GraphPad Software, La Jolla, Calif). Data are expressed as means ± SE. Statistically significant differences were defined as P values of less than 0.05, less than 0.01, and less than 0.001.

**RESULTS**

**T cells from lung biopsy specimens of asthmatic patients display distinct IL-22–producing T-cell subsets**

T-cell lines were generated from lung biopsy specimens of asthmatic patients and analyzed by using multicolor flow cytometric staining to characterize IL-22–producing T cells in asthmatic patients. On iomyocytcin/pherbol 12-myristate 13-acetate stimulation, the majority of lung-derived T cells produced IFN-γ (Fig 1, A and B). The frequency of IL-22+ T cells was comparable with that of IL-17+ and IL-4+ infiltrating T cells (Fig 1, A and B, and see Fig E1 in this article’s Online Repository at www.jacionline.org). Only a minority of total lung IL-22+ T cells coexpressed IL-17, confirming that IL-22 and IL-17 are weakly associated in human tissues (Fig 1, A). 

**IL-22 and IFN-γ mediate reciprocal antagonistic effects on primary bronchial human epithelial cells**

To understand the potential pathologic mechanism of IL-22 and IFN-γ cocrystation in the context of airway inflammation, we investigated the gene expression profile of primary human bronchial epithelial cells (NHBE cells) exposed to either IL-22 or IFN-γ alone or in combination. Genes regulated more than 2-fold compared with untreated cells were hierarchically clustered (Fig 2, A–C) and filtered for P value (P ≤ 0.05). Hierarchic gene expression analysis highlighted clusters of antagonism between IL-22 and IFN-γ (Fig 2, A–C). IL-22 was antagonizing genes regulated by IFN-γ (Fig 2, B, and see Tables E10 and E11 in this article’s Online Repository at www.jacionline.org).

**TABLE I. Study subjects’ characteristics**

<table>
<thead>
<tr>
<th>Healthy control subjects</th>
<th>Asthmatic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>5/6</td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.6 ± 11.7</td>
</tr>
<tr>
<td>Duration of asthma (y)</td>
<td>NA</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>94.8 ± 8.1</td>
</tr>
<tr>
<td>FEV1/FVC ratio (%)</td>
<td>79.3 ± 5.4</td>
</tr>
<tr>
<td>Bronchodilator response</td>
<td>NA</td>
</tr>
<tr>
<td>BDP equivalent (µg/d)</td>
<td>NA</td>
</tr>
<tr>
<td>Atopy</td>
<td>NA</td>
</tr>
<tr>
<td>Total IgE (IU)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values represent means ± SDs.

**LAL** Bronchoalveolar lavage; **BDP** beclomethasone dipropionate; **F** female; **M** male; **NA** not applicable.

*P < 0.05; †P < 0.01; and ‡P < 0.001 compared with healthy subjects.

*Measured as percentage increase in FEV1 after 400 mg of aerosolized albuterol.
Repository at www.jacionline.org), and conversely, IFN-γ inhibited IL-22–regulated genes (Fig 2, C, and see Tables E8 and E9 in this article’s Online Repository at www.jacionline.org). IFN-γ treatment induced 2920 genes in NHBE cells compared with untreated cells (see Tables E4 and E5 in this article’s Online Repository at www.jacionline.org). Among those genes, many proinflammatory mediators were upregulated, such as the Tα1 chemoattractants CXCL10, CXCL9, and CXCL11 and the eosinophil-recruiting chemokines, CCL5, CCL2, CCL7, and CCL8, thus supporting the propagation of inflammation (see Table E4). In contrast, IL-22 significantly regulated only 118 genes (see Tables E2 and E3 in this article’s Online Repository at www.jacionline.org). Consistent with its role in host defense, IL-22 specifically induced the antimicrobial peptide psoriasin (S100A7) in NHBE cells. Furthermore, IL-22 modulated several genes involved in cell survival and remodeling, such as SERPINB3 and SERPINB4 involved in mucin secretion and cell survival, and VWA1 involved in matrix deposition.
Asprv1, at least 2-fold induction compared with untreated cells by IL-22 and IFN-γ.

HLA molecules (see inflammatory IFN-γ Tables E8 and E9 and remodeling genes regulated by IL-22 (see article’s Online Repository at www.jacionline.org).

Antagonistic gene regulation in primary human bronchial epithelial cells by IL-22 and IFN-γ. A, Hierarchical clustering analysis of genes regulated at least 2-fold induction compared with untreated (N.T.), B and C, Representative cluster of IFN-γ–regulated genes suppressed by IL-22 (Fig 2, B) and IL-22–induced genes suppressed by IFN-γ (Fig 2, D). D and E. Percentage of the number of IFN-γ–regulated genes significantly antagonized by IL-22 (Fig 2, B) and IL-22–regulated genes significantly antagonized by IFN-γ (Fig 2, E). *P<.05.

Asprv1 involved in skin regeneration, the transcription factor NF-E2 involved in cell maturation (see Fig E2 in this article’s Online Repository at www.jacionline.org).

The IFN-γ/IL-22 combination regulated 2623 genes compared with untreated cells (see Tables E6 and E7 in this article’s Online Repository at www.jacionline.org). Consistent with antagonistic features, IFN-γ inhibited the expression of both host defense and remodeling genes regulated by IL-22 (see Fig E2 and Tables E8 and E9), whereas IL-22 inhibited most of the proinflammatory IFN-γ–regulated genes, such as chemokines and HLA molecules (see Fig E3 and Tables E10 and E11 in this article’s Online Repository at www.jacionline.org).

Among the genes significantly regulated, IFN-γ inhibited 57 (48.3%) of 118 genes regulated by IL-22 (Fig 2, F), whereas IL-22 inhibited 2203 (74.9%) (Fig 2, D) of 2920 genes modulated by IFN-γ (Fig 2, B-D), and see Figs E2 and E3).

Taken together, these data suggest that IL-22 and IFN-γ can act antagonistically on bronchial epithelium.

IL-22 impairs proinflammatory activities of IFN-γ

On IFN-γ exposure, epithelial cells upregulate a plethora of proinflammatory cytokines, chemokines, and adhesion molecules that promote recruitment of immune cells and T cell–mediated damage. IFN-γ induced many proinflammatory mediators in primary bronchial epithelial cells (see Table E4). Consistent with the antagonistic activities of IL-22 on IFN-γ, IL-22 diminished the induction of HLA haplotypes, CD54/ICAM-1, and the chemokines CXCL10, CXCL9, CXCL2, CXCL17, CXCL16, CXCL11, CCL8, CCL5, CCL7, and CCL2 by IFN-γ in the microarray assay. Confirming observations at the level of gene expression, IL-22 inhibited the IFN-γ–mediated expression of CCL5/RANTES at the protein level (Fig 4, A). To confirm the MHC-I and MHC-II regulation observed in the microarray, we performed flow cytometric analysis of ICAM-1 (Fig 4, C), the MHC-II haplotype HLA-DR (Fig 4, B), and the MHC-I haplotypes HLA-A, HLA-B, and HLA-C (Fig 4, D). Consistently, IL-22 suppressed the IFN-γ–mediated induction of MHC-I (Fig 4, D), MHC-II (Fig 4, B), and ICAM-1 (Fig 4, C) on the surface of both NHBE and DHBE cells (see Fig E4). The IL-22 inhibitory effects were reverted by blocking IL-22R (see Fig E6 in this article’s Online Repository at www.jacionline.org).

Taken together, these data suggest that IL-22 suppresses IFN-γ–mediated inflammation.

Inhibition of IFN-γ–mediated MHC-I expression leads to a reduced CD8-dependent cytotoxicity

MHC-I, MHC-II, and CD54/ICAM-1 upregulation on epithelial cells enables CD8- and CD4-mediated cytotoxicity. To investigate the functional consequences of the IL-22–mediated inhibition of MHC-I and MHC-II upregulation, we measured the T cell–mediated cytotoxicity. Untreated epithelial cells were slightly prone to CD4- and CD8-mediated cytotoxicity, whereas IFN-γ–treated epithelial cells upregulated MHC-I and MHC-II and became highly susceptible to T cell–mediated cytotoxicity. Treatment of primary epithelial cells with the combination of IFN-γ and IL-22 greatly reduced CD8-mediated cytotoxicity compared with IFN-γ–treated NHBE cells (Fig 4, F). A similar but not significant tendency was observed for CD4+–mediated cytotoxicity (Fig 4, E).

Together, these results suggest that by impairing IFN-γ–mediated expression of MHC-I, MHC-II, and ICAM-1 molecules, IL-22 can protect the lung epithelium from T cell–mediated damage.

IL-22 expression inversely correlates with IFN-γ–dependent proinflammatory mediators in vivo

To investigate the expression and in vivo relevance of IL-22–mediated suppression of IFN-γ, we performed immunohistochemical staining of sections from lung biopsy specimens from healthy and asthmatic subjects and measurement of BALF content (Table I). Immunohistochemistry showed IL-22–producing cells infiltrating the lung epithelium.
the epithelium of the bronchial mucosa in asthmatic patients, whereas few IL-22+ cells were detected in healthy subjects (Fig 5, A). Because cytokines are normally undetectable in neat BALF of asthmatic patients, we measured cytokine levels in concentrated BALF. We corrected the values for concentration factor and expressed them as representative of the unconcentrated BALF. The expression of IL-22 was increased in BALF of asthmatic patients compared with that of healthy control subjects (Fig 5, B). To evaluate whether IL-22 might have a suppressive effect on IFN-γ-mediated inflammation in vivo, we examined whether there was a correlation between the IL-22/IFN-γ ratio and the content of the IFN-γ-inducible chemokines CXCL10/IP-10 and
CCL5/RANTES (Fig 5, C). In line with the in vitro data, the IL-22/IFN-γ ratio but not other ratios investigated inversely correlated with the expression of CXCL10/IP-10 and CCL5/RANTES. Taken together, these results suggest that an IL-22–dominated environment might suppress the IFN-γ–mediated inflammation in vivo.

**DISCUSSION**

The current study identifies a regulatory role for IL-22 in the context of IFN-γ–mediated inflammation. We demonstrated that IL-22 and IFN-γ mediate antagonistic effects in lung epithelial cells. These antagonisms become manifest in impaired protective and remodeling activities of IL-22, as well as in dampened IFN-γ–mediated upregulation of proinflammatory molecules. To our knowledge, IL-22 is the first T-cell cytokine described to inhibit IFN-γ–mediated proinflammatory effects on human primary epithelial cells.

In murine models IL-22 shows either proinflammatory or anti-inflammatory properties. Consistently, 2 reports in animal models of lung inflammation conclude that IL-22 can act as an anti-inflammatory cytokine in the absence of IL-17, whereas in the presence of IL-17, IL-22 contributes to the recruitment of
that the interplay between IFN-like receptors 2 and 3 induced by IFN-γ affects the innate related molecules. In fact, molecules such as Toll-like receptors and chemokines like CCL11 and CCL5 and consecutive eosinophil infiltration.

IL-22 in an OVA mouse model of asthma impaired the secretion of mucin and other inflammatory mediators involved in mucin secretion and matrix deposition.

Similar to reports studying the skin, IL-22 is produced by some T cells producing IL-22. Both CD4+ and CD8+ T cells can produce IL-22. Both CD4+ and CD8+ T cells were detected. As expected, IL-22 was produced by some Tc17 cells but primarily by Tc1 cells. An additional source of IL-22 in the lung is the recently described Tc22 subset. Tc22 cells were originally described in the skin on the basis of their expression of CCR10, CCR6, and CCR4. However, this chemokine receptor repertoire is also adequate for migration of T cells to the lung. Thus Tc22 seems to be a tissue-restricted rather than a skin-restricted T-cell subset.

In line with previous reports, IL-22 induces antimicrobial peptides in airway epithelial cells, epithelial migration, and genes involved in mucin secretion and matrix deposition. IFN-γ antagonized the majority of IL-22-mediated effects. In fact, antifibrotic properties were previously assigned to IFN-γ, such as inhibition of collagen synthesis, cell-cycle arrest, induction of apoptosis, and activation of natural killer cell cytotoxicity.

The role of IL-22 in this context remains to be investigated.

The current study demonstrates that IL-22 impairs IFN-γ-induced chemokine release in airway epithelial cells, which confirms previous studies showing that exogenous IL-22 administration in a mouse model of lung fibrosis reduces inflammation through inhibition of CXCL9, CXCL10, and CXCL11 expression.

Consistently, it was shown that administration of IL-22 in an OVA mouse model of asthma impaired the secretion of CCL11 and CCL5 and consecutive eosinophil infiltration. Thus IL-22 antagonism of IFN-γ might explain the anti-inflammatory effects of IL-22 in mice and human subjects.

Interestingly, the IL-22/IFN-γ antagonism only partially affects the innate related molecules. In fact, molecules such as Toll-like receptors 2 and 3 induced by IFN-γ on epithelial cells (see Table E4) were not inhibited by IL-22. This observation suggests that the interplay between IFN-γ and IL-22 results in a different rather than diminished response to microbial invasion compared to the effects of the single cytokines.

Notably, IL-22 is the first T-cell cytokine that diminishes IFN-γ-induced expression of MHC-I and MHC-II on primary epithelial cells and reduces CD8-mediated cytotoxicity. This finding is consistent with reports showing that IL-22 is an effective cytokine for the clearance of extracellular, but not intracellular, pathogens in which MHC-I recognition by CD8+ cells is critical.

In fact, it was previously demonstrated that lower lung viral titers were observed after treatment with anti–IL-22 in a mouse model of influenza virus replication.

Viruses play an important role in asthma exacerbation and are implicated in the development of chronic asthma. In this context, IL-22 might have a Janus-faced role: on the one hand, IL-22 can protect the lung from an excessive IFN-γ-mediated inflammation, and on the other hand, it might be involved in the propagation, perpetuation, or both of virus-mediated asthma and unwanted remodeling of the airway structure.

IFN-γ-mediated inflammation and MHC-I recognition are of critical relevance in several medical fields, such as tumor immunology, transplantation immunology, autoimmunity, and intracelular infectious diseases. Further studies are needed to clarify the role of IL-22 in MHC-I-dependent diseases and to further investigate its role in pathogen-dependent aggravation of inflammation.

The present report links the murine evidence to human data and investigates in detail how and in what context IL-22 might act as an anti-inflammatory cytokine.

In summary, IL-22 might control the extent of IFN-γ-mediated lung inflammation and thereby play a regulatory role in tissue inflammation. These results raise possibilities for new therapies using recombinant IL-22 to limit tissue inflammation and anti–IL-22 to enhance IFN-γ-mediated tissue immunity.

We thank Gaby Pleyle-Wignicki and Juliette Kranz for excellent technical assistance.

Key messages

- Tc22 cells infiltrate the lung during asthma.
- IL-22 and IFN-γ exert tissue-restricted antagonistic functions in the lung.
- IL-22 protects the epithelium from T-cell-mediated cytotoxicity by reducing the IFN-γ-induced MHC-I and MHC-II expression on bronchial epithelial cells.
- IL-22 might limit tissue inflammation, whereas anti–IL-22 might enhance IFN-γ-mediated tissue immunity.

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