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Interleukin-17 is a key cytokine in first-line defence of the skin

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Table of content

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[*] Both authors contributed equally to this work.

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Used abbreviations

%	Percent
Ab	Antibody
AE	Atopic eczema
APC	Antigen-presenting cell(s)
APE	aqueous pollen extract
APT	Atopie Patch Test
Aqua dest.	Destilled water
Bet.	Betula alba
BFA	Brefeldin A
°C	Degree Celsius
cAMP	Cyclic Adenosine-monophosphate
CCR	Chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte associated antigen
cpm	Counts per minute
DC	Dendritic cell(s)
DNA	Desoxy-ribonukleic acid
EBV	Ebstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
Fig	Figure
FcεRI	high affinity IgE receptor
Foxp3	Forkhead box p3
g	Gravitation
GM-CSF	Granulocyte-Macrophage colony stimulating factor
gr	greek
h	Hour(s)
HHV	Human Herpes virus
ICAM-1	Intercellular adhesion molecule 1
IDEC	Inflammatory dendritic epidermal cell(s)
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
LC	Langerhans cell
LMP-1	Latent membrane protein 1
MACS	Magnetic antibody column separation
MHC	Major histocompatibility complex
min	Minute(s)

ml	Milliliter
mM	Millimolar
MTP	Mikrotiterplate
mRNA	messenger Ribo-Nucleic-Acid
nm	Nanometre
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
PBMC	Peripheral blood mononuclear cell(s)
pDC	Plasmacytoid dendritic cell(s)
PHA	Phytohemagglutinin (= Lectin)
PI	Proliferation index
PPT	Pollen Patch Test
Phl.	Phleum pratense L.
PP	Poly-Propylen
µg	Mikrogram
µl	Mikrolitre
µm	Mikrometre
R	Receptor
rpm	Rounds per minute
REM	Scanning electron microscop
sec	Second(s)
Stat	signal transducer and activator of transcription
Tc1	Cytotoxic type 1 T cell(s)
Tc2	Cytotoxic type 2 T cell(s)
TCR	T cell receptor
Th1	T helper type 1 cell
Th2	T helper type 2 cell
TNF-α	Tumor necrosis factor α
T-reg	T regulatory cell(s)
TRAIL	TNF related apoptosis inducing ligand
TSLP	Thymus stroma lymphopoeitin
U	Unit(s)
UV	Ultra-violet light

1 Introduction

T lymphocytes are central mediators of adaptive immunity. Increasing knowledge regarding morphology, secretional profile and chemokine receptor repertoire of T cells reveals that distinct T cell subpopulations fulfil specialised tasks. Among these T cell subpopulations, much attention has been focused on interleukin-17 (IL-17) producing Th17 cells in the last years. IL-17, also produced by other T cell subsets and NKT cells, seems present especially in inflammatory and autoimmune diseases of epithelial tissue in the human organism. Accordingly, many *in vitro* studies demonstrate important tissue-instructing functions for IL-17.

In this context, the presented thesis illuminates that IL-17 is a central mediator for protection of the human organism against microbials at barrier-defining organs. This essential capacity is illustrated in two human diseases that are characterized by chronic and selective infections of the skin – the orphan syndrome chronic mucocutaneous candidiasis (CMC) and the common inflammatory skin disease atopic eczema (AE). This manuscript illustrates that CMC patients suffer from a generally diminished IL-17 response. In contrast, in AE IL-17 can be triggered efficiently by microbial-derived stimuli, but the local microenvironment in the skin partially inhibits an effective IL-17 response. In both cases, the absent or ineffective IL-17 signal results in a failed instruction of an innate immune response by epithelial cells, which can explain a selective inability to clear skin infections.

Taken together, this work describes that defence against extracellular microorganisms at barrier-organs such as the skin is critically mediated by IL-17, thus indicating that within T helper cell subsets the Th17 cells may be classified as “tissue-signaling leukocytes”.

1.1 Differentiation of CD4+ T cells

CD4+ T cells orchestrate the adaptive immunity by mediating CD8+ cytotoxicity and phagocytosis by macrophages, by enhancing antibody production of B cells and by recruiting other leukocytes such as neutrophil, eosinophil and basophil granulocytes to sites of infection. They do so by the production of an arsenal of cytokines and chemokines – amongst these IL-17 (Figure 1). So far, at least eight distinct CD4+ T cell subsets are known. Th1 cells produce IFN-γ, induce apoptosis and are proinflammatory, Th2 cells are characterised by production of IL-4 and induce an eosinophil and mast cell immune response[1,2]. Nothing is known about the function of Th9 cells that differentiate out of Th2 cells and are characterised by secretion of IL-9[3]. Th17 cells co-produce IL-17 and IL-22, and they accumulate in several autoimmune diseases[4]. Recently, a T cell population characterised by the production of IL-22, but not of IFN-γ, IL-4 nor IL-17 was discovered. These so-called Th22 cells are associated with inflammatory skin diseases[5,6,7]. Finally, T cellular immune responses are limited by a further independent entity of so-called regulatory T cells. These comprise naturally occurring CD4+CD25+ T regulatory cells (nTreg), inducible Treg cells (iTreg) and CD4+CD25- Tr1 (producing TGF-β) and Th3 cells (producing IL-10). While naturally occurring Tregs suppress effector functions of other T cells via contact-dependent mechanisms, iTreg, Tr1 and Th3 cells exert their suppressive activity via secreted cytokines[8].

All known CD4+ T cell populations with the exception of Th9 cells develop out of so-called naive T cells under the influence of a distinct cytokine combination (Figure 1). Increasing evidence suggests different T cell subpopulations differentiate to fulfil specialised tasks under specific pathogen-associated molecular patterns that create

a distinct microenvironment. Such a scenario has recently been described by several groups for the homeostasis between Th17 and iTreg cells[9,10]. A central role for regulating this homeostasis plays the commensal microflora of the gut that seems to enhance Th17 differentiation[11,12].

However, despite existence of distinct T cell subtypes, co-secretion of several lineage-indicating cytokines (so-called T cell plasticity) is a commonly observed phenomenon. T cells co-producing IFN-γ and IL-4 are called Th0, cells that co-produce IFN-γ and IL-17 are called Th1/IL-17 and Th2/IL-17 cells co-secrete IL-4 and IL-17.

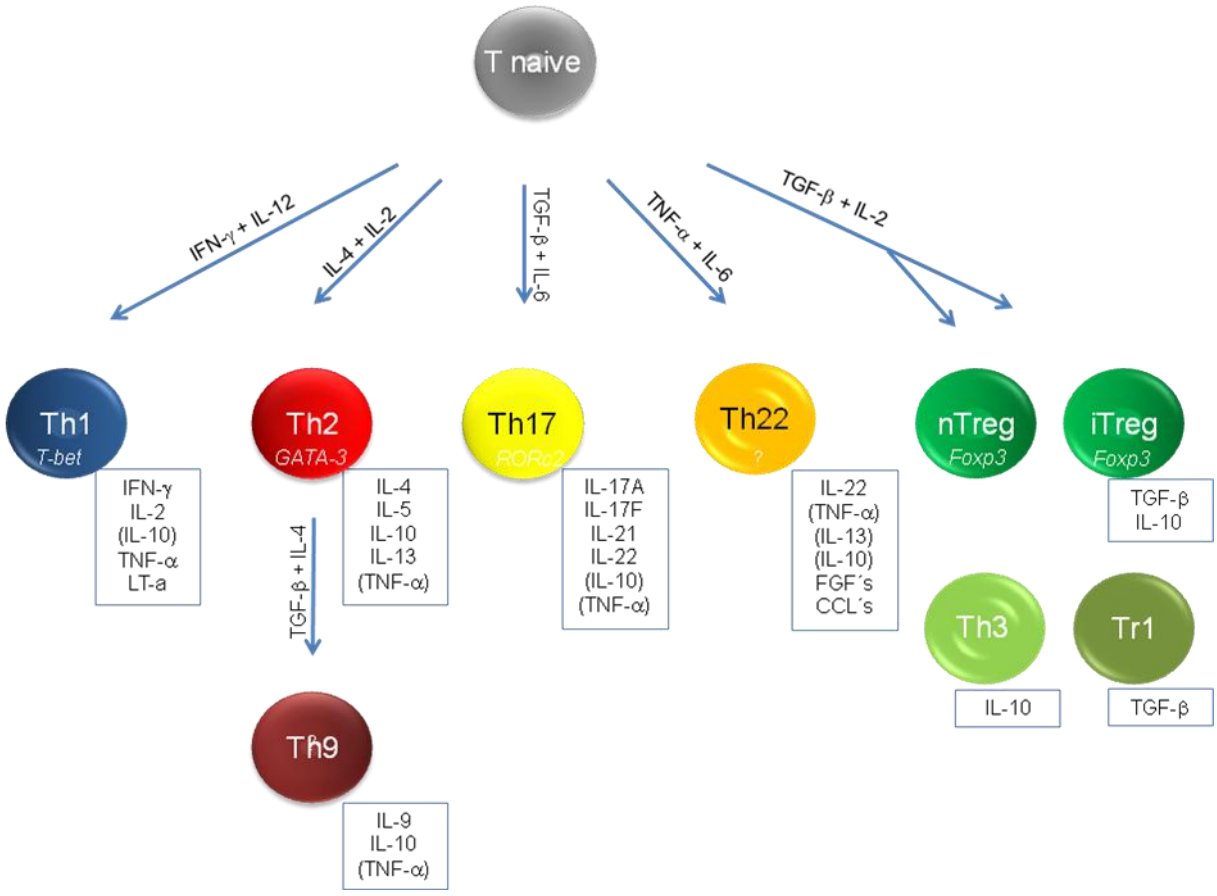


Figure 1. Differentiation, key transcription factors (shown in italic font) and indicating effector cytokines of CD4+ T cell subsets identified so far.

1.2 Non CD4+ T cell sources of IL-17 in the skin

Besides Th17, Th1/IL-17 and Th2/IL-17 cells, a broad variety of other leukocytes releases IL-17 upon adequate microenvironmental stimuli (Table 1).

All IL-17 producing leukocytes most likely share expression of the transcription factor RORc, the human analogue to mouse RORγt, shown to be essential for IL-17 production[13].

While human αβT cells (Th17; CD8+ IL-17 producing T cells) and NK cells (NKT) as sources of IL-17 have been extensively described in the last years, little is known about whether human γδT cells[14] and granulocytes produce this cytokine. In contrast, plenty of reports describe an essential role for IL-17 producing γδT cells in the initial phase of diverse infectious[15,16,17] and immune-mediated[18,19] disease models in the mouse system. Furthermore, indirect evidence exists that neutrophil granulocytes can produce IL-17 in mice, as SCID mice also develop neutrophilic responses associated with IL-17[20].

However, given the quantitiy of CD8+, NKT and other non CD4+ T cells secreting IL-17 within the cellular infiltrate of inflamed skin, these cells contribute most likely marginally to the overall quantity of IL-17 in the skin. The main source of IL-17 in inflamed skin are CD4+ lymphocytes.

Well characterised in human system				
Cell	Other secreted factors	Evolution	Transcript. factor	Surface phenotype
Th17	IL-21, IL-26, TNF-a, (IL-10), CCL20	differentiation: naïve T cell +TGF-β/IL-1β/IL-6; IL-21; IL-23	RORc	CD4+ CCR4+ CCR6+ CXCR3- CD161+ IL-23R+
NKT	IFN-γ		ROR	CD3+ CD56+
LTi	TNF-α, Lymphotoxin	unknown (early NK cell?)	RORc	CD3- CD56- NKp44+ CD117+ CD127+ CD161+
NK22	TNF-α, Lymphotoxin, IL-26, leukaemia inhibitory factor	LTi cells (?)	RORc	CD3- CD56+ NKp44+ CD117+ CD127+ CD161+
CD8+IL-17+ (human)/ Tnc17		Mouse: CD8+ T cell +TGF-β/IL-6 or +IL-1/IL-23	Mouse: RORγt	CD3+ CD8+ CD45RO+
Only single reports or described only in mice				
γδT cell		naïve T cell		CD3+ CD4- CD8- CD27- CD25+ CD122-
T follicular helper cells	IL-21	Naïve T cell +IL-21 +IL-6 +ICOSL	Unknown (BCL6?)	CD4+ ICOS+ CXCR5+
Monocytes/ macrophages			RORγt	CD11b+ CD68+
Neutrophil granulocyte				
Paneth cells	TNF-a, GM-CSF, iNOS, Matrilysin			

Table 1. Identified cellular sources of IL-17.

1.3 Th17 cells and known effects of IL-17 in the skin

Th17 cells have first been described in the year 2005 in the context of experimental autoimmune encephalitis[21]. Differentiation and expansion of Th17 cells are regulated stepwise by different cytokines (Figure 2): differentiation of Th17 cells out of naive T cells requires the cytokines TGF-β, IL-1β and IL-6[22,23,24]. A positive amplification loop of this process is created by ICOS-induced c-Maf via the production of IL-21[25]. In a third step, the cytokine IL-23 promotes a stable Th17 phenotype and expansion of Th17 cells[26]. Discovery of IL-23 preceded the first description of Th17 cells[27].

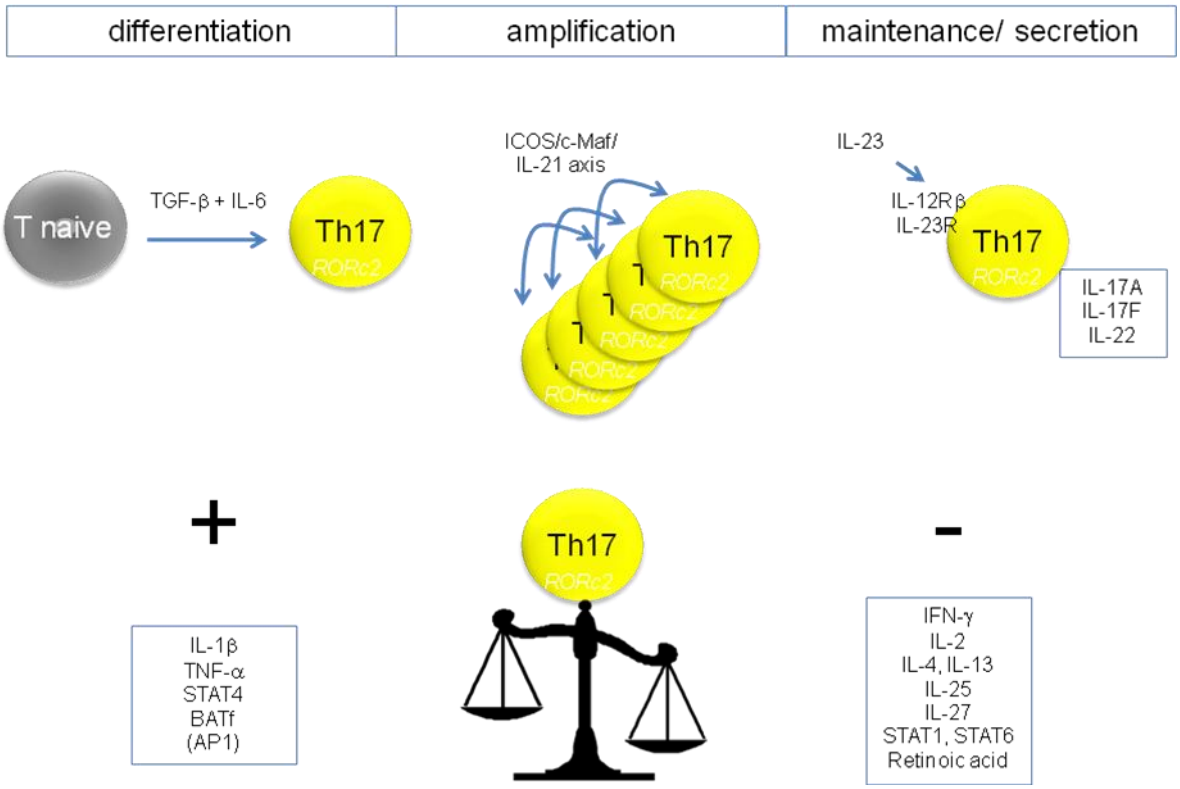


Figure 2. Regulation of T helper 17 cells.

Th17 cells are characterised by the production of IL-17A, IL-17F, IL-21, IL-22, IL-26 and the chemokine CCL20, which recruits CCR6+ cells4. Th17 cells in turn express CCR6[28]. A second recently described surface marker for Th17 cells is CD161, a molecule previously described to be expressed on Natural Killer cells[29,30].

Concerning effector functions of Th17 cells, increasing evidence suggests a strong pro-inflammatory role in several human diseases. Th17 cells are associated with autoimmune diseases like rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease on the one and defence against several bacteria and fungi on the other hand[31].

First evidence that IL-17 could be involved in host defence against *Candida* was given by Huang et al in 2004, showing that mice lacking IL-17 suffered from severed *Candida* infections[32]. An association between *Candida* and Th17 cells was demonstrated both in the mouse and the human system in 2007[33,34]. Recently, IL-17 was identified as the main cytokine responsible for oral Candidiasis[35].

Apart from *Candida albicans*, also other pathogens are related to a Th17 immune response. Among these are the bacteria *Propionibacterium acnes*, *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Bacteroides* and *Borrelia* spp. and *Mycobacterium tuberculosis*[31].

A main pathway for antimicrobial effector functions of Th17 cells in the skin is through induction of innate immunity. IL-17 induces secretion of IL-8 in human keratinocytes, which represents a strong stimulus for migration of neutrophil granulocytes[36,37]. Furthermore, IL-17 and IL-22 induce secretion of antimicrobial peptides, the so called defensins, by human keratinocytes[38]. Defensins are critical for killing microorganisms[39], and they induce migration of CCR6+ cells[40], thus opening a pro-inflammatory circle by recruiting more Th17 cells into the skin.

1.4 The skin as a first-line defence organ of the organism

Epithelial cells build the interface between the human organism and its environment. Besides this mechanical protection that is warranted by continuous regeneration of the epithelial layer from the basal membrane, keratinocytes as main component of

the human skin secrete an arsenal of immune-mediating cytokines and chemokines (Figure 3).

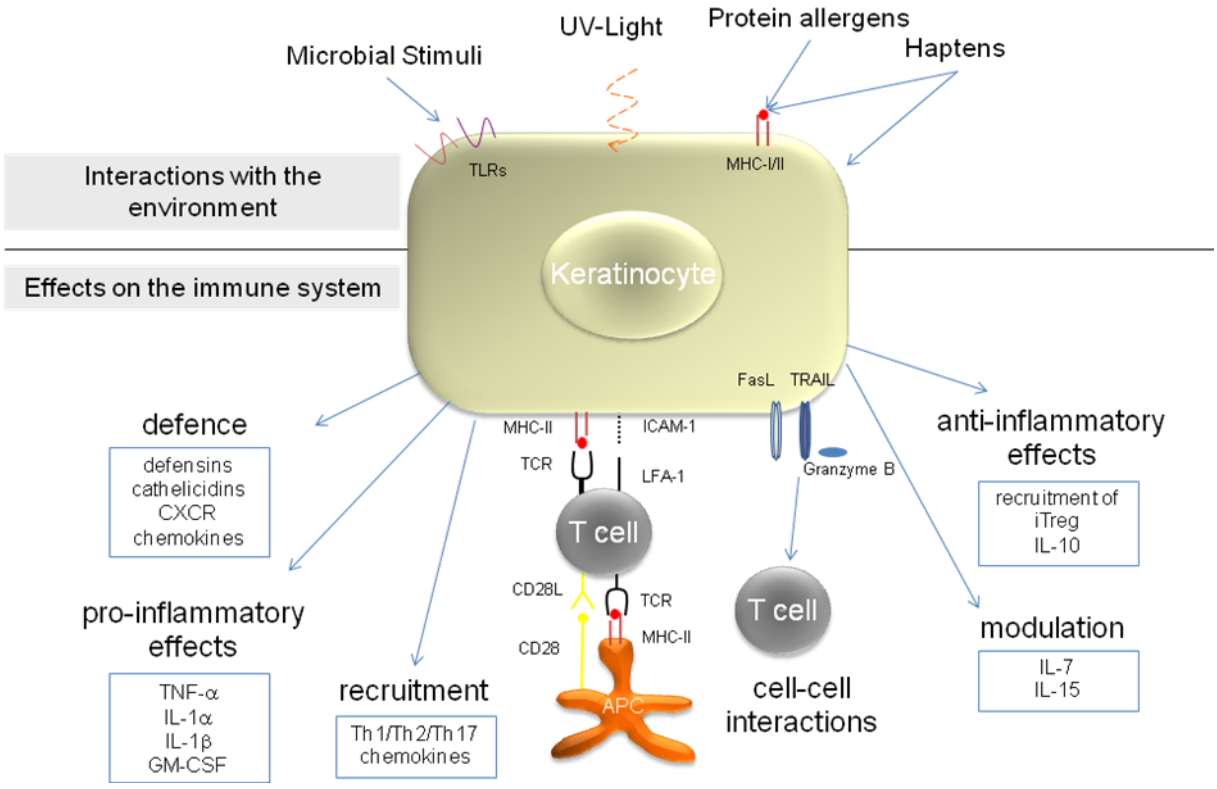


Figure 3. Human keratinocytes orchestrate innate and adaptive immunity in response to a wide range of external signals.

Keratinocytes contribute to the inflammatory reaction

To guarantee an adequate response, keratinocytes express a variety of sensing structures to detect microbial danger signals. Among these so-called "pattern recognition receptors", toll-like receptors (TLR) binding to bacterial, fungal or viral structures play a central role. Human keratinocytes express TLR 1-6, 9 and 10; however, only 3,4,5 and 9 seem to be functional[41].

TLR-mediated activation of keratinocytes results in production of antimicrobial peptides, the so-called defensins and cathelicidins. Besides these direct antimicrobial effects that allow a control of skin colonization under normal conditions[42],

keratinocytes recruit non-resident immune cells to sites of infection by secretion of a variety of chemokines[41].

However, not only microbial stimuli activate keratinocytes. It has been shown that small molecules like haptens, that can elicit allergic reactions after binding to self-proteins, induce a pro-inflammatory response in keratinocytes that results in upregulation of ICAM-1, IL-1α, IL-1β, IL-1RA, TNF-α, CCL2 and CCL5[43].

As well as to external stimuli, keratinocytes respond to stimuli of the immune system. They constitutively express the receptor for IFN-γ. Binding of IFN-γ to keratinocytes induces expression of surface molecules (ICAM-1, HLA-ABC, HLA-DR, CD40), secretion of cytokines like IL-1, IL-18, IL-6, IL-15, GM-CSF, TNF-α, TGF-β and chemokines (CCL2, CCL3, CCL4, CCL5, CCL18, CCL22, CXCL8 und CX3CR ligands)[44,45]. Thus keratinocytes do not only contribute to a pro-inflammatory microenvironment, they also recruit additional immune cells.

Among recruited immune cells are neutrophil granulocytes via secretion of CXCL-8, CXCL-1, CCL-20 and IL-18[46,47], and various T cells. T cells expressing the skin homing molecule CCR10 are recruited via expression of CCL-27[48], CXCR3+ Th1 cells via CXCL-9, CXCL-10 and CXCL-11, and CCR4+ Th2 cells via CCL-17 and CCL-22[49].

Interestingly, keratinocytes do not only recruit pro-inflammatory effector T cells, but also naturally occuring $CD4^+CD25^+FoxP3^+$ regulatory T cells or IL-10 producing Tr1 cells, especially via secretion ot CCL-1[50]. Thus keratinocytes do not only aggravate an inflammatory reaction, but also contribute to its limitation.

Keratinocytes as target cells in inflammation

Besides their role as active mediators of the immune reaction in the skin, keratinocytes are central target cells in the pathophysiology of an eczematous

reaction. Upon stimulation with IFN-γ, keratoinocytes upregulate adhesion molecules like ICAM-1, HLA-ABC, HLA-DR and CD40 that allow a close morphological interaction with effector immune cells like T cells[51]. Such a close interaction leads to loss of epidermal integrity (cleavage of e-cadherin) and keratinocyte apoptosis, which results in edema and the clinical picture of eczema[52,53].

Immune-modulatory capacity of keratinocytes

Keratinocytes also express the T cell-modulating cytokines IL-12, IL-15 and IL-18. Secretion of IL-15 results in a extended life span especially of natural killer cells and some T cells in the skin and thus promotes a cytotoxic immune response[54].

IL-12 on the other hand promotes the differentiation of type 1 T cells. Interestingly, among the two subunits of IL-12, the one shared with IL-23 (IL-12p35) is constitutively expressed in keratinocytes, while IL-12p40 is induced by e.g. contact allergens or haptens. This could explain type 1-dominated immune reactions during allergic contact dermatitis[55]. IL-12 also influences the effects of IL-18; in presence of IL-12, IL-18 promotes a type I differentiation, while in absence of IL-12 a type 2 reaction is enhanced[56].

Taken together, keratinocytes are potent players in immunity against (extracellular) microorganisms by sensing external and internal danger signals and responding with an arsenal of cytokines, chemokines and antimicrobial peptides.

1.5 Chronic mucocutaneous candidiasis

Candida albicans is a ubiquitous, opportunistic yeast colonizing membranes of human skin and mucosal surfaces. The yeast causes infections (candidiasis) only, if the homeostasis between virulence of the microbe and resistance of the host

immune system is disturbed. Chronic mucocutaneous Candidiasis (CMC) is a collective term for a complex group of disorders characterised by persistent or recurrent infections of the skin, nails and mucosal tissues. Patients with CMC rarely develop disseminated or systemic infections with *Candida*[57]. The first case of CMC was described by Thorpe and Handley in 1929[58], followed by other reports in the 1950s[59,60]. The term “chronic mucocutaneous candidiasis” was introduced in the late 1960s[61]. Today, CMC still is diagnosed clinically and by *in vitro* isolation and cultivation of *Candida* from smear tests. Additionally, diagnosis can be confirmed by mutational analysis in subgroups with known underlying genetical defects.

Heterogeneity and prevalence of CMC

The complex group of CMC syndromes can be subclassified according to distribution (local candidiasis versus generalized mucocutaneous candidiasis) and to underlying pathomechanism (primary versus secondary syndromes) (Table 2).

Notably, inherited CMC syndromes are often associated with autoimmune diseases of endocrine glands. This is the case in the autosomal-recessive “autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome” (APECED or APS-1)[62,63], where monogenic defects in the autoimmune regulator gene (AIRE)[63,64,65] have been described, or in distinct syndromes of dominantly inherited CMC[66] with endocrinopathies, where either the underlying genetic defect has been mapped on chromosome 2p[67] or associations with a variant in the lymphoid protein tyrosine phosphatase have been reported[68], respectively. For other primary forms of CMC the genetical basis is unknown. For all known mutations, however, the link between mutation and immune defect(s) remains unclear. In contrast, secondary CMC syndromes are usually the consequence of local or systemic immune-suppression due to infections (especially AIDS, where candidiasis is of prognostic value[69]),

reduced micro-perfusion in diabetes or immune-suppressive long-term medication. Another predisposing factor for secondary *Candida* infections is a disturbed microenvironment, e.g. after long-term antibiotic treatment or around dentures (Table 2).

Name	Pathomechanism
Primary immunodeficiencies	
Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED, also APS1)	mutation in AIRE gene; associated dysfunctions of endocrine glands
Autosomal-recessive CMC	mutation in PTPN22 gene; associated with autoimmune endocrinopathies and antibody deficiency
Autosomal-recessive CMC	unknown mutation(s)
Autosomal-dominant CMC	mutation mapped on chromosome 2p; associated with thyroid gland malfunction
Autosomal-dominant CMC	unknown mutation(s)
Autosomal-dominant hyper-IgE syndrome	mutation in STAT3 gene; associated with
Secondary CMC	
Chronic infection (HIV)	Immune-suppression
Metabolic disease (Diabetes, obesity)	
Long-term medication (corticosteroids, immunosuppressive drugs)	
Long-term antibiotic treatment	Alteration of local microenvironment
Denture	

Table 2. Clinical syndromes underlying chronic mucocutaneous Candidiasis.

Concerning the prevalence of CMC syndromes, only data on APECED exist. APECED is most common in the small populations with high consanguinity of Iranian

Jews (about 1:9.000)[70], Sardinians (1:14.400)[71] and Finnish (1:25.000)[72]. Both in the Jewish and the Finnish population old founder mutations responsible for almost all cases were detected[73]. Prevalence in Norway is estimated around 1:90.000[74]. In other parts of the world only sporadic cases of APECED have been reported. Similarly, no information is available on how frequent (or, rather orphan) non-APECED CMC syndromes are.

Pathogenesis of CMC

Defence against *Candida* requires an orchestrated immune response involving both innate and adaptive mechanisms. Though the pathogenesis of CMC is complex and may be heterogeneous, increasing evidence suggests that an altered T cell cytokine secretion is a central event.

In contrast, innate immunity generally seems functional in CMC patients[75]. However, single reports exist demonstrating defects in innate immunity also. This is true for phagocytic cells such as neutrophil granulocytes, where a serum-dependent functional defect has been published[76]. However, recent studies indicate a normal candidicidal capacity and migratory behaviour of neutrophils in CMC patients[77,78]. A subtle impairment in activation and migration of other phagocytic cells such as macrophages or monocytes[79] has also been reported in single CMC patients[57,80], though these might be secondary effects due to an altered cytokine production. Furthermore, some studies suggest a defect in natural killer (NK) cells in CMC, either stating they were decreased[81] or functionally impaired[82].

There seems to be no general defect in humoral immunity also, as most CMC patients show normal serum concentrations of immunoglobulins and high titres of specific antibodies against *Candida* species[57,83]. Again, within the heterogeneous

group of CMC patients, a small subgroup seems to suffer from recurrent respiratory infections accompanied by deficiency of the IgG subclasses 2 and 4[84].

However, protection against mucocutaneous *Candida* infections seems to rely mainly on cell-mediated immunity, in particular on T cells. Evidence for that hypothesis is given by the fact that patients lacking T cells due to a severe combined immunodeficiency or DiGeorge syndrome often suffer from oral candidiasis and *Candida* infections of skin and nail, but very rarely from systemic candidiasis[85]. The ability of T cells to proliferate to *Candida* antigen is discussed controversially: some studies describe a diminished proliferation both to *Candida* and to mitogens[86], others state a specific defect in the proliferation to *Candida* or a normal T cell proliferation[77]. Cytokine secretion of T cell subtypes rather than proliferation seems a more critical parameter in the pathogenesis of CMC. An impaired Th1 immune response leads to an increased susceptibility to severe *Candida* infections[87] while reduction of IL-10 increases resistance against these infections[88]. Furthermore, the T helper subset Th17 is essential in *Candida* resistance in mice and humans[89,90,91]. In fact, numerous studies show that CMC patients suffer from a deregulated T cell cytokine production, with a diminished production of type 1-cytokines[77,92,93,94] such as IFN-γ, IL-12 and IL-2 and an increased secretion of IL-10 or IL-4[95]. The observed effects, however, are not highly reproducible nor impressive. Whether deregulated T cell cytokine production is due to a direct T cell defect or a disturbed interaction with APC remains to be elucidated, as evidence exists that dendritic cells of CMC patients show an abnormal maturation[96] while normal distribution of pattern recognition receptors[97].

Clinical course of CMC

Impaired clearing of *Candida* is the basis for the main clinical symptoms of CMC, a chronic local inflammation, erosion/ulceration and hyperproliferation/ squamation of

skin and mucosal epithelia that ranges from mild angular cheilitis to severely inflamed thick plaques and crusts (Figure 4). Predisposed areas are oral and oesophageal mucosa, trunk (especially axillary and vaginal region) and hands and nails (onychomycosis or candidal paronychia). Primary CMC syndromes usually show an early onset within the first years of life, while secondary CMC occurs in later life stages.

Clinically, CMC patients suffer from high psychological stress, dissatisfying aesthetical appearance and uncomfortable itch, burning sensations or pain.

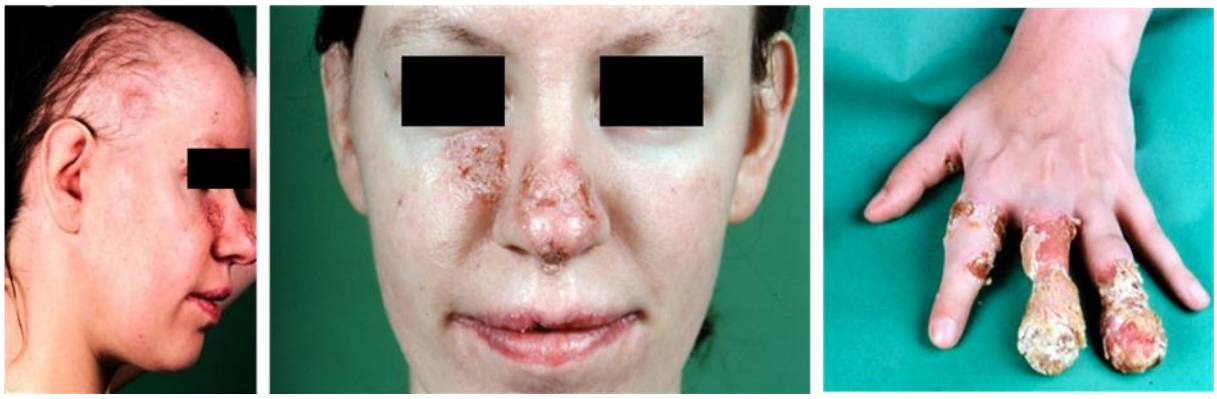


Figure 4. Clinical manifestation of chronic mucocutaneous candidiasis at the skin.

Furthermore, *Candida* plaques can cause severe clinical complications. Due to lesion expansion, local *Candida* plaques (*Candida granuloma*) can massively debilitate the use of hands. Another frequent and dangerous complication due to volume expansion and/or scarring after chronic inflammation is stricture of the oesophageus that has to be treated with balloon dilatation or stenting[98]. A secondary consequence of chronic plaques in the gastrointestinal tract can be maldigestion or malabsorption with consecutive iron and vitamin deficiency or even excessive loss of weight and cachexia that requires intraveneous nutrition. A third group of complications comprises metaplasia or neoplasia such as development of oral squamous cell carcinoma reported in several cases of severe CMC[99,100,101]. Potentially, neoplasia

develops as a consequence of the chronic inflammation, as previously described for gastro-oesophageal reflux disease[102] and cutaneous inflammation[103].

Therapy of CMC

Historically, CMC was treated with immune-stimulating or -restoring agents such as adoptive leukocyte transfer[104] or thymus transplantation[105]. After development of systemic anti-fungal drugs, however, long-term medication with azole antimycotics became the standard therapy of *Candida* infections[106] (Table 3). Azoles inhibit ergosterol synthesis, thereby acting static against *Candida*. Today fluconazole is recommended as first-line systemic drug in a dosage of 100-200mg/day. However, sensitivity against fluconazole often decreases over time[107]. Regular microbial sensitivity tests (antibiogram) of isolated *Candida* strains are therefore essential in the treatment of CMC. Itraconazole, voriconazole or posaconazole[108] are newer azole-antifungals that can substitute fluconazole.

Another class of systemic antifungal drugs applicable against *Candida* infections are the echinocandins[109] caspofungin[110], micafungin[111] and anidulafungin. They act against fungi by inhibition of glucan synthesis and therefore cell wall formation. The main disadvantage of echinocandins is lack of oral formulation. Echinocandins and azoles are comparably efficient against *Candida* infections. Though no long-term safety analyses are available, echinocandins seem relatively safe and show potential synergism with other antimycotics.

A third class of antifungal agents are polyenes that bind to ergosterol. The only polyene currently recommended for systemic anti-*Candida* treatment is amphotericin B; however, since it has to be administered intravenously and it shows severe side effects (in particular nephrotoxicity), amphotericin B has to be considered a third-line agent, although new lipid-associated formulations show lower toxic side effects[112].

Topical amphotericin B, however, is recommended as safe long-term treatment of local infections, as resistances are rarely observed.

Class/ name	Recommended dosage	Additional information
Azoles (Triazoles)		
fluconazole (first-line drug)	100-200mg/day; systemic infections up to 800mg/day; pediatric 6-12mg/kg/day	Oral administration; Candida-static activity; sometimes resistances (esp. fluconazole); liver enzymes ↑, drug-drug-interactions
itraconazole	200(-400)mg/day	
voriconazole	400mg/day oral or 8mg/kg/day intravenous	
posaconazole	600-800mg/day	
ravuconazole	No official recommendations yet (in clinical phase I and II studies)	
Echinocandins		
caspofungin	70mg/ first day, then 50mg/day (dose reduction in liver dysfunction)	Intravenous administration; Candidicidal activity; low side-effects (infusion reactions, liver enzymes ↑)
micafungin	100-150mg/day; prophylaxis: 50mg/day	
anidulafungin	200mg/first day, then 100mg/day	
Polyenes		
local Amphotericin B	2g/day	Local Candida infections (oral Candidiasis), safe and few resistances
liposomal amphotericin B	3mg/kg/day	Intravenous administration; rarely infusion-related side effects and nephrotoxicity

Table 3. Recommended anti-Candida drugs.

1.6 Atopy and atopic eczema

Atopic eczema (AE) is a chronic relapsing-remitting inflammatory skin disorder beginning mostly in early childhood. It is often associated with other atopic diseases such as allergic asthma and allergic rhinitis. The three atopic diseases can overlap or manifest at different life stages in the same individuum, which is called the atopic march[113]. AE is highly pruritic and severely affects life quality of the single individual and it´s environment[114]. The incidence of AE is continuously increasing[115,116], which implies a high socioeconomic impact[117].

The underlying pathogenesis of AE is a complex interaction of genetical predisposition and environmental factors (Figure 5). Defining hallmarks are the relapsing-remitting cutaneous inflammation, a disturbed epidermal barrier with a consecutive high transepidermal water-loss that results in dry skin, and a hyper-reactive immune system with IgE-mediated sensitisations against environmental allergens. Recently, a strong association with loss-of-function mutations in the gene filaggrin was reported[118,119], which could be critically involved in the observed epidermal barrier dysfunction. Other known defects in building an accurate epidermal barrier in AE patients are mutations in the lipid metabolism that result in decreased ceramides and alterations in the SPINK5/LEKTI genes.

In between epidermal barrier dysfunction and immunological changes, psychosomatic aspects and physical factors determine the outcome of AE. Psychological pressure and stress worsen AE, and adjuvant mental relaxing techniques have been shown to improve skin inflammation and life quality of patients. As for physical factors, moderate doses of UV light improve cutaneous inflammation in AE, but extreme temperature and sweating have opponent effects. In general, AE patients take benefit from a rehabilitation sojourn in a low-allergen environment like the seaside or mountains, supporting a role both for physical and for allergic factors.

AE is often associated with type I (Th2-dominated) immune hyperreactivities mediated by allergen-specific IgE to common environmental or food allergens114. The atopy patch test (APT) has been widely accepted as a model for allergen-specific induction of an acute atopic eczema by type I allergy inducing proteins such as pollen or house dust mite derived allergens[120]. AE and APT reactions share histological similarities with delayed type hypersensitivity responses, with the exception that in acute AE and APT lesions T helper 2 cytokines like IL-4, IL-5 and IL-13 are abundantly present[121,122].

Beyond deregulations within the adaptive branch of the immune system, innate immune responses are critical for the outcome of AE. Evidence exists that antimicrobial peptides, the so-called defensins, are reduced as compared to other immune mediated skin diseases like psoriasis, and this may be responsible for the high *Staph. aureus* skin colonisation[123].

The role of skin-colonising microorganisms in atopic eczema

More than 85% of AE patients are affected by skin colonisation with facultative pathogenic microbials like *Staph. aureus*[124]. Although *Staph. aureus* usually doesn´t elicit imminent clinical signs of infection, its colonisation contributes to allergic sensitisation and inflammation. Infection of eczematous lesions with *Staph. aureus* is strongly associated with an increased disease severity[125]. A suspected underlying mechanism is the frequent production of exotoxins by *Staph. aureus* species colonising AE skin[125].

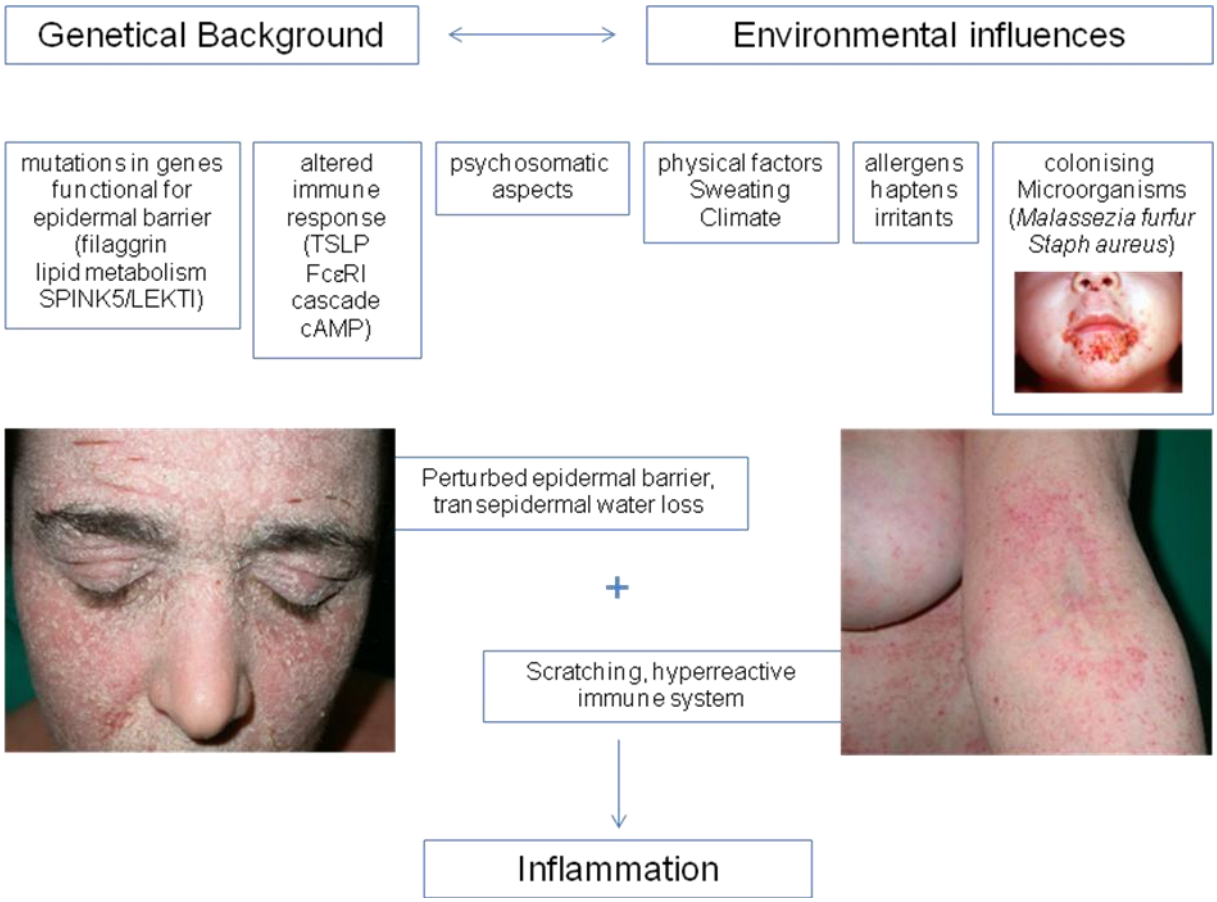


Figure 5. The pathogenesis of atopic eczema is based upon complex interactions between genetical predispositions and environmental influences.

These exotoxins stimulate T cells bearing particular T cell receptor Vβ chains regardless of their specificity and are therefore called superantigens[126]. Recently, the superantigen Staphylococcal enterotoxin B (SEB) was shown to enhance house dust mite-induced patch test reactions in AE patients, while only a minor proportion of AE patients developed an eczematous reaction to SEB alone. This study illustrates an enhancing role for bacterial-derived enterotoxins in the pathogenesis of AE[127].

2. Aim of the study

The aim of this study is to investigate the role of IL-17 in the first-line defence of the human organism against colonising and transient microorganisms. Since knock-out models are not available in the human system, two diseases are chosen that are frequently associated with an inability to clear skin infections – the orphan syndrome “chronic mucocutaneous candidiasis” and the common disease “atopic eczema”.

The following questions will be addressed to gain insight in the role of IL-17:

1. Is the IL-17 pathway disturbed in patients with chronic mucocutaneous candidiasis?
   a) Do these patients have normal counts of IL-17 producing T cells in peripheral blood?
   b) Do T cells of CMC patients react with an adequate secretion of IL-17 in response to *Candida* antigen or to mitogen?
   c) Is there a defect in Th17 cell differentiation?
2. Why do patients with atopic eczema express only low amounts of antimicrobial peptides and consecutively suffer from chronic skin infections?
   a) Are IL-17+ T cells detectable in inflamed skin of AE patients and how is their pasticity?
   b) Can IL-17 secretion be efficiently triggered in T cells capable of IL-17 production?
   c) Is there an intrinsic defect in response to IL-17 in human keratinocytes?
   d) Does the Th2-dominated microenvironment of acute eczematous AE reactions inhibit IL-17 effects?

3. Materials and methods

3.1 Patients

For the characterisation of the T cell immune response of CMC patients, we included four patients with this orphan disease (Table 4). Diagnosis was made clinically and confirmed by typical laboratory alterations. CMC patients were compared to four patients suffering from an (at the time of investigation) untreated *Candida* infection without any obvious immune suppression (Table 5) and to healthy volunteers (n=9).

Initials	MG (I)	MF (I)	CB (I)	MS (III)
Age (years)	27	37	48	8
Sex	♀	♀	♀	♂
Clinical symptoms	Chronic oral, esophageal, vaginal and cutaneous candidiasis, paronychia			
Onset (at age)	Early (3)	Early (birth)	Early (birth)	Early (birth)
Endocrinology/ Immunology	Normal	Normal/ squamous cell carcinoma, ANA mildly positive		Morbus Addison, polyendocrinopathy syndrome/ normal
Laboratory markers	Iron deficiency anemia	CRP constantly elevated (3,5mg/dl)	Normal	Normal
Electrophoresis	Albumin 50,9%↓, γ-globulin 22,8%↑	Albumin 56,9%↓, γ-globulin 22,0%↑	Albumin 55,9%↓, γ-globulin 20,4%↑	n.d.

Candida-Abs serum	IgG 258U/ml, IgM 1172U/ml	IgG <40U/ml, IgM <60U/ml	IgG 170U/ml, IgM <60U/ml	n.d.
Immunoglobulins serum	Normal, IgG4 0,20g/l↓	Normal	IgG 1635mg/dl↑, IgA 940mg/dl↑	n.d.
Phenotyping of T cells	Normal	Lymphopenia	Lymphopenia	n.d.

Table 4. Clinical and laboratory characteristics of CMC patients included into the study.

Initials	UD	WH	DU	LK
Age (years)	47	65	39	51
Sex	♀	♂	♀	♀
Clinical symptoms	Chronic paronychia and onychodystrophia			Vaginal Candidosis
Onset (at age)	Late (45)	Late (60)	Late (30)	Late (58)
Endocrinology/ Immunology	Normal	Normal	Normal	Diabetes mellitus/normal
Laboratory markers	Iron deficiency anemia	Normal	Normal	Normal

Electrophoresis	Albumin 63,2%→, γ-globulin 18,8%↑	Albumin 60,0%→, γ-globulin 16,4%→	n.d.	n.d.
Candida-Abs serum	IgG 52U/ml, IgM <60U/ml	IgG <40U/ml, IgM <60U/ml	n.d.	n.d.
Immunoglobulins serum	Normal	Normal	Normal	Normal
Phenotyping of T cells	Normal	Normal	Normal	Normal

Table 5. Clinical and laboratory characteristics of immune-competent patients with chronic Candida *infection included into the study.*

Before blood was taken, each participant had given his informed consent. The study was approved by the ethical committee of the Technical University Munich, following the guidelines of the Helsinki declaration[128].

For the second model disease, three patients suffering from atopic eczema (AE) included into the study. Diagnosis was confirmed according to the criteria of Hanifin and Rajka[129]. Furthermore, all patients suffered from a relevant allergy to house dust mite, as diagnosed with a positive prick test to *Dermatophagoides pteronyssinus* (Der p), RAST class ≥ 3 to Der p 1 and a positive Atopy Patch Test (APT) to Der p.

Before blood or skin samples were taken, each participant gave his informed consent. The study was approved by the ethical committee of the Istituto Dermopatico Dell'Immacolata.

3.2 Materials

Biologic material	
Albumin, bovine (BSA)	Sigma, Munich, A-8806
Candida albicans	Sigma, Munich, IRMM354
Dermatophagoides pteronyssinus	Indoor Biotechnology, UK
Natural affinity purified	NA-DP1
Recombinant	RP-DP1
Fetal bovine serum (FBS),	Perbio, Bonn
Human male AB Serum	Sigma, Munich, H-4522
LPS	Sigma, Munich, L-4391
Staphylococcal enterotoxin B	Sigma, Munich, S-4881
Cell culture material	
96-well plates flat bottom sterile	Nunc, Roskilde, Denmark, 167008
96-well plates U-bottom sterile	Nunc, Roskilde, Denmark, 163320
96-well maxisorp plates flat bottom	Nunc, Roskilde, Denmark, 449824
Clustertubes 1,2ml	Abgene, Surrey, UK, AB-0672
Cryo tubes 1,8ml	Nunc, Roskilde, Denmark, 375418
Eppendorf tubes	Eppendorf, Hamburg, 0030 015.002
Falcon Polypropylen tubes	Becton Dickinson, NJ, USA
15 ml/50 ml	352070 / 2096
Heat sealing paper (β-Counter)	Perkin Elmer, Rodgau-Jügesheim, 1450-467
LS columns (MACS)	Miltenyi, Bergisch-Gladbach, 130-041-306
Melti Lex TMA (β-Counter)	Perkin Elmer, Rodgau-Jügesheim, 1450-441
Printed Filtermat A (β-Counter)	Perkin Elmer, Rodgau-Jügesheim, 1450-421
Sterile filter units 500ml	Millipore, FCGVUO5RE
Surgical scissor Aesculap	Braun, Melsungen, BC107R

Chemicals

2-Mercapto-Ethanol	Sigma, Munich, M-7522
Adenine	Sigma, Munich, A-9795
Antibiotic-Antimycotic Solution	PAA, Linz, Austria, P11-002
Aqua ad injectabilia	Delta-Select, Pfullingen
CFSE	Molecular Probes, Leiden, Netherlands, C-1157
Cholera toxin	Sigma, Munich, C-3012
DMSO	Baker, Griesheim, 7157
DMEM	Invitrogen, Paisley, UK, 41966-029
DPBS Ca/Mg	Invitrogen, Paisley, UK, 14040174
DPBS w/o Ca/Mg	Invitrogen, Paisley, UK, 14190094
EDTA	Sigma, Munich, ED4SS
EDTA 0,5M	Invitrogen, Paisley, UK, 15575-020
Epidermal growth factor (EGF)	Sigma, Munich, E-4127
Gentamycin	Invitrogen, Paisley, UK, 15710049
Glucose	Sigma, Munich, G-7528
HAM´s F12	Sigma, Munich, N-6760
HBSS w/o Ca/Mg	Invitrogen, Paisley, UK, 24020091
Heparin 250.000U	Ratiopharm, Ulm, PZN-7833909
Hydrocortisone	Sigma, Munich, H-0135
Insulin	Sigma, Munich, I-1882
Keratinocyte medium	Promocell, Wien, Austria, C-20211
L-Glutamine	Invitrogen, Paisley, UK, 25030024
Lymphoprep	Progen Biotechnik, Heidelberg, 111-4545
Mitomycin C	Sigma, Munich, M-4287
Nickel sulphate	Sigma, Munich, N-4882
Non-essential amino acids	Invitrogen, Paisley, UK, 11140-35
Penicillin-Streptomycin	Invitrogen, Paisley, UK, 15140130
PHA (Lectin)	Sigma, Munich, L-9132
Reverse transcriptase	Roche, Mannheim
RNA extraction buffer (PeqGold)	Peqlab, Erlangen
RPMI 1640 + L-Glutamine	Invitrogen, Paisley, UK, 31870-025

Sodium pyruvate	Invitrogen, Paisley, UK, 11360-039
SYBR green master mix	Bio-Rad, Munich, Germany
Transferrin	Sigma, Munich, T-5391
Trypan blue 0,4% solution	Sigma, Munich, T-8154
Trypsin 0,05% EDTA	Invitrogen, Paisley, UK, 2530054

Cytokines and antibodies

anti-CCR4 PE	R&D systems, clone 205410
anti-CCR6 PE	BD pharmigen, clone 11A9
anti-CD14 FITC	BD bioscience, clone MφP9
anti-CD1a FITC	BD pharmigen, clone HI149
anti-CD28	BD bioscience, clone 37.51
anti-CD3	BD bioscience, clone 145-2C11
anti-CD4 PE	BD bioscience,clone SK3
anti-CD4 FITC	BD bioscience, clone SK3
anti-CD56 FITC	BD bioscience, clone NCAM16.2
anti-CD8 PE	BD bioscience,clone SK1
anti-CD8 FITC	BD pharmigen, clone RPA-T8
anti-CD83 FITC	BD pharmigen, clone HB15e
anti-CD86 FITC	BD pharmigen, clone 2331FUN1
anti-CXCR3	R&D systems, clone 49801.111
anti-HLA-DP	R&D systems, 347730
anti-HLA-DQ	R&D systems, 347450
anti-HLA-DR	R&D systems, 347360
anti-IL10 PE	BD pharmigen, clone JES3-19F1
anti-IL4 FITC	BD pharmigen, clone MP4-25D2
anti-IFN-γ FITC	BD pharmigen, clone B27
anti-IFN-γ APC	BD pharmigen, clone B27
anti-TNF-α FITC	BD pharmigen, clone Mab11
anti-IL22 PE	R&D systems, clone 142928
anti-IL4 PE	R&D systems, clone 3007.11
anti-IL-17	R&D systems, clone AF-317
anti-IL17A PE	eBioscience, clone SK3
GM-CSF	Schering Plough

IFN-γ	BD bioscience, 554617
IL-13	R&D Systems, 213-IL-005
IL-17	R&D Systems, 317-ILB-050
IL-1β	R&D systems, 201-LB-005
IL-2	Novartis, Munich
IL-23	R&D Systems, 1290-IL-010
IL-4	R&D Systems, 204-IL-010
IL-6	R&D Systems, 206-IL-010

ELISA systems and kits

HBD-2	Phoenix pharmaceuticals
Iotest Beta Mark Repertoire Kit	Beckman Coulter
IFN-γ duoset	R&D systems, DY285
IL-4 duoset	R&D systems, DY204
IL-10 duoset	R&D systems, DY217B
IL-13 duoset	R&D systems, DY213
IL-17 duoset	R&D systems, DY317
IL-22 duoset	R&D systems, DY782
TNF-α duoset	R&D systems, DY210

Machines

Camera	WILD MPS 52, Leitz-Leica, Wetzlar
Centrifuge “Biofuge 13”	Heraeus, Hanau
Centrifuge “Megafuge 1.0R”	Heraeus, Hanau
FACS“Calibur“	Becton Dickinson, Heidelberg
FACS“Aria“	Becton Dickinson, Heidelberg
Homogeniser	Ultra Turrax T 25 basic, IKA Werke, Staufen
Light microscope	Aristoplan, Leitz-Leica, Wetzlar
Light microscope	Axiovert 25, Zeiss, Jena
Multi-channel pipette	Eppendorf, Hamburg
Pipettes „reference“	Eppendorf, Hamburg
Precise weighing machince	MC1 Research, Sartorius, Göttingen
Real-time PCR “ABI Prism 7000”	Applied biosystems, Foster City, CA

Shaker	Titramax 100, Heidolph, Schwabach
Weighing machine	MC1Labor, Sartorius, Göttingen
Water bath „type 1003“	GFL, Burgwedel

Used media

3T3-medium

DMEM, 10% FCS (30min at 56° C de-activated), 5ml Pen/Strept.

Stored at 4-8°C, used within 10 days

Antibiotic-antimycotic solution for keratinocyte isolation

solution I: 500ml DMEM, 15ml gentamycin, 20ml *Antibiotic-Antimycotic solution*

solution II: 250ml Lösung I + 250ml DMEM

solution III: 250ml MEM + 2,5ml *Antibiotic-Antimycoticsolution*

Sterile filtered, stored at -20°C

Feeder-/ keratinocyte medium

300ml DMEM, 150ml HAM´s F12, 10ml glutamin, 50ml Hyclone II FCS, 5ml senicillin/streptomycin, 1ml adenin (= 1g), 1ml hydrocortisone (= 1g), 0,5ml Trijodthyronine (= 1mg), 0,5ml Cholera toxin (= 1mg), 0,5ml EGF (Epidermal Growth Factor; = 0,1mg), 0,5ml insulin (= 100mg), 0,5ml transferrin (= 10mg)

Sterile filtered, stored at 4°C, used within 14 days

Mitomycin solution

Mitomycin C 2mg

Stock solution (500µg/ml): dissolved in 4ml DPBS (+Ca^{2+}/Mg^{2+}).

Stored at 4°C in the dark

Ready-to-use solution: 2% (diluted in RPMI 1640) Medium sterile filtered

MACS buffer

500ml DPBS Dulbecco´s w/o Ca/Mg, w/o Sodium bicarbonate, 2ml EDTA (2mM, diluted from Invitrogen 0,5M EDTA, pH 8,0), 2,5g bovine serum albumin (0,5%)

Sterile filtered, oscillated in ultrasound-bath before usage for 15min

Proliferation medium (RPMI complete 5% human serum)

450ml RPMI 1640 Medium with L-Glutamin (Gibco™, Lot No. 3063498), 28ml human serum, 5ml L-glutamin, 5,6ml non-essential amino acids, 5,6ml sodium pyruvate, 500µl 50mM 2-Mercapto-ethanol
Sterile filtered, stored at 4°C, used within 7 days

EBV-Medium

RPMI complete with 50ml FCS (=10%), no human serum

cloning medium

RPMI complete with 25ml human serum (= 5%), 50ml FCS (30min at 56°C heat inactivated) (= 10%), 5ml *antibiotic-antimycotic solution*
Sterile filtered, stored at 4°C, used within 7 days

3.3 Methods

Methods for part A (Th17 deficiency in CMC patients)

Isolation of peripheral blood mononuclear cells (PBMC)

For isolation of PBMC, venous blood was taken from the forearm of patients and healthy volunteers in a syringe containing heparin. Blood was diluted 1:2 in DPBS and then transferred to a density gradient (15ml Lymphoprep medium in a 50ml tube) and centrifuged at 470xg for 20 minutes without brake. After centrifugation, the separated band of PBMC was recovered with a 5ml pipette and washed three times in DPBS (409xg for 15 minutes, then two times 301xg for 10 minutes). An aliquot of PBMC was counted in a 0,5% Trypan blue solution, then the rest of the PBMC was resuspended in proliferation medium in a defined concentration prior the experiments.

Separation of CD4+ and CD8+ T cells

For some experiments, CD4+ and CD8+ T cells were purified from isolated PBMC using magnetic bead labeling. 20μl anti-CD4 or anti-CD8 microbeads and 80μl MACS buffer per 10^7 PBMC were incubated for 15 minutes at 4° C and separated by positive selection through a magnetic column. Cells were then washed, counted and resuspended in proliferation medium.

Stimulation and co-culture experiments

PBMC were cultured in flat-bottom 96well plates ($3,5*10^5$/well) and stimulated with either *Candida* antigen (100µg/ml), Phytohemagglutinin (PHA) (10µg/ml) or coated antiCD3/antiCD28 antibodies. For isolation of total RNA PBMC were stimulated for 6 hours, for quantification of secreted cytokines into supernatant by ELISA PBMC were stimulated for 60 hours.

Flow cytometry analysis

PBMC were stimulated with PMA (20 ng/ml) and ionomycin (1ng/ml) for 6 hours and examined for intracellular cytokine accumulation.

To prevent cytokine secretion, the stimulation was performed in the presence of Monensin (from the beginning) and Brefeldin A (10 g/ml) was added for the final 4 hours. T cells were fixed (2% paraformaldehyde), permeabilized (0,5% saponin), and stained with PE-conjugated anti human IL-17 antibody or isotype-matched control antibody. Acquisition and analysis was done using a FACS Calibur.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IFN-γ, IL-4, IL-10, IL-17, IL-22, IL-1β, and IL-6 in cell-free culture supernatant were quantified using commercially available sandwich ELISA kits.

RNA isolation and Real time PCR

Total mRNA from stimulated PBMC was extracted using PeqGold RNA extraction buffer. RNA was reverse transcribed using oligo(dT) primers and AMV reverse transcriptase. PCR reactions were performed with synthetic oligonucleotides reacting specifically against IL-1β, IL-6, IL-17A, IL-17F, IL-22, and IL-23A (sequences see Table 6). SYBR green mastermix was added.

Name of gene	Sequence
IL-1β	forward 5'-TTC GAC ACA TGG GAT AAC GA-3'
	reverse 5'-TCT TTC AAC ACG CAG GAC AG-3'
IL-6	forward 5'-ATG CAA TAA CCA CCC CTG AC-3'
	reverse 5'-GAG GTG CCC ATG CTA CAT TT-3'
IL-17A	forward: 5'-CTC GAT TTC ACA TGC CTT CA-3'
	reverse 5'-GAG GGG CCT TAA TCT CCA AA-3'
IL-17F	forward 5'-AGT TGG AGA AGG TGC TGG TG-3'
	reverse 5'-CCA TCC GTG CAG GTC TTA TT-3'
IL-22	forward 5'-GAG GAA TGT GCA AAA GCT GA-3'
	reverse 5'-GCT TTG GGG CAT CTA ATT GT-3'
IL-23A	forward 5'-CAG TTC TGC TTG CAA AGG AT-3'
	reverse 5'-ATC TGC TGA GTC TCC CAG TG-3'

Table 6. Real-time PCR primers used in the study. Primer sequences were obtained from www.realtimeprimers.com

PCR reactions were run on an ABI Prism 7000 Sequence Detection System using the following program: 10 min at 94°C followed by 45 cycles of 15 s at 95°C, and 60 s at 58°C. 18 s RNA served as housekeeping gene.

Statistical analysis

Statistical analysis was performed by the software programme SPSS 14.0. Differences between the CMC group and healthy controls or immune competent *Candida*-infected patients were analysed using Mann-Whitney-U test. Statistically significant differences between CMC patients and controls were defined as $p<0,05$.

Methods for part B (IL-17 in AE patients)

Isolation and characterisation of skin-derived T cells

To analyse T cells at clonal level, T cells derived from positive Atopy Patch Tests were cloned by limiting dilution.

Atopy Patch Tests containing house dust mite (*Dermatophagoides pteronyssinus*, Der p) were applied in large Finn chambers (11 mm in diameter) on the back of the patients using petrolatum as carrier. Petrolatum alone served as negative control. After 48 hours the Finn chambers were removed and tested areas were marked. The test was evaluated after 48 and 72 hours. Positive reactions were classified according to the European Task Force on Atopic Dermatitis (ETFAD) 2000 reading key[130].

T cells were then isolated from positive patch test lesions (Figure 6). For that purpose, punch biopsy specimens (4mm in diameter) were taken from positive APT reactions from the centre of the patch test areas after local anaesthesia had been administered (1% lidocaine). Biopsies were immediately cultured in proliferation medium containing 60 IU/ml recombinant IL-2).

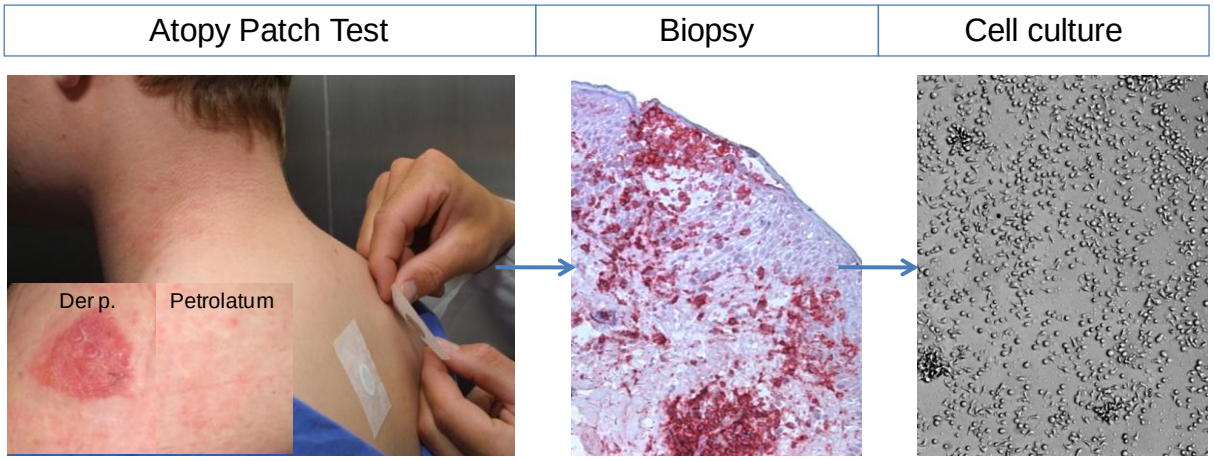


Figure 6. Isolation of T cells from inflammatory skin reactions. Acute eczematous reactions were induced in sensitized patients (APT), biopsied and migrated cells were cultured in T cell medium.

After three days, half the medium was replaced by fresh medium containing IL-2. After seven days, migrated cells were collected and cloned by limiting dilution. T cells were counted and diluted to a concentration of 0,6 cells/well in 96-well U-bottom microplates in cloning medium on a feeder layer (450.000/well) of irradiated PBMC, 30U/ml IL-2 and 1% PHA. Fresh medium containing IL-2 was added three times a week and clones were restimulated using irradiated feeder PBMC every three to four weeks.

Growing T cell clones were expanded and characterised regarding cytokine secretion profile and Der p 1 reactivity. Clones were stimulated with either coated anti-CD3/anti-CD28 or autologous dendritic cells and Der p 1, respectively. After 48 hours, cell-free supernatant was obtained and the content of TNF-α, IFN-γ, IL-4, IL-10, IL-13, IL-17 and IL-22 was determined by ELISA. Proliferation was investigated using the ^{3}H-Thymidine assay. After 48 hour stimulation, 2μCi/ml ^{3}H-thymidine was added to the cultures for additional 12 hours. Cells were then harvested on a filter, dried and afterwards reactivity was measured in a β-counter. In this setting, the

amount of incorporated thymidine directly correlates with the proliferation of the T cells.

Definition of T cell subtype

T cell clones were classified according to their relative cytokine secretion into supernatant of the lead cytokines IFN-γ (Th1), IL-4 (Th2), IL-10 (Th3), IL-17 (Th17), and IL-22 (Th22) after 48 hour stimulation with anti-CD3/anti-CD28. Table 6 illustrates the relative cytokine profile of each T cell population.

	IFN-γ	IL-4	IL-10	IL-17	IL-22
Th1	>80%	<5%	<20%	<5%	<20%
Th2	<20%	>50%	<50%	<5%	<20%
Th3	<20%	<5%	>80%	<5%	<20%
Th17	<20%	<5%	<20%	>50%	<50%
Th22	<20%	<5%	<20%	<5%	>80%

Table 7. Definition of T cell subpopulations according to their relative cytokine secretion of lineage-indicating cytokines.

Isolation and generation of antigen-presenting cells (APC)

Dendritic cells were generated from CD14+ monocytes[25]. CD14+ monocytes isolated by magnetic labelling and positive selection out of PBMC were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 0.5 mM 2-Mercaptoethanol, 20 µg/ml gentamycin 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 50µg/ml LPS for 24 hours. Immediately before coculture experiments, cells were harvested and characterized for maturation markers (CD83, CD86, HLA-DR) and CD1a using flow cytometry.

Suspension and culture of primary human keratinocytes

To establish an autologous model of eczema, after isolating T cell clones keratinocytes were isolated from the same individuals using the method of suction blister[26]. Blisters were induced by generating a vacuum on normal skin of the forearms. Epidermal sheets were obtained from blister roofs, treated with 0.05% trypsin (Invitrogen) to obtain single-cell suspension and seeded on a feeder layer of irradiated 3T3/J2 fibroblasts in modified Green's medium. At 70–80% confluence, keratinocytes were detached with 0.05% trypsin, aliquoted, and cryopreserved in liquid nitrogen. Keratinocytes of second and third passage were used in experiments.

Stimulation and co-culture experiments

10^5 T cells and between 100 and 10000 immature or mature DC were co-cultured in flat-bottom 96well plates in RPMI complete 5% human serum for 36 hours with 5µg/ml Der p 1, 10µg/ml PMA/ 1µg/ml ionomycine, 5µg/ml SEB or full medium as negative control, respectively. Cell-free supernatant was obtained and T cell proliferation was analysed by ^{3}H thymidine incorporation as described above.

Patch testing of house dust mite and SEB in vivo

To investigate the SEB/IL-17/HBD-2 axis *in vivo*, commercially available Der p was applied at the two forearms of an AE patient. 36 hours after allergen application, 50µg/cm² SEB was added on one forearm. Sixty hours after allergen application, epidermal sheets and blister fluid of induced eczematous reactions were obtained by the method of suction blister. RNA was prepared from epidermal roofs as described above, suction blister fluid was analysed using the Luminex method and by HBD-2 ELISA as described above (Figure 7).

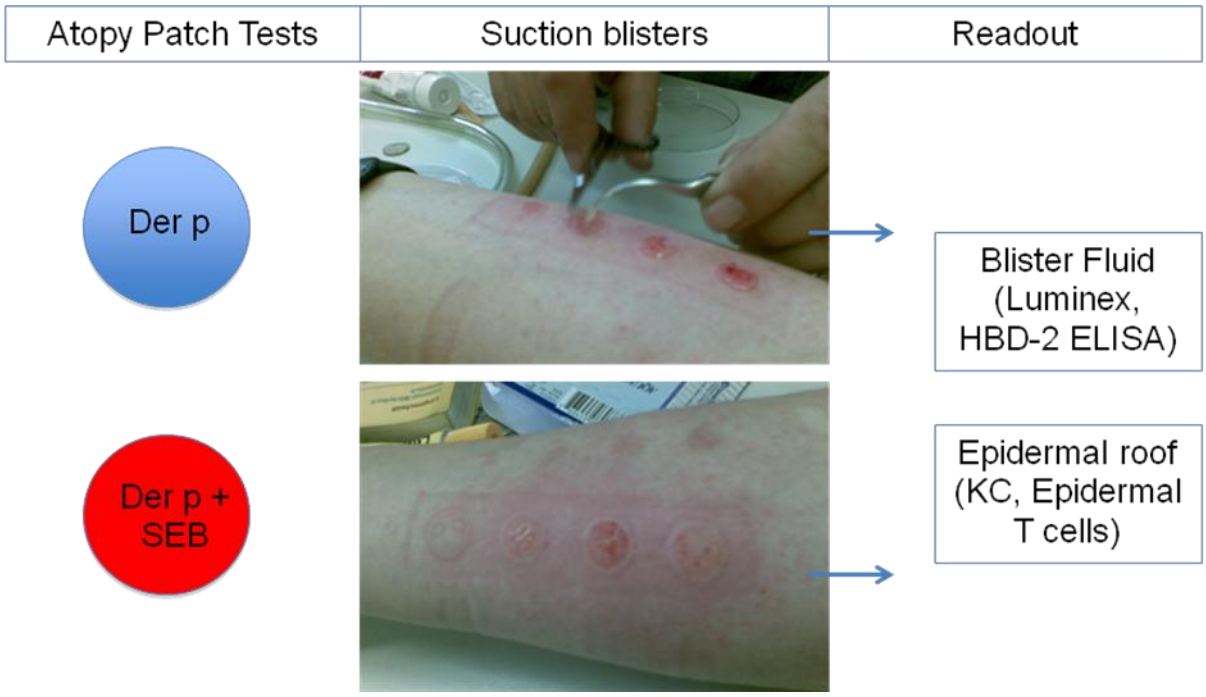


Figure 7. Application of house dust mite and SEB in vivo*.*

Flow cytometry analysis

T cell receptor Vβ repertoire of selected T cell clones was analysed using the "Iotest Beta Mark Repertoire Kit" following the instructions of the manufacturer.

Surface marker staining of skin-derived T cell lines or resting T cell clones was performed with 105 T cells for 15 mintes on ice and in the dark. After the incubation period, cells were washed in FACS buffer and acquisition was performed using a FACS calibur.

For intracellular cytokine analyses, skin-derived T cell lines or resting T cell clones were stimulated with PMA (20 ng/ml) and ionomycin (1ng/ml) for 6 hours. To prevent cytokine secretion, Monensin was added in the beginning and Brefeldin A (10 g/ml) was added for the final 4 hours of stimulation. T cells were then fixed (2% paraformaldehyde), permeabilized (0,5% saponin), and stained with conjugated

antibodies (IFN-γ, IL-4, IL-17, IL-22, TNF-α) or isotype-matched control antibodies. Acquisition and analysis was done using a FACS Calibur.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-22, TNF-α and human β-defensin2 in cell-free culture supernatant were quantified using commercially available sandwich ELISA kits of indicated companies.

RNA isolation and Real time PCR

Total cellular mRNA was extracted from stimulated keratinocytes employing the phenol/chloroform method. RNA was then reverse transcribed using oligo(dT) primers and AMV reverse transcriptase. PCR reactions were performed with synthetic oligonucleotides reacting specifically against HBD-2 (sequences see Table 8). SYBR green mastermix was added.

Name of gene	Sequence
HBD-2	forward: 5'-TCCTCTCGTTCCTCTTCATATT-3'
	reverse: 5'-TTAAGGCAGGTAACAGGATCGC-3'

Table 8. Real-time PCR primers used in the study. Primer sequences were obtained from www.realtimeprimers.com

PCR reactions were run on an ABI Prism 7000 Sequence Detection System using the following program: 10 min at 94°C followed by 45 cycles of 15 s at 95°C, and 60 s at 58°C. GAPDH was housekeeping gene.

Statistical analysis

Differences in cytokine production and secretion in T cell clones and keratinocytes were analysed using a two-tailed Student´s t-test. Statistically significant differences were defined as $p<0,05$.

4. Results

4.1 CMC patients suffer from an impaired secretion of IL-17 and IL-22

PBMC of CMC patients show a strong decrease in IL-17 and IL-22 secretion upon stimulation

Since a T cellular immune defect was hypothesised by us and others in patients with CMC, we stimulated PBMC of four CMC patients with *Candida albicans* as well as with mitogen (Phythemagglutinin). Immune competent healthy volunteers (n=9) and immune competent patients with an acute *Candida*-infection (n=4) served as controls. As a first step, we investigated the cytokine induction in PBMC at genetic level. For that purpose, we stimulated PBMC for 12 hours and then isolated total mRNA, reverse transcribed and amplified it in real time PCR and compared the cytokine mRNA level with that of un-stimulated PBMC (Figure 8).

Indeed we observed that IL-17F and IL-22 were significantly induced in PBMC of healthy (non-*Candida* infected) controls and immune competent Candidiasis patients, whereas CMC patients failed to upregulate the expression of these cytokines on mRNA level (Figure 8). Differences in upregulation of IL-17 and IL-22 mRNA in response to both *Candida albicans* and to PHA were significant ($p>0,05$) between CMC patients and both immune competent control groups, while no significant differences were observed between healthy volunteers and Candidiasis patients.

The impaired upregulation of IL-17 and IL-22 mRNA was irrespective of the stimulus, as both specific stimulation with *Candida albicans* and mitogenic stimulation with PHA did neither induce IL-17 nor IL-22 (Figure 8). IL-17A was not upregulated in CMC patients and healthy controls in the monitored time course (data not shown).

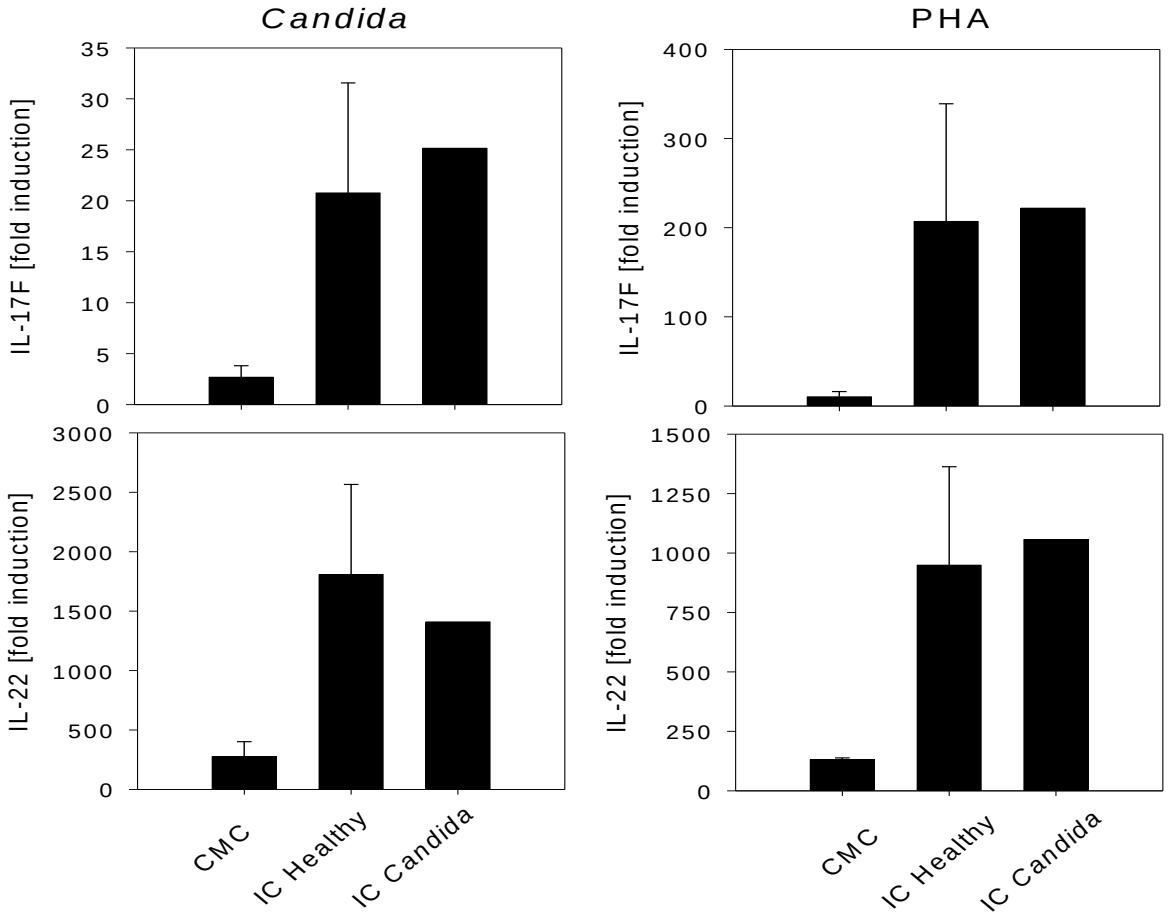


Figure 8. CMC patients fail to upregulate IL-17 and IL-22 mRNA. Upregulation of IL-17 (upper panel) and IL-22 (lower panel) is given at the y-axis. Shown is the mean of three independent experiments (3 CMC patients, 4 healthy controls, 1 Candidiasis patient in total). Error bars indicate mean +/- SEM. (CMC: chronic mucocutaneous candidasis; IC healthy: immune competent volunteers; IC Candida: immune competent Candidiasis patients)

An impaired IL-17 and IL-22 immune response in CMC patients as observed on transcriptional level was confirmed at secretion level of proteins. PBMC of CMC patients released very low amounts of IL-17A/F and IL-22 into culture supernatant after stimulation with either *Candida albicans* or PHA as measured by ELISA after 72 hours stimulation (Figure 9). In contrast, healthy volunteers secreted significantly higher amounts of Il-17A/F and IL-22. Even more impressive was the production of IL-17 and IL-22 in immune competent patients with current *Candida* infection that was significantly higher than that of CMC patients and of healthy volunteers (Figure 9).

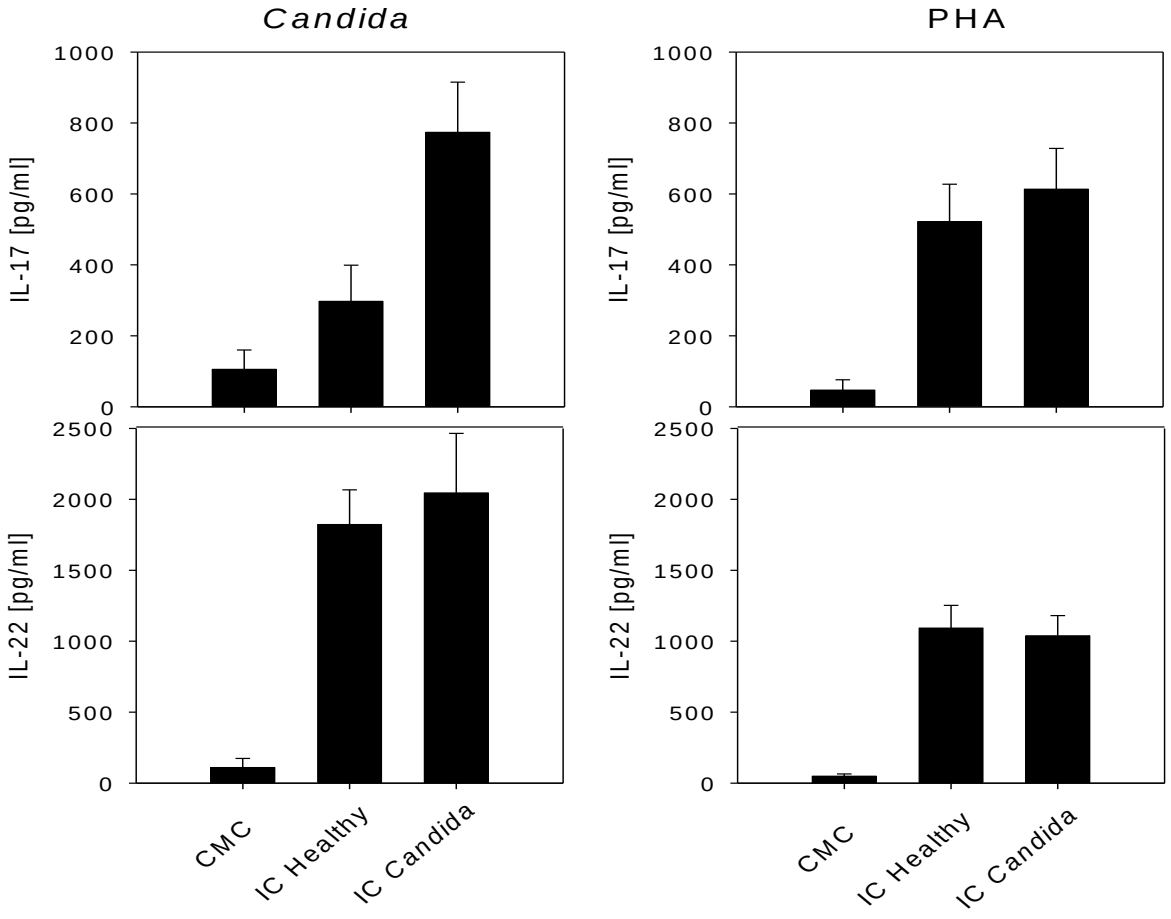


Figure 9. Impaired IL-17 and IL-22 secretion in PBMC of CMC patients. Secretion of IL-17 (upper panel) and IL-22 (lower panel) is given at the y-axis. Shown is the mean of five independent experiments (4 CMC patients, 9 healthy controls, 4 Candidiasis patients in total). Error bars indicate mean +/- SEM. (CMC: chronic mucocutaneous candidasis; IC healthy: immune competent volunteers; IC Candida: immune competent Candidiasis patients)

In the next step, we wanted to analyse whether the observed diminished secretion of IL-17 and IL-22 in PBMC was the consequence of a direct T cell defect or of an impaired antigen presentation by dendritic cells (DC). We therefore stimulated PBMC from CMC patients and immune competent controls with anti-CD3/anti-CD28, thus directly stimulating the T cells. Again, we observed a clearly diminished induction at mRNA level and secretion at protein level in CMC patients (Figure 10). This finding points to a defect in the T cell compartment rather than in antigen-presentation or absent APC-derived signals.

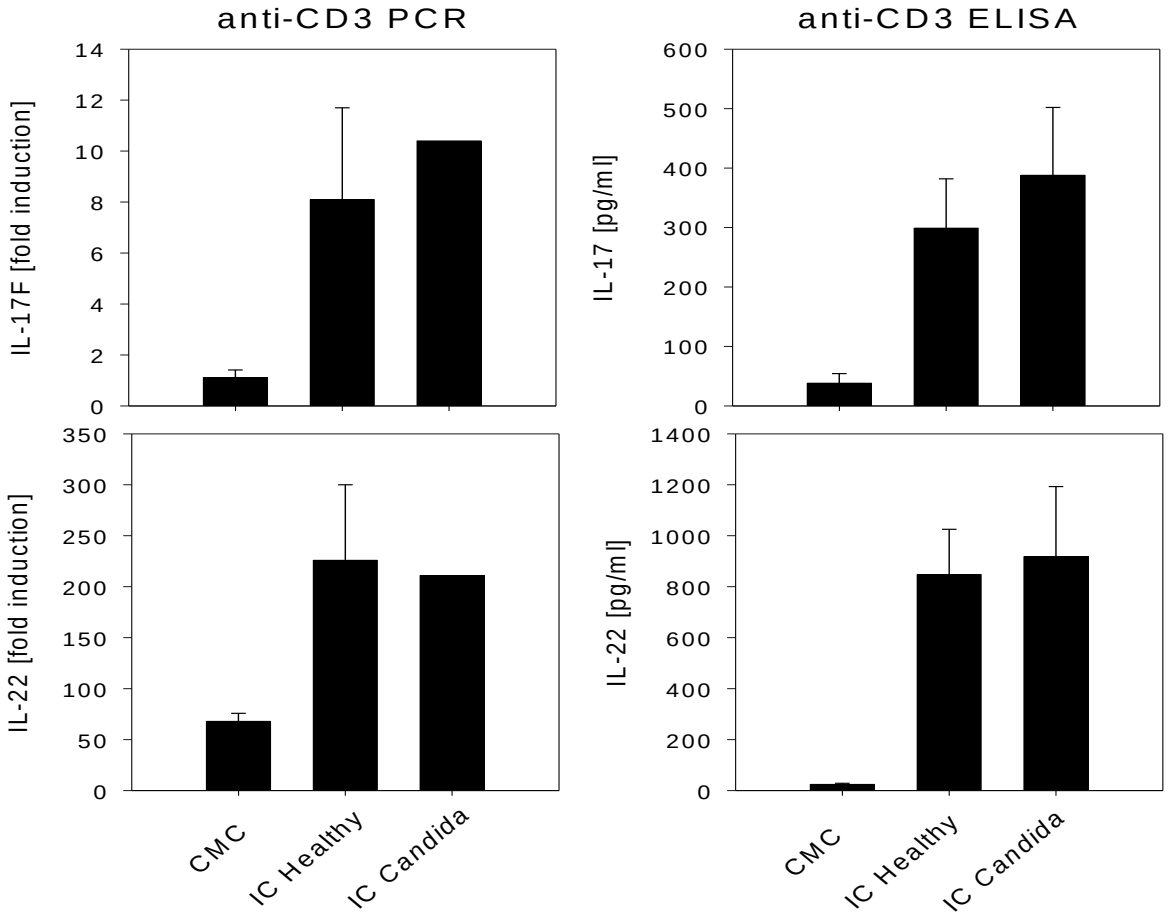


Figure 10. Directly stimulated T cells of CMC patients secrete diminished levels of IL-17 and IL-22. Secretion of IL-17 (upper panel) and IL-22 (lower panel) is given at the y-axis. Shown is the mean of three independent experiments (3 CMC patients, 8 healthy controls, 2 Candidiasis patients in total). Error bars indicate mean +/- SEM. (CMC: chronic mucocutaneous candidasis; IC healthy: immune competent volunteers; IC Candida: immune competent Candidiasis patients)

To investigate the main source of IL-17 within the T cell compartment, we isolated purified CD4+ and CD8+ T cells and co-cultured them with autologous monocytes as antigen-presenting cells and *Candida albicans*. These experiments revealed that IL-17 and IL-22 are predominantly derived from CD4+ T cells. In contrast, only a very low amount of IL-17 and IL-22 was secreted by the CD8+ T cell compartment. Thus, the disability of CMC patients to produce IL-17 and IL-22 is mainly attributed to CD4+ T cells (Figure 11).

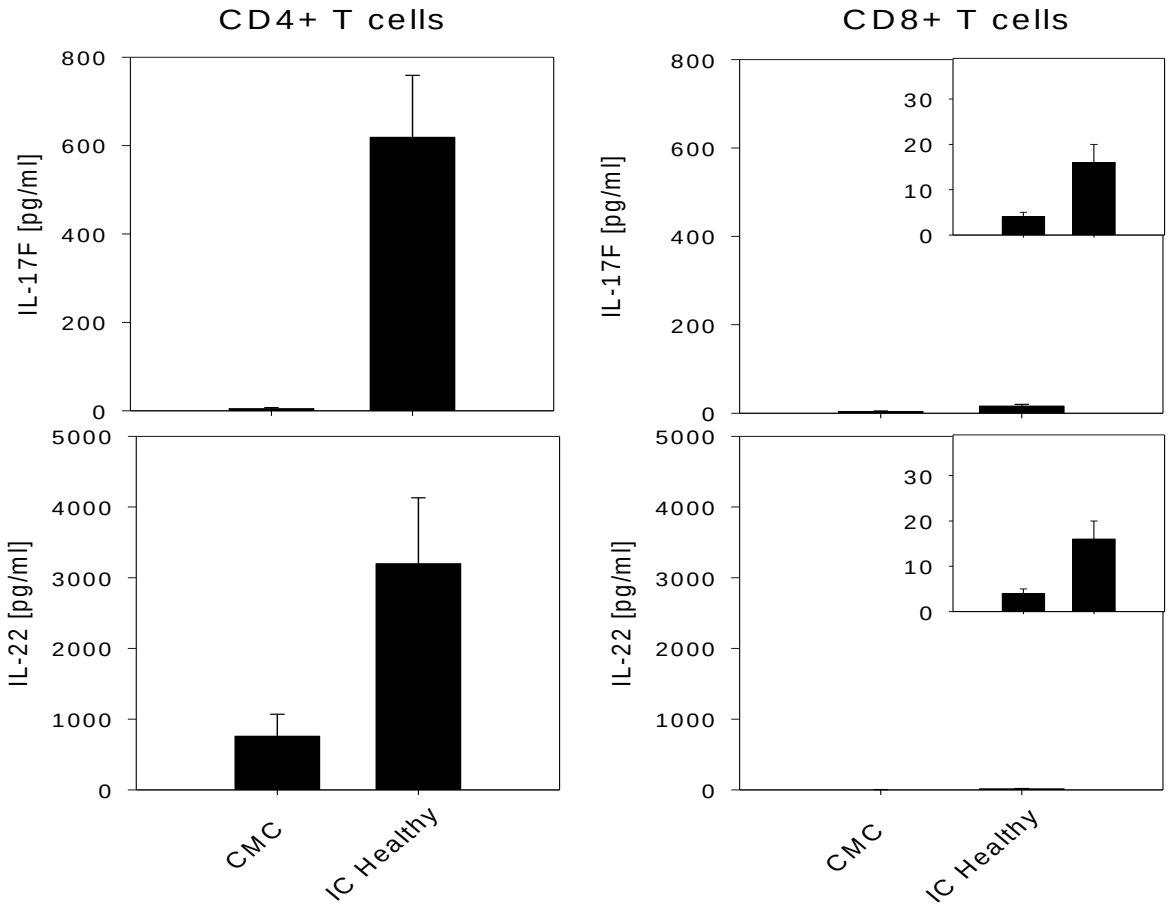


Figure 11. CD4+ T helper cells are the main source of IL-17 and IL-22. Secretion of IL-17 (upper panel) and IL-22 (lower panel) of CD4+ (left panel) and CD8+ (right panel) T cells is given at the y-axis. The inset in the right panel shows cytokine secretion in pg/ml in a lower scale. Shown is the mean of three independent experiments (2 CMC patients and 5 healthy controls in total). Error bars indicate mean +/- SEM. (CMC: chronic mucocutaneous candidasis; IC healthy: immune competent volunteers)

CMC patients exhibit reduced total number of IL-17 producing T cells but normal amounts of CCR4+/CCR6+ T cells

To answer the question whether CMC patients lack Th17 cells, we quantified IL-17 producing T cells in CMC patients and healthy volunteers. Since CCR4 and CCR6 are described to be good markers for Th17 cells *in vitro*, we first stained PBMC of CMC patients and healthy controls for CCR4 and CCR6 (Figure 12).

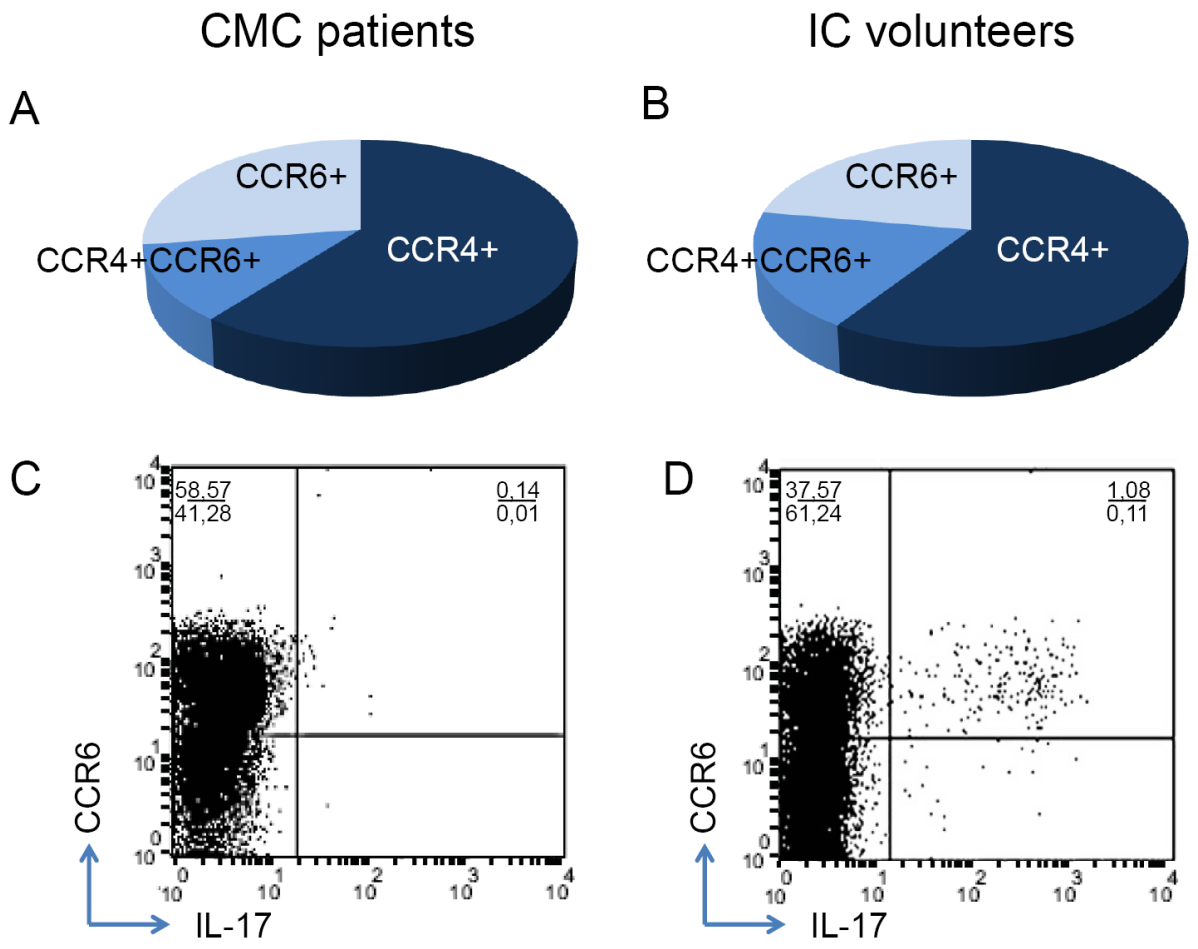


Figure 12: The CCR6+ IL-17A+ cell population is strongly decreased in CMC patients, while the number of CCR4+CCR6+ cells is not diminished. PBMC of CMC patients and healthy controls were stained intracellularly after PMA/ionomycine stimulation for IL-17 (C,D) (one representative experiment of 3 performed is shown) and analysed for the expression of the surface markers CCR4 and CCR6 (A,B) by flow cytometry (CMC patients n=3 and healthy controls n=4).

We observed no differences in the relative frequency of CCR4+, CCR6+ or double positive T cells on total PBMC (Figure 12A,B) between CMC and immune competent controls.

However, CCR4+CCR6+ T cells from CMC patients did not produce any IL-17 protein. In contrast, a proportion of CCR6+ cells of healthy controls showed an intracellular accumulation of IL-17 after PMA/ionomycine stimulation (Figure 12C,D). Thus, in line with the secretion profile observed in ELISA, CMC patients show a dramatic decrease of IL-17 producing T cells.

PBMC of CMC patients are able to secrete Th17-differentiating and -maintaining cytokines

The absence of IL-17 and IL-22 producing T cells in CMC patients could be the consequence of an impaired differentiation of these cells. Since IL-1β and IL-6 are important for the differentiation of human IL-17 producing T cells, we analysed the expression of IL-1β and IL-6 at the transcriptional level by real time PCR. PBMC of CMC patients stimulated with *Candida albicans* for 12 hours tended to show higher mRNA expression of IL-1β and IL-6 compared to healthy controls (Figure 13). Thus, the observed defect in IL-17 producing T cells in CMC does not involve Th17-differentiating cytokines IL-1β and IL-6.

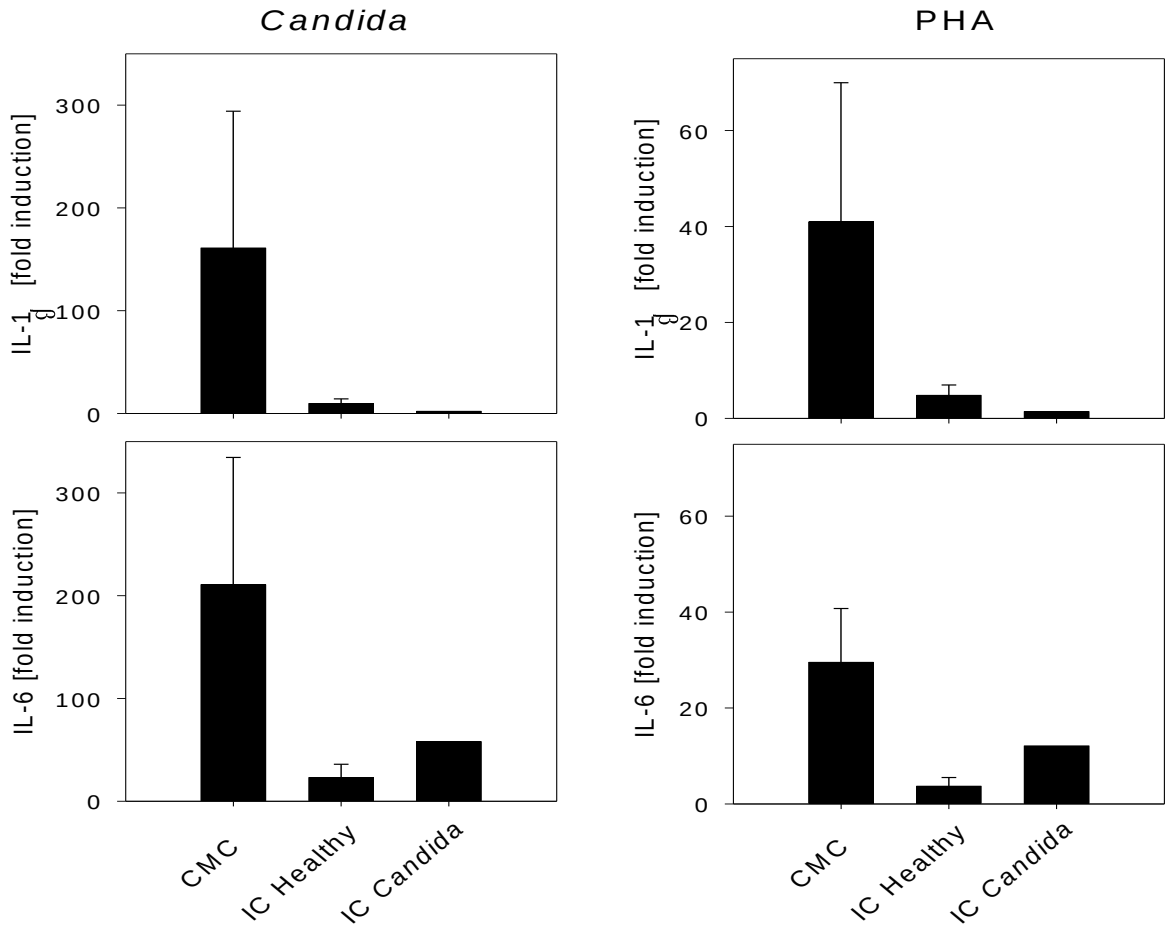


Figure 13: No significant differences in induction of Th17-differentiating cytokines between CMC patients and immune competent controls. Induction of IL-1β (upper panel) and IL-6 (lower panel) mRNA is given at the y-axis. Shown is the mean of three independent experiments (3 CMC patients, 4 healthy controls, 1 Candidiasis patient in total). Error bars indicate mean +/- SEM. (CMC: chronic mucocutaneous candidasis; IC healthy: immune competent volunteers; IC Candida: immune competent Candidiasis patients).

4.2 IL-17 is involved in a pro-inflammatory *circulus vitiosus* in atopic eczema

IL-17 producing T lymphocytes are infiltrating the skin during an APT reaction: newly characterised Th2/IL-17 subset

AE patients characteristically suffer from chronic skin colonisation with *Staphylococcus aureus*, and the density of colonisation directly correlates with disease severity. Since we observed in the first part of this study that the lack of IL-17 and IL-22 results in chronic skin infections (like in CMC patients), we investigated whether also PBMC from AE patients might show a defect in the production of IL-17 and IL-22. For that purpose, three AE patients with documented type I hypersensitivity to *Dermatophagoides pteronyssinus* (Der p) were challenged with Atopy Patch Tests (APT) of Der p. Biopsies were taken from the resulting eczematous reactions and infiltrating T cells were isolated and characterised by intracellular cytokine staining using flow cytometry techniques. In line with the hypothesis of a Th2 domination in early AE, the majority of skin derived T cells activated *in vitro* by PMA plus ionomycine expressed high levels of IL-4 (Figure 14). Moreover, about 9% (9% +/- 3%) of all infiltrated T cells were capable of producing IL-17. IL-17 and IL-22 were not necessarily co-expressed (Figure 14). Interestingly, about one third of IL-17 releasing T cells co-expressed IL-4 (Th2/IL-17 T cells) or IL-4 plus IFN-γ (Th0/IL-17 T cells). 50% of IL-17 producing T cells were pure Th17 T cells, a minor proportion coproduced IL-17 and IFN-γ (Th1/IL-17) (Figure 14).

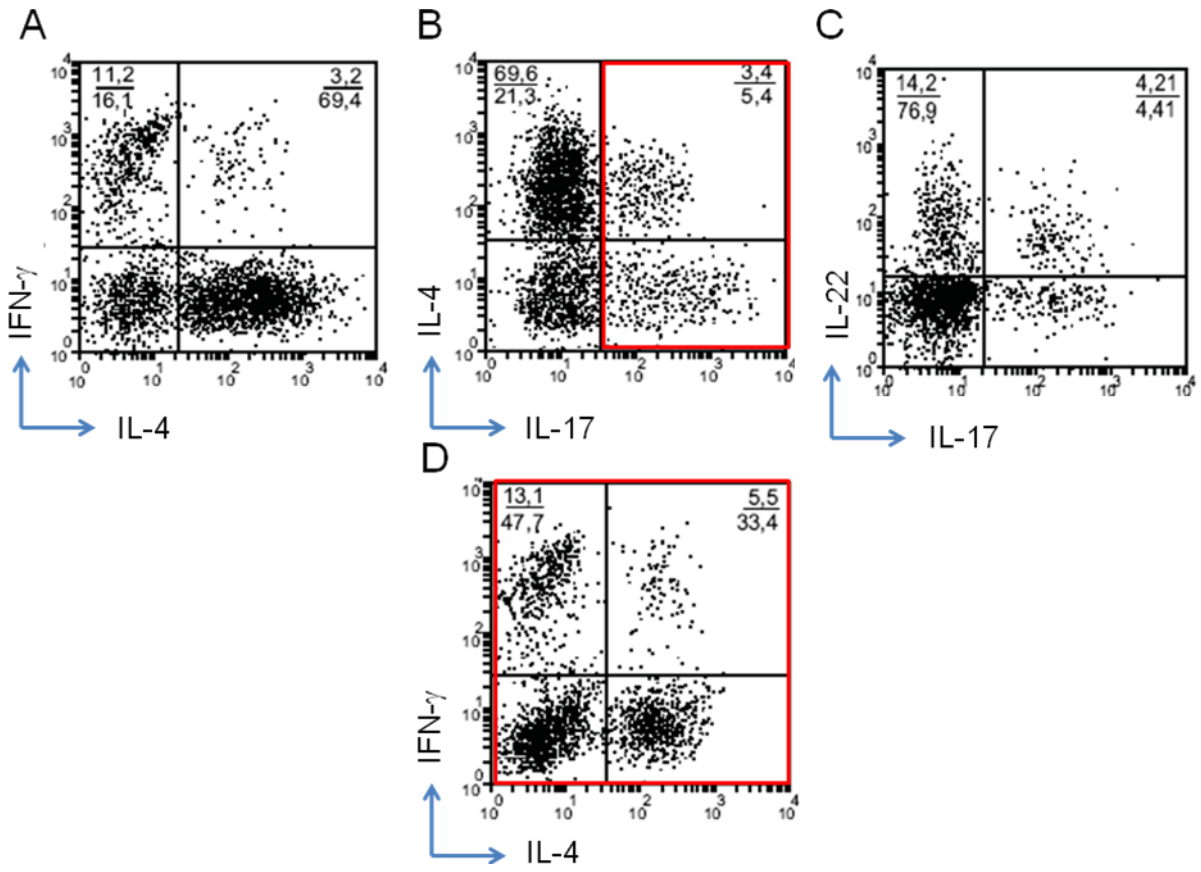


Figure 14: Distinct populations of IL-17 producing T cells in the skin infiltrate of a positive APT reaction. A: Intracellular cytokine staining of skin-derived lymphocytes with IL-4/IFN-γ (left dot plot), IL-4/IL-17 (middle dot plot) and IL-17/IL-22 (right dot plot). Gating on IL-17+ cells after triple staining with IL-4, IFN-γ and IL-17 (dot plot lower panel). Percentage of cells is indicated for each quadrant. Shown is one representative experiment.

A subpopulation of Der p 1 specific T cells has the capacity to produce IL-17

In order to further characterise skin-derived IL-17 producing T lymphocytes, T cell lines isolated from biopsied positive APT reactions to Der p were cloned by limiting dilution. Expanded T cell clones (in total 142 T cell clones obtained from 3 AE patients) were characterized for Der p 1 specificity, chemokine receptor expression, as well as the release of IFN-γ, IL-4, IL-10, IL-13, IL-17, IL-22 and TNF-α (Table in the appendix). Consistent with the observations obtained from the APT derived T cell line, a high number of APT derived T cells (24% +/- 1,9%) was capable of producing IL-17 after activation with PMA plus ionomycine. The newly identified subset Th2/IL-17 was identified also on clonal level. The relative distribution of IL-17 producing

subpopulations was comparable to the results obtained from T cell lines (Figure 15), with more than 40% pure Th17 phenotype (41% +/- 4,1%), one third Th2/IL-17 (32% +/- 3,6%) and one fourth Th1/IL-17 cells (26% +/- 4,5%). Representative intracellular cytokine stainings for each IL-17 producing subtype are shown in figure 16.

Pure Th17 cells were not specific for Der p 1. Likewise the skin-derived T cell lines, a correlation of IL-17 with IL-22 was not obvious on clonal level. 69% (+/- 4,8%) of skin infiltrating T cell clones were capable of producing IL-22 (Figure 15, Table in the appendix). A characterisation of IL-17 producing T cell clones is shown in table 9 and table 10.

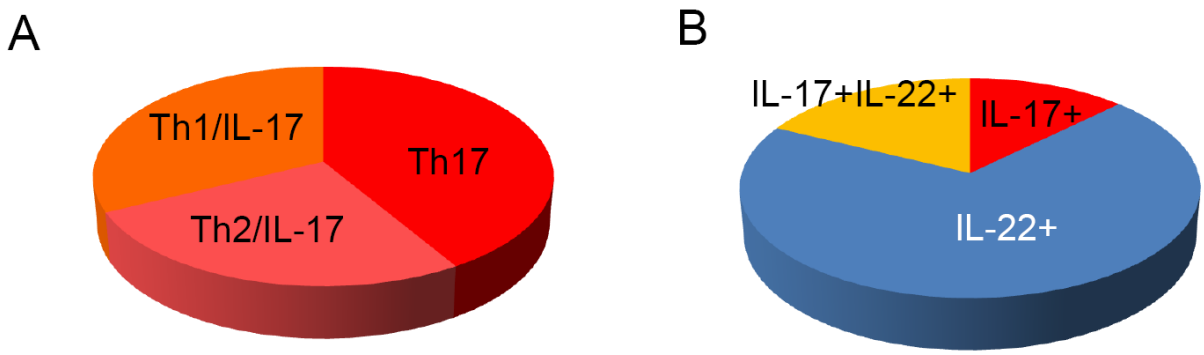


Figure 15. Cytokine production of T cell clones. A: relative distribution of pure Th17, Th1/IL-17 and Th2/IL-17 clones on total IL-17+ clones. B: percentage of IL-17 secreting, IL-22 secreting and double secreting T cell clones.

Clone	Proliferation		Cytokine Profile (after PMA/ionomycine stimulation)							Subtype
	SI to Der p 1	SI to SEB	IFN-γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF-α	
3	75	45	496	4285	13484	13738	12494	2931	7779	Th2/IL-17
27	1	20	0	3	1578	947	13788	2595	1596	Th17
60	25	30	5385	2922	10623	13074	11000	126	5348	Th0/IL-17
91	20	15	0	3252	16260	18317	3519	7000	4620	Th2/IL-17
96	40	45	9946	3477	16159	14302	2394	3568	3840	Th0/IL-17
141	40	1	0	4710	1149	20472	5500	219	861	Th2/IL-17

Table 9. Der p 1 specificity and cytokine secretion profile of IL-17 producing T cell clones obtained from AE patients.

Clone	TCR Vβ chain	Surface markers (in resting state)				
		CD4	CD8	CCR4	CCR6	CXCR3
3	14	94%	0%	73%	85%	14%
27	12	98%	0%	95%	99%	1%
60	3	96%	2%	85%	90%	67%
91	14	98%	0%	89%	50%	3%
96	n. det.	90%	0%	1%	54%	45%
141	n. det.	99%	0%	45%	55%	1%

Table 10. TCR analysis and expression of surface markers of IL-17 producing T cell clones obtained from AE patients. Shown is the percentage of positive cells on total cells as compared to isotype control. "n. det." = clone expresses a T cell receptor Vβ chain that is not detectable by the Iotest Beta Mark Repertoire Kit.

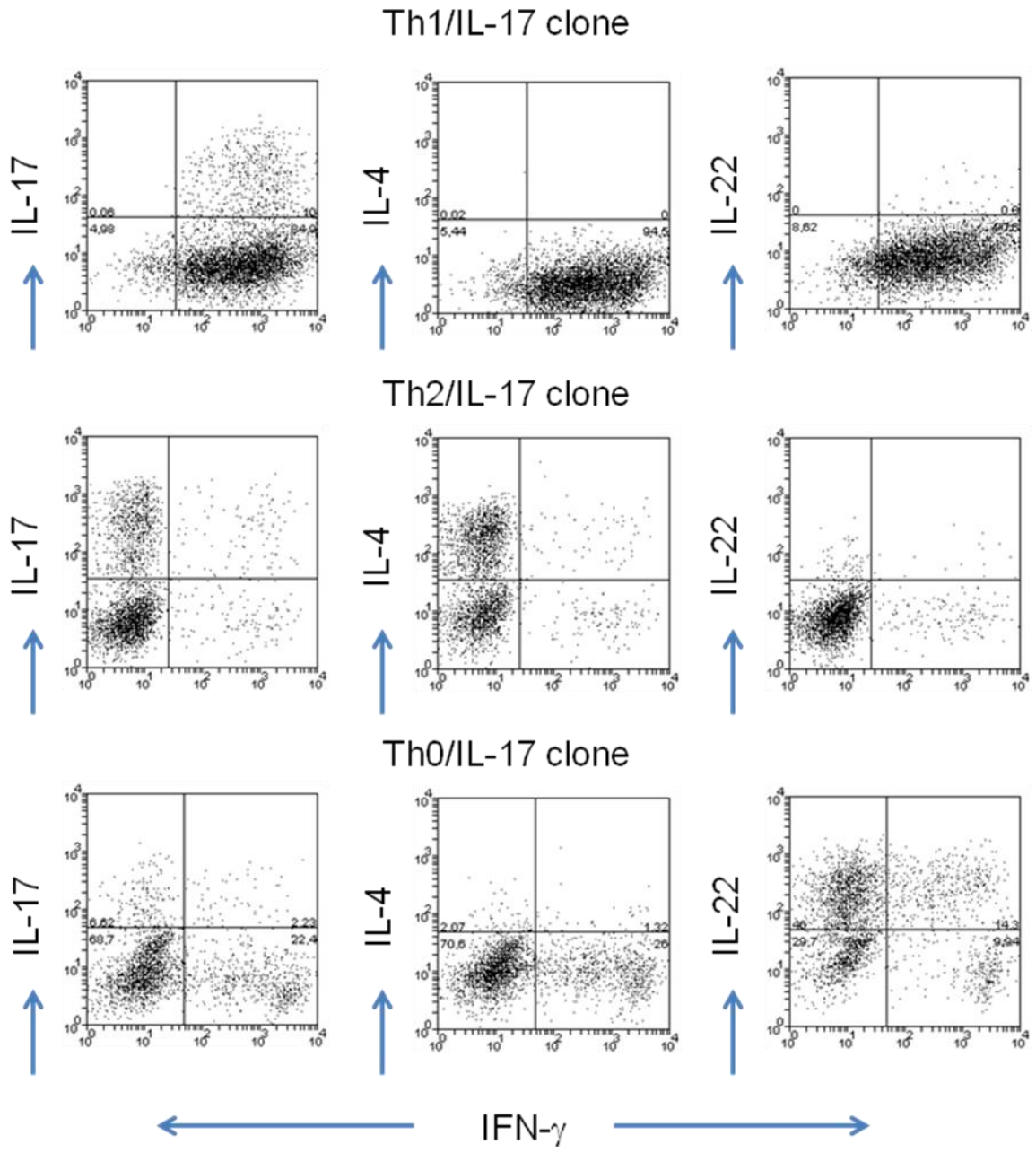
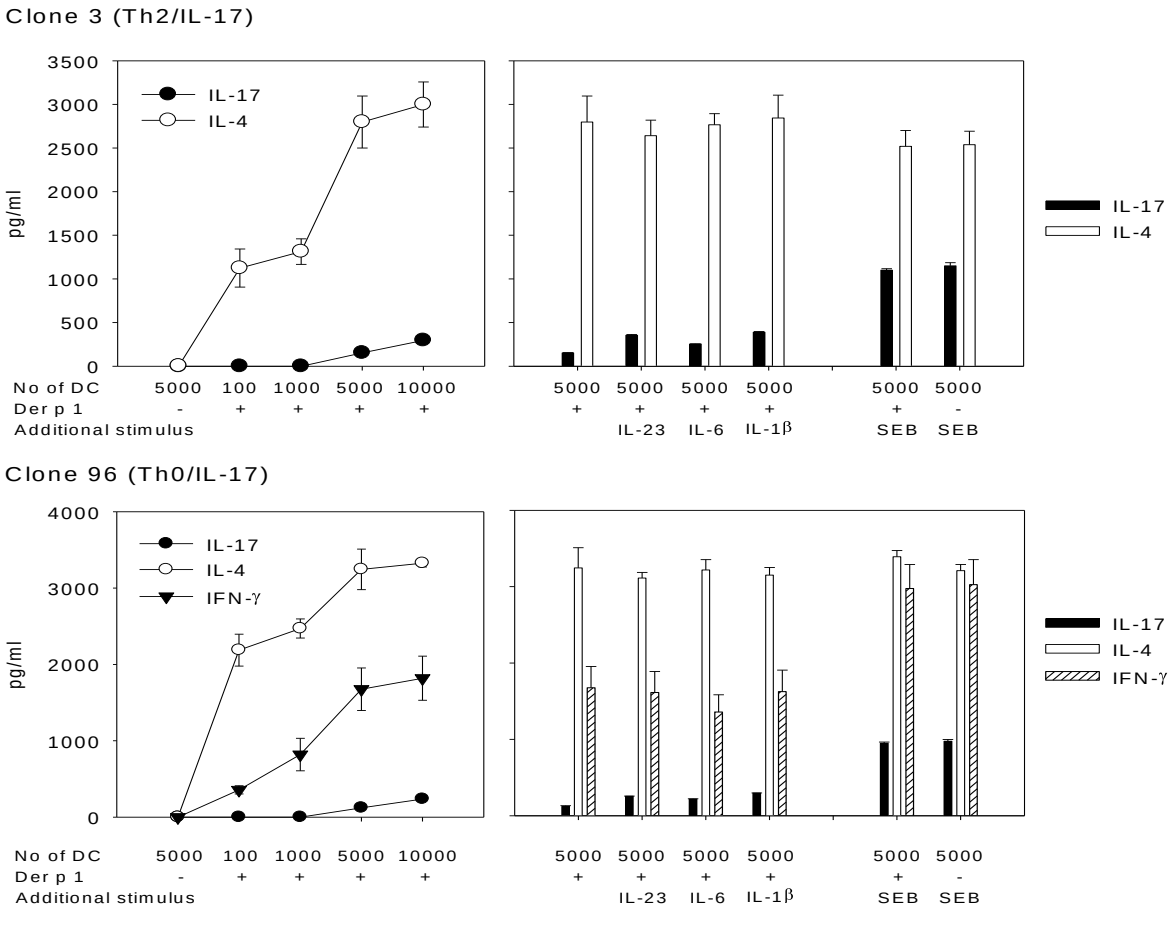


Figure 16. Intracellular cytokine staining after PMA/ionomycin stimulation of a representative Th1/IL-17, Th2/IL-17 and Th0/IL-17 clone.



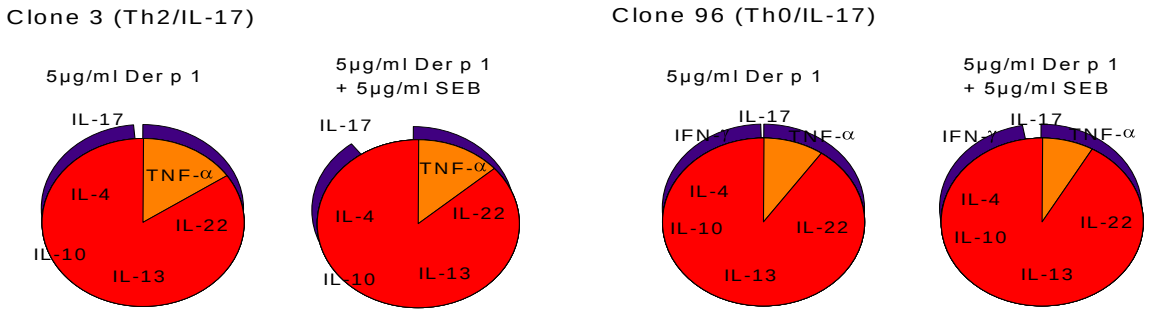


Figure 17. IL-17+ Der p 1-specific T cell clones secrete IL-17 upon stimulation with SEB, but not with Der p 1. Dose-dependent secretion of IL-17/IL-4/IFN-γ, addition of cytokines and SEB (upper panel). Type of APC: immature DC. Error bars indicate SD of one representative experiment performed in triplicates. Relative cytokine profile (mean % of three independent experiments) of clones is shifted towards IL-17 after stimulation with SEB (lower panel).

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

We then analysed the physiologic reaction pattern of specific skin-derived Th2/IL-17 and Th0/IL-17 T cell clones stimulated with native or recombinant Der p 1 in the presence of different antigen presenting cells (APC). Surprisingly, IL-17 was not or only marginally secreted by Der p 1-specific Th2/IL-17 and Th0/IL-17 cells when the allergen was presented by varying numbers of immature DC (Figure 17). This picture did not change when we co-cultured T cell clones with mature DC (Figure 18) or CD14+ monocytes (Figure 19).

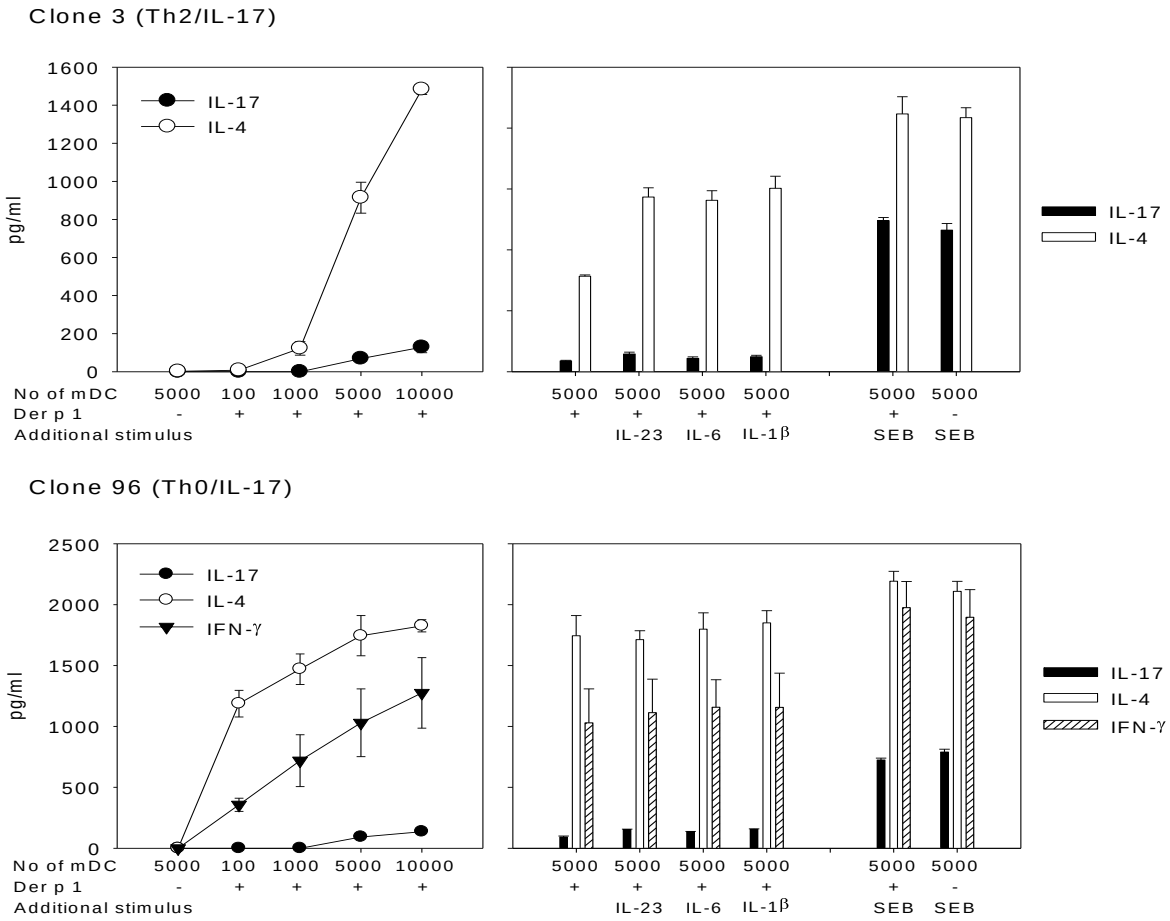


Figure 18. IL-17+ Der p 1-specific T cell clones secrete IL-17 upon stimulation with SEB, but not with Der p 1. Dose-dependent secretion of IL-17/IL-4/IFN-γ, addition of cytokines and SEB (upper panel). Type of APC: mature DC. Error bars indicate SD of one representative experiment performed in triplicates.

Increasing the T cell receptor stimulation intensity by stimulation with higher allergen concentration (Figure 20) or the number of APC only marginally upregulated IL-17 secretion (Figures 17-19). In contrast, even at low levels of stimulation intensity (DC: T cell ratio 1:1000 and 5μg/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, physiological TCR triggering failed to upregulate the release of IL-17, albeit capable of inducing Th2 cytokines which predominate the early phase of AE.

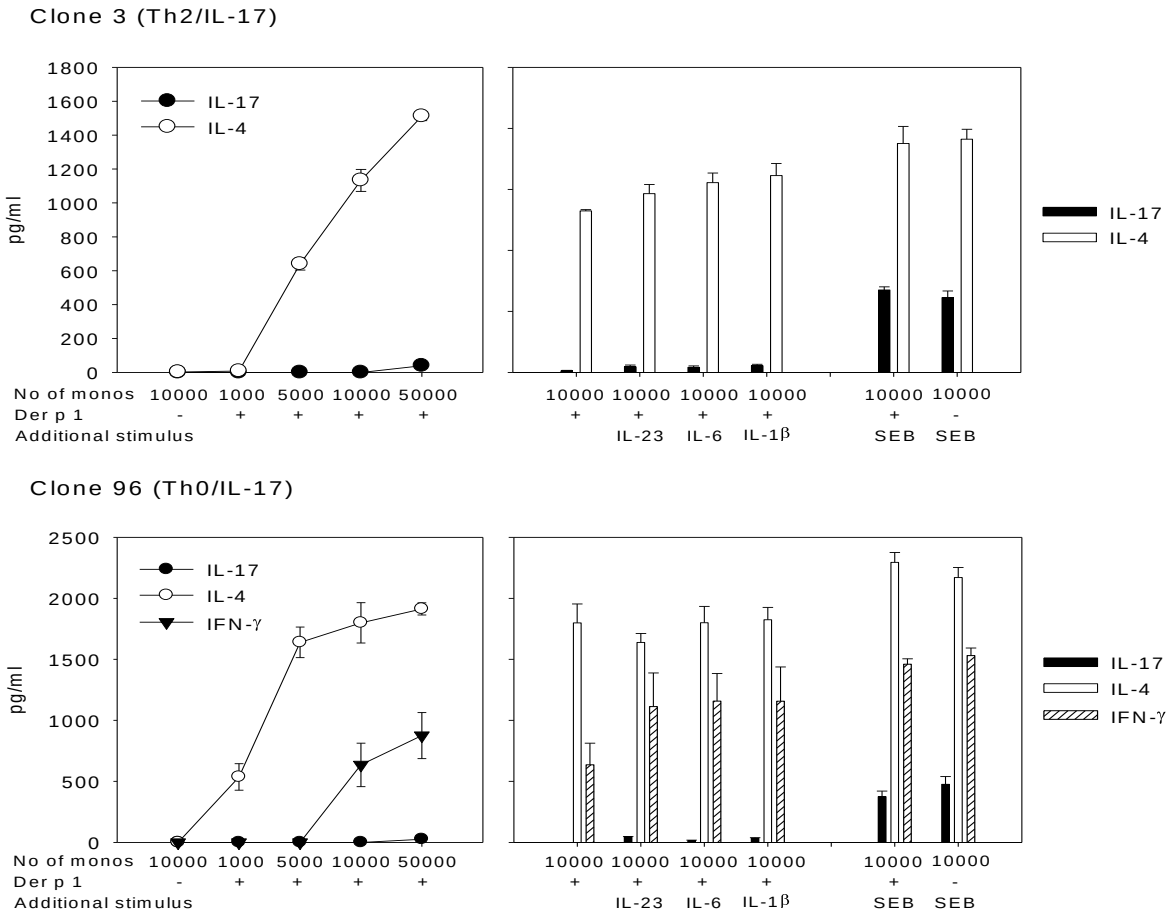


Figure 19. IL-17+ Der p 1-specific T cell clones secrete IL-17 upon stimulation with SEB, but not with Der p 1. Dose-dependent secretion of IL-17/IL-4/IFN-γ, addition of cytokines and SEB (upper panel). Type of APC: monocytes. Error bars indicate SD of one representative experiment performed in triplicates.

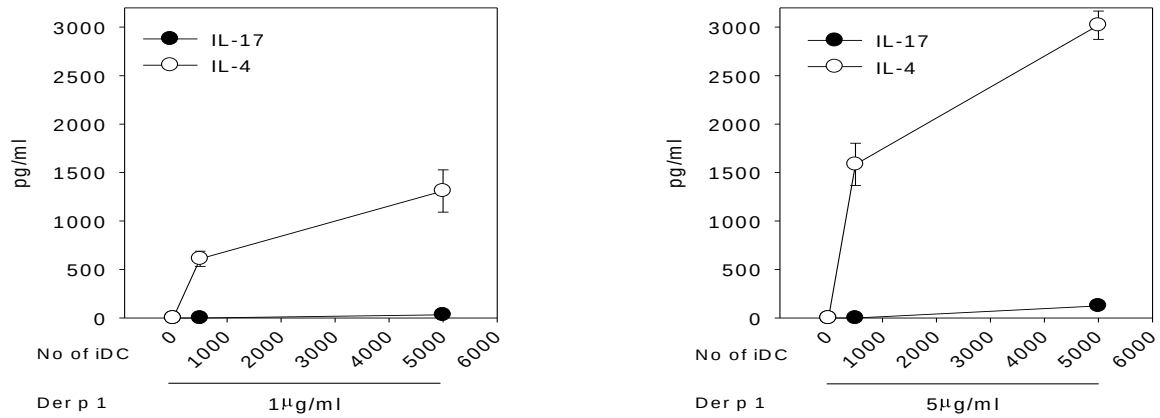


Figure 20. IL-4 secretion of Der p 1 specific Th2/IL-17 clone is dependent on allergen dose, but IL-17 secretion is not. Shown is the cytokine production of clo ne 3 (Th2/IL-17) after stimulation with increasing amounts of immature DC (x-axis) and the cognate allergen Der p 1 (panels). Error bars indicate standard error of the mean.

Th17 associated cytokines IL-1β, IL-6 and IL-23 do not increase IL-17 secretion in allergen-specific stimulated effector T cell clones

To explain the divergence between the capacity of T cell clones to produce IL-17 and the *de facto* secretion upon cognate antigen recognition, we tried to identify tissue-derived stimuli that could induce IL-17 secretion in Th2/IL-17 and Th0/IL-17 cells. In a first step, we investigated the effect of so far identified cytokines known to be involved in differentiation and maintenance of human Th17 cells. Neither addition of IL-1β, IL-6 nor IL-23 increased IL-17 secretion after stimulation with DC and Der p 1 (Figures 17-19), thus these cytokines seem unlikely to play a role in cytokine secretion of differentiated effector T cells.

Staphylococcal enterotoxin B induces high secretion of IL-17 by Der p 1-specific T cells

Since a defect in IL-17 secretion results in recurrent infections of skin and mucosal membranes (chronic mucocutaneous candidiasis, see 4.1), we investigated if

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. While 50μg/ml LPS did not alter the cytokine secretion of T cell clones, addition of 5μg/ml SEB to DC-T cell co-culture strongly promoted IL-17 release by T cells expressing SEB-sensitive TCR Vβ chains (four out of five), (Figures 17-19).

Secretion of IL-10 and of IFN-γ, but not that of IL-4, was also affected in these clones. However, increase of IL-17 secretion was by far most prominent, resulting in an increased percentage relative to other T cell cytokines in all clones examined (Figure 17 lower panel). A predominating Vβ chain was not detected in IL-17 producing T cell clones (Table 10). In line with the literature, blocking TCR by adding neutralizing antibodies against MHC class II molecules abrogated SEB stimulation almost completely (Figure 21). This finding indicates that secretion of inflammatory IL-17 by T lymphocytes is tightly regulated, and requires additional stimulation beyond cognate antigen presentation by professional APC. In AE, such hyper-stimulation could be provided by microbial-derived superantigens.

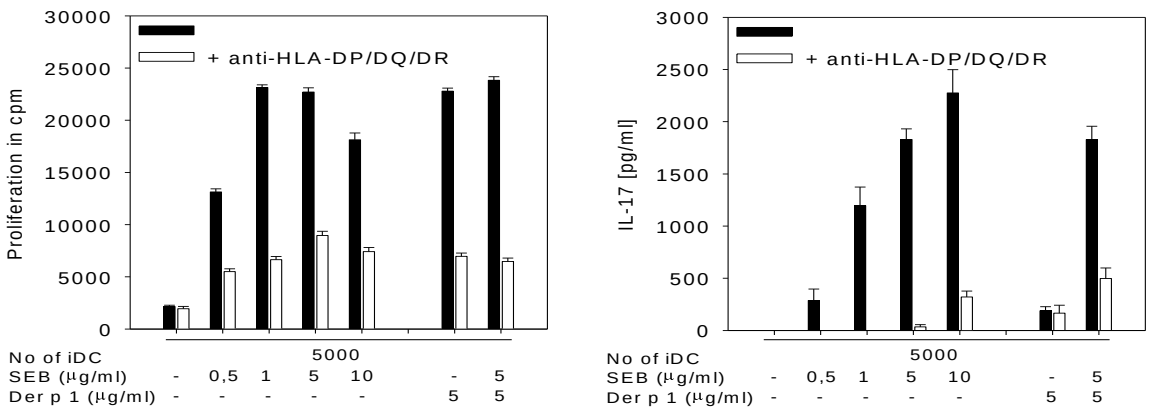


Figure 21. SEB activates T cells through a TCR dependent mechanism. Clone 3 was stimulated with increasing amounts of SEB and immature DC. Experimental endpoints were proliferation (left panel) and IL-17 secretion (right panel). Addition of anti-HLA-DP/DQ/DR almost completely abrogated SEB stimulation (white bars). Error bars indicate SEM of two independent experiments.

IL-17 strongly induces HBD-2 in vitro, but this effect is diminished in AE.

To clarify whether the ineffective upregulation of antimicrobial peptides by keratinocytes in AE is due to intrinsic defects in AE keratinocytes or due to inhibitory effects of the microenvironment, we stimulated primary keratinocytes from AE patients (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* (Figure 22). However, IL-17-induced HBD-2 upregulation was partially inhibited by addition of the Th2 cytokines IL-4 and IL-13. Accordingly, experiments performed with supernatants from APT-derived T cell clones demonstrated that neither Th2 nor Th0 could induce HBD-2 release by AE keratinocytes. Th17-derived supernatant was the most effective in HBD-2 induction, whereas the co-expression of IL-4 in the supernatant of Th2/IL-17 partially, but not completely, blocked the induction of HBD-2 release. Finally, pre-incubation of Th17 and Th2/IL-17 supernatant with a neutralizing antibody against IL-17 abrogated HBD-2 induction almost completely (Figure 22).

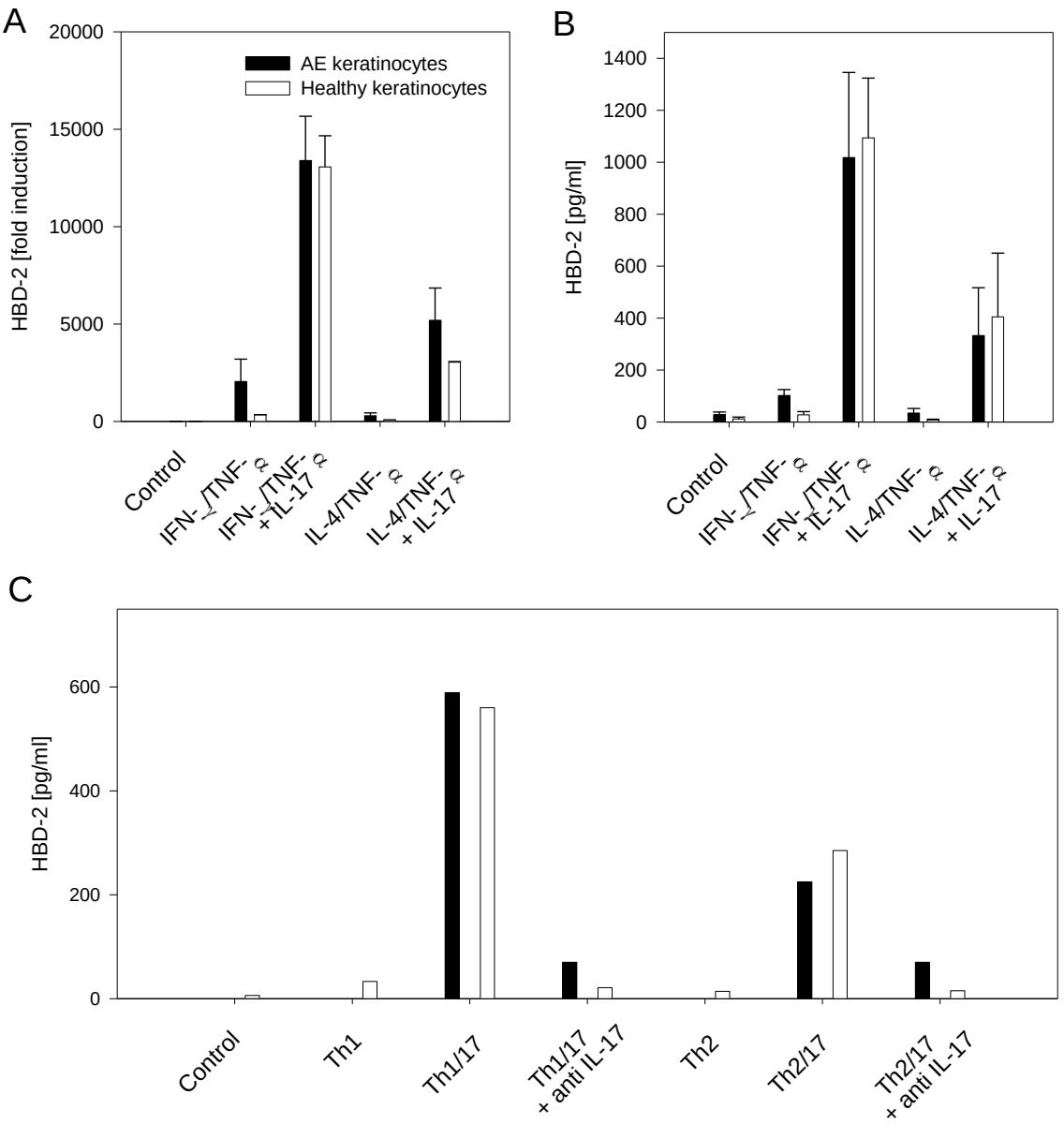


Figure 22. T cell-derived IL-17 induces β-defensin2 in primary human keratinocytes, IL-4 and IL-13 partially block this effect. Induction of HBD-2 mRNA (left panel) and protein (middle) in AE and healthy keratinocytes in response to recombinant cytokines and to cell-free supernatant obtained from stimulated T cell clones (right panel). Error bars indicate SEM of three independent experiments.

These data indicate that keratinocytes from AE patients are not hypo-responsive to IL-17, but rather the Th2-dominated microenvironment impairs important effector functions of IL-17.

SEB strongly upregulates HBD-2 mRNA and protein release in Der p-induced atopic eczema in vivo

To confirm the role of superantigens in triggering the IL-17/HBD-2 axis *in vivo*, we applied Der p on the two forearms of an AE patient. 36 hours after allergen application, we added 50μg/cm² SEB on one forearm. In concordance with previous reports[127], the clinical reaction was severely aggravated (classified as "+++++" vs. "+++" according to the European Task Force on Atopic Dermatitis [ETFAD] 2000 reading key[130]) and maintained 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 the method of suction blister and suction blister fluid was investigated for cytokine content.

In line with our *in vitro* results, IL-17 was induced more than two fold in the SEB challenged Patch Test site, while IFN-γ was unchanged. IL-4 was below detection level, and IL-10 was marginally increased (Figure 23). Consequently, HBD-2 mRNA was two fold increased in keratinocytes from SEB-exposed epidermal sheets. Increased HBD-2 concentration in the SEB-treated APT reaction was confirmed at protein level by ELISA assays performed on the blister fluids (Figure 23).

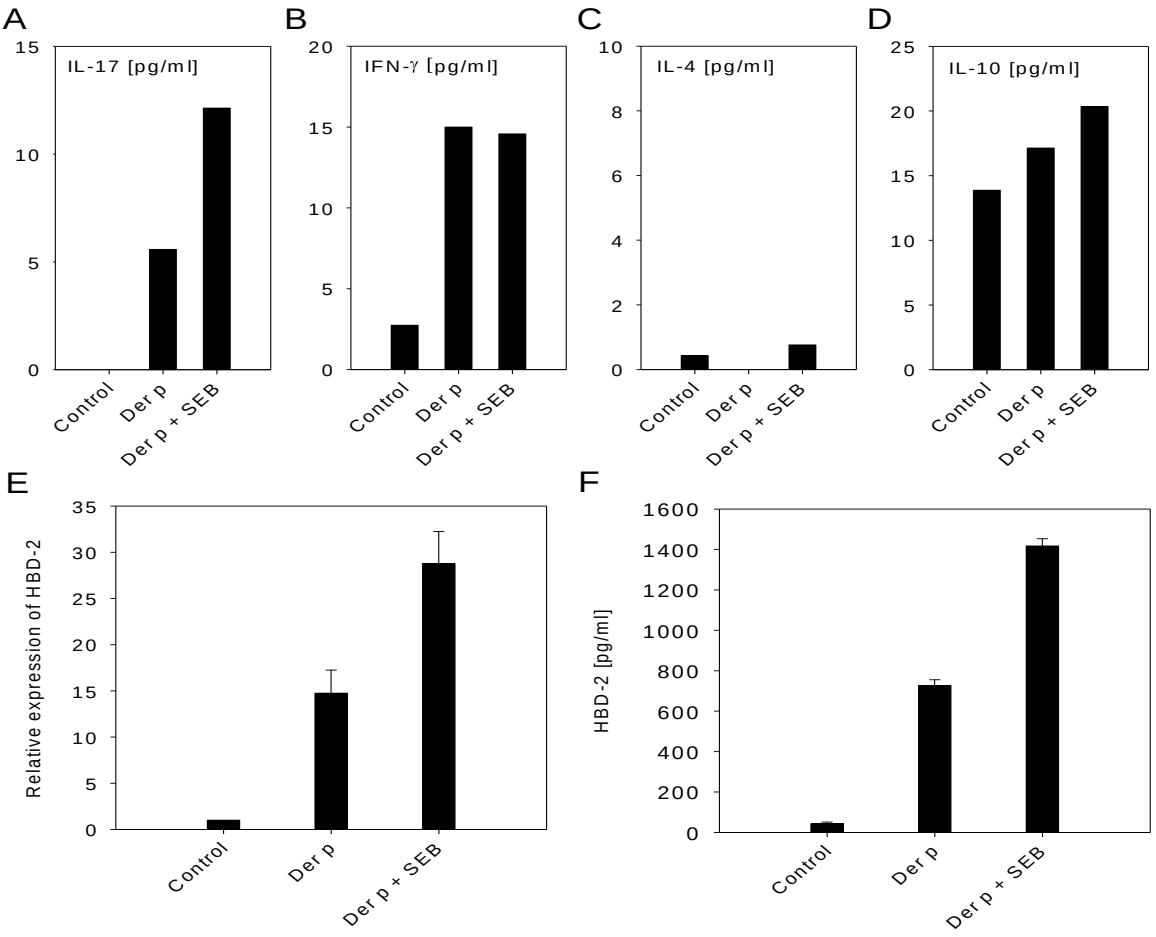


Figure 23. SEB induces expression of IL-17 and HBD-2 in vivo. Levels of IL-17 (A), IFN-γ (B), IL-4 (C) and IL-10 (D) in suction blister fluids as detected by Luminex analysis. E: relative induction of HBD-2 mRNA (E) and protein content (F). Error bars indicate SD of one experiment performed in triplicates.

These data indicate that microbial-derived products might play an essential role in inducing IL-17 *in vivo*.

5 Discussion

This work highlights an essential role for IL-17 in the first-line defence of barrier organs. It illustrates that loss of IL-17 results in chronic infections limited to skin and mucosal membranes, which is the underlying pathogenesis of the orphan human disease “chronic mucocutaneous candidiasis”. Mechanisms by which IL-17 acts are characterised in the second part of this manuscript, the description of the role of IL-17 in atopic eczema. It reveals that IL-17 is important in coordinating an effective immune response by instructing epithelial cells to an innate immune response and by recruiting immune cells to the site of infection; in atopic eczema, however, such effects are outbalanced by inhibiting effects of the Th2-dominated microenvironment, which results again in chronic infections of the skin.

5.1 CMC patients suffer from an impaired Th17 immune response

Patients suffering from CMC show a characteristic pattern of infection with the yeast Candida: while skin and mucosal membranes are heavily attacked, no systemic *Candida* infections are reported. Recent data suggest that the inability to clear *Candida albicans* in CMC patients is based upon a complex heterogeneity of immune defect(s), probably characteristic for various disease subgroups. In the first part of this thesis, a main pathogenic mechanism of CMC is elucidated – a deficiency in the production of the Th17-associated cytokines IL-17 and IL-22. Interestingly, mediators important for differentiation (IL-6 and IL-1β) and maintenance (IL-23) of the Th17 lineage were enhanced or not altered.

The role of Th17 cells in Candida *infections*

First evidence for the importance of IL-17 in clearing *Candida* infections has been provided in the mouse system: IL-17A receptor knockout mice showed a dose-dependent, substantially reduced survival in a murine model of systemic candidiasis[32] . Recent data revealed that infection with *Candida albicans* leads to an induction of murine IL-17 producing T cells[131]. Furthermore, the hyphal form of *Candida albicans* triggers IL-17 production of freshly isolated human CD4+ T cells of healthy donors *in* vitro[34].

An important role for IL-17 producing T cells in clearing *Candida* infections is confirmed by results demonstrated in this work, as PBMC of immune competent patients suffering from *Candida* infections showed a significantly higher secretion of IL-17 and IL-22 after *in vitro* stimulation with *Candida albicans* compared to healthy (non-Candida infected) donors. In concordance with previous reports[28,131,143], the source of IL-17 in PBMC was almost exclusively limited to CD4+ CCR-6+ T cells. T cell receptor specific stimulation of PBMC and stimulation of isolated CD4+ T cells with autologous monocytes showed comparable amounts of secreted IL-17, indicating that IL-17 was predominantly derived from CD4+ T cells. Flow cytometry analysis of PBMC revealed nearly all cells positive for IL-17 in intracellular staining were also CCR-6 positive.

CMC patients suffer from an impaired Th17 immune response

However, even though they had been chronically exposed to *Candida albicans*, PBMC from CMC patients secreted significantly lower amounts of IL-17 and IL-22 than PBMC from healthy donors and patients with current *Candida* infection after stimulation with *Candida albicans in vitro* - both on the mRNA and on protein level. The underlying immune defect was not specific for the stimulus *Candida*,as mitogen

stimulation (PHA) and T cell receptor specific stimulation (anti-CD3/anti-CD28) also resulted in a reduced secretion of IL-17 and IL-22 in CMC patients. This decrease was due to a strongly diminished total number of IL-17 producing T cells, as detected by surface CCR-6 and intracellular IL-17 staining of PBMC by flow cytometry. The weaker secretion of IL-22 was less pronounced than that of IL-17 as compared to immune competent patients either infected or non-infected with *Candida*. This could be explained by the fact that IL-22 production is not limited to Th17 cells, but is also produced by other activated T cell subtypes[35,132].

Th17-differentiating cytokines are not diminished in CMC patients

The so far identified differentiation factors for human IL-17 producing T cells are IL-1β and IL-6[22] , whereas IL-23 seems to be important for maintaining the production of IL-17 and IL-22 in mouse[133,134,144], but to a lesser degree in human IL-17 producing T cells[22] (see 1.1). IL-21 and TGF-β are important differentiation factors in the mouse, but their role is still controversial in humans. While IL-21 does not seem to play a role in the human system[135], the role of TGF-β is still under debate with some authors arguing it is not required for differentiation[22] and others observing a dependence of Th17 differentiation on presence of TGF-β[24]. To investigate if CMC patients lack one of these Th17-associated cytokines, we measured the induction of IL-1β, IL-6 and IL-23 after stimulation of PBMC with both specific and mitogenic stimuli. We observed a strong enhancement of IL-1β and IL-6, but not of IL-23. The secretion of these cytokines in PBMC of patients suffering from CMC was not diminished. In contrast, mRNA expression of IL-1β and IL-6 was induced much stronger in CMC patients, resulting in slightly higher release of proteins after 72 hours. This could indicate a

defect in differentiation or survival of IL-17 producing T cells downstream of IL-1β and IL-6.

Mechanisms of candidicidal activity of IL-17

Concerning the mechanism of the candidicidal effects of IL-17 producing T cells, two possible pathways could be involved in clearing infection: there is, on the one hand, the strong neutrophil recruiting capacity of IL-17 via the induction of IL-8 in human keratinocytes[36]. On the other hand, IL-17 and IL-22 synergistically induce β-defensins in human keratinocytes[38] that are able to kill *Candida albicans*[136,137].

More than the described strongly decreased levels of the tissue-instructing cytokines IL-17, IL-22, and IFN-γ, type-2 cytokines such as IL-4 and IL-10 are over-expressed in PBMC of CMC patients[77]. Importantly, these cytokines further promote an infection of epithelium with *Candida* by counteracting the effects of IL-17[138,139] regarding the induction of antimicrobial peptides (see 4.2) and failed recruitment of phagocytic cells like neutrophil granulocytes by epithelial cells (Figure 24). Taken together, a decrease in the absolute number of IL-17 producing T cells and the resulting diminished stimulation of epithelial cells[140] could explain why Candidiasis is limited to skin and mucosal membranes in CMC patients – and help to understand why they do not suffer from a systemic Candidiasis.

In summary this study underlines the importance of IL-17 producing T cells for the clearance of *Candida* infections. Furthermore our data suggest that an impaired IL-17 and IL-22 response seems to be at least in part responsible for the pathogenesis of CMC. The hypothesis that a failed immune response of “tissue-signaling leukocytes” like Th17 cells leads to chronic infections limited to skin and mucosal membranes is strengthened by a recent report on an impaired Th17 immune response in

autosomal-dominant hyper-IgE syndrome – a second orphan disease regularly associated with *Candida* infections limited to skin and mucosa[141].

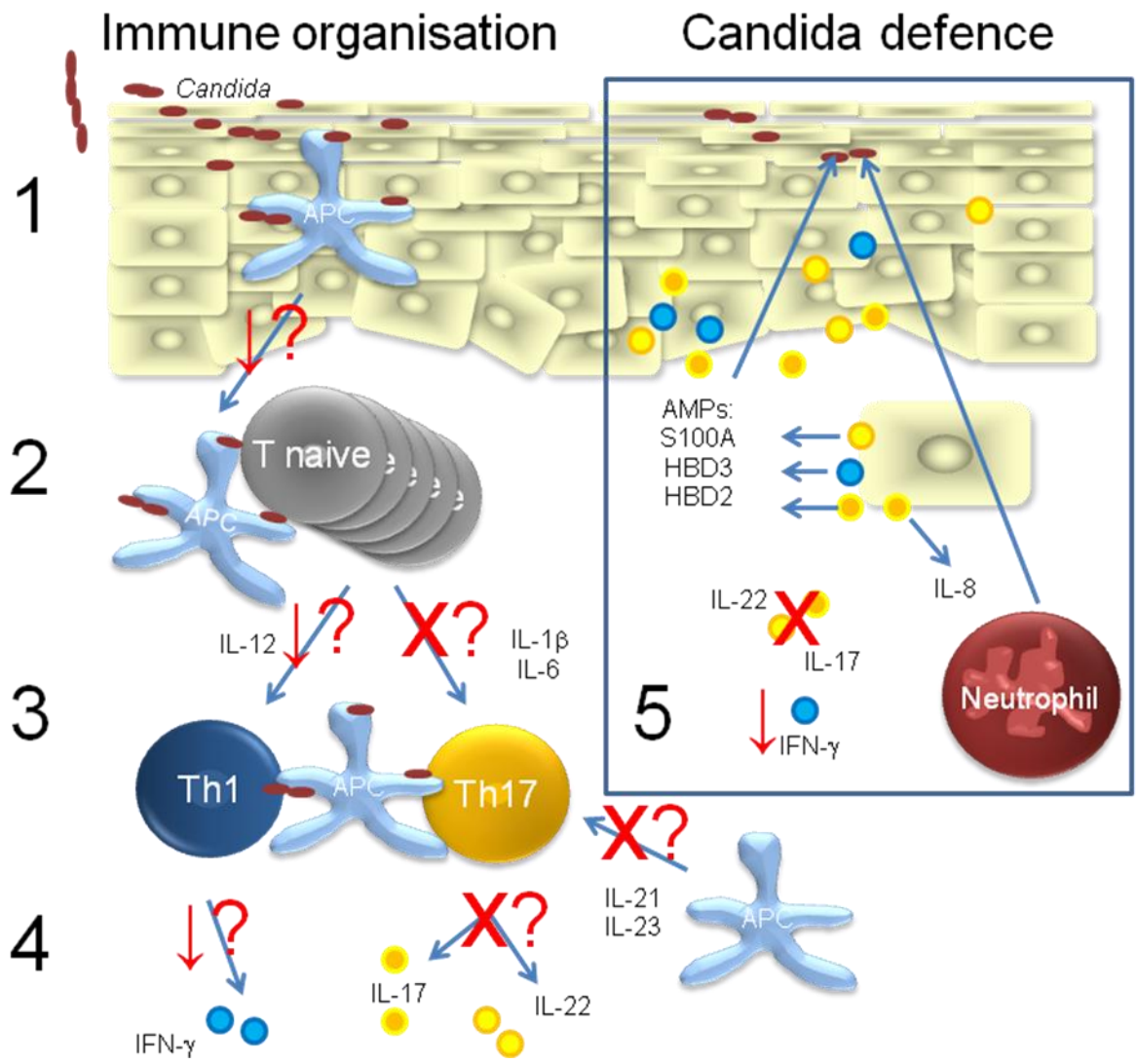


Figure 24. The pathogenesis of CMC. Upon encounter of Candida, antigen presenting cells take up and process antigens (1), then migrate to regional lymph nodes and present them to specific naive T cells (2), which undergo clonal expansion and differentiation towards memory effector cells mainly of the Th1 or Th17 phenotype (3). A second stimulation of T cells with Candida leads to secretion of IFN-γ or IL-17 and IL-22, respectively (4). These cytokines induce secretion of antimicrobial peptides (AMPs) and neutrophil-recruiting IL-8 in epithelial cells (5). Lack of IL-17 and IL-22 and diminished IFN-γ results in CMC. The potential defects in this cascade underlying CMC are marked with red "X" or "↓".

5.2 The IL-17 mediated host defence is partially impaired in AE patients

Our results on the pathogenesis of “chronic mucocutaneous candidiasis” suggest a central role of IL-17 in encompassing host defence against microorganisms at surface barriers. This led us to investigate the role of IL-17 in atopic eczema (AE), since almost all AE patients suffer from a chronic skin-colonisation with the bacterium *Staphyloccocus aureus*. Furthermore, disease severity in AE positively correlates with the densitiy of *Staphylococcus aureus* on the skin. This observation led us to investigate IL-17 in the pathogenesis of AE.

IL-17 producing T cell populations infiltrating AE lesions

In a first step, 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 type-2 cytokines abundantly present in AE skin microenvironment.

By isolating and characterising the lymphocytic infiltrate in APT reactions, we directly demonstrate 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 PCR in AE skin[142]. When we further characterised skin infiltrating T cells, we identified distinct subpopulations of IL-17 producing T cell clones: besides the previously published pure Th17 and Th1/IL-17 cells that co-express IFN-γ[36,143], a newly described population coproducing IL-17 and type 2 cytokines was classified as Th2/IL-17.

Secretion of IL-17 in T cells is tightly regulated

While in the APT lesion no Der p 1 specific Th17 cells were found on clonal level, we were surprised to observe that stimulation of Der p 1 specific Th2/IL-17 and Th0/IL-17 cells with their cognate antigen resulted in a strong induction of proliferation and of IL-4 secretion, but IL-17 was poorly secreted. Neither Th17 differentiating cytokines IL-1β and IL-6[22] nor IL-23, described to maintain survival and cytokine secretion of mouse[144], but to a lesser degree also of human Th17 cells[22], strongly increased secretion of IL-17 in our differentiated effector T cells stimulated with DC and their cognate antigen.

In order to find an *in vivo* stimulus for substantial IL-17 secretion, we investigated whether microbial derived products could be adequate stimuli. Consensus exists that *Staph. aureus* colonisation of AE skin significantly aggravates intensity and accounts for persistence of eczematous reactions[124,125], however the mechanisms remain unclear. Under natural exposure conditions, *Staph. aureus* derived superantigens, like SEB, could contribute to the amplification of the inflammatory reaction by stimulating infiltrating T cells bearing particular T cell receptor Vβ chains[125,126]. Indeed, when we stimulated SEB-sensitive Th2/IL-17 and Th0/IL-17 clones with SEB, secretion of proinflammatory IFN-γ, but especially of IL-17, was strongly enhanced compared to cognate TCR triggering alone both *in vitro* and *in vivo*. Thus, our data underline a role of the microenvironment in triggering full effector functions of tissue-infiltrating T cells.

The role of the local microenvironment for the induction of HBD-2 in keratinocytes

However, despite availability of SEB-triggered IL-17, which is a very efficient stimulus for HBD-2[38], expression of HBD-2 in AE was reported to be diminished in comparison to Th1-mediated skin immune diseases, such as psoriasis[123]. We therefore

investigated whether AE keratinocytes show an intrinsic defect in responding to IL-17 or whether co-expressed type 2 cytokines could account for the diminished HBD-2 induction, as reported for the IFN-γ and TNF-α induced expression of antimicrobial peptides[145,146]. We found that AE keratinocytes are not hypo-responsive 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 colonisation of AE skin with *Staph. aureus* that represents a continuous trigger of cutaneous inflammation.

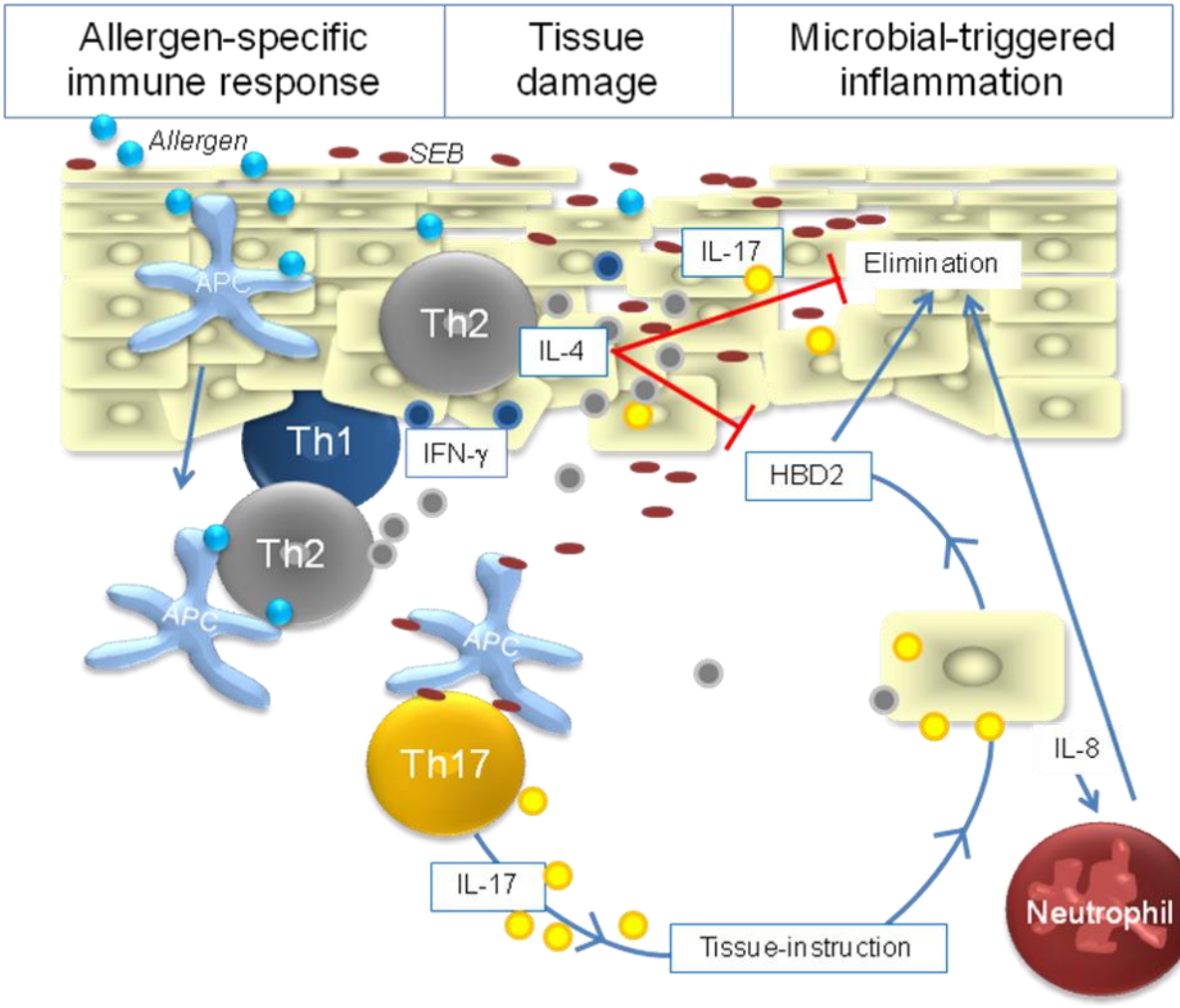


Figure 25. A new concept for the pathogenesis of AE. An initial allergen-specific Th2-dominated immune response causes epidermal barrier disruption and consequent triggering of an IL-17 immune response by microbial-derived substances. IL-17, however, does not signal effectively to epithelial cells due to inhibitory effects of the type 2-dominated microenvironment.

A new concept on the pathogenesis of atopic eczema

Atopic eczema is a common inflammatory skin disorder based upon complex pathogenic mechanisms that altogether result in a disturbed epidermal barrier and cutaneous hyper-inflammation. Our *in vivo* and *in vitro* observations demonstrate that IL-17 is involved in a previously unknown pro-inflammatory *vicious circle* contributing to both epidermal barrier damage and hyper-inflamed skin (Figure 25).

IL-17 producing T cells are recruited into inflamed AE skin, mainly as Th2/IL-17 and Th0/IL-17 subsets. Cognate antigen recognition of causative allergens (e.g. house dust mite) by infiltrating T cells strongly upregulates Th2 cytokines, which dominate the early phase of AE[147], but only marginally regulates IL-17 secretion. Tissue-derived additional stimuli are required to arm IL-17 releasing T cells. A prerequisite for such a strong stimulation is the loss of the epidermal barrier integrity that builds up the physical and chemical protection against the environment. In the case of AE, the epidermal barrier is altered due to predisposing mutations even in steady state. During an immune reaction in the skin, this barrier is further damaged by two independent cascades: first, the abundantly present type 2 cytokines (IL-4, IL-13) downregulate genes important for building an intact epidermal barrier (e.g. filaggrin)[148]. Interestingly, filaggrin deficiency in turn promotes a Th17 immune response[149]. Second, the immune reaction in the skin results in keratinocyte apoptosis[150] and early disruption of E-cadherins[151]. Both events lead to a complete loss of epidermal integrity and thus to contact of resident and recruited immune cells with microorganisms (*Staph. aureus*) that are colonising the skin of most AE patients.

A second inflammatory wave is now initiated, since *Staph. aureus*-derived superantigens (e.g. SEB) effectively induces IL-17 production in capable T cell clones and thereby initiates the IL-17/HBD-2 axis in the skin. Whereas this

mechanism may occur as a natural protective function of IL-17, the IL-17/HBD-2 axis is only marginally effective in AE skin, due to inhibitory effects of Th2 associated cytokines in AE microenvironment[145,146]. The incomplete clearance of microbial-derived triggers leads to a *vicious circle* responsible for the continuous release of pro-inflammatory IL-17 and other T cell cytokines and persistence of the eczematous reaction.

6 Summary

This manuscript demonstrates that IL-17 is crucial in the first-line defence of the human organism by identifying an absent or impaired IL-17 signaling cascade in two human diseases characterised by recurrent infections limited to skin and mucosal membranes.

In the first part we identify the main immune defect in the orphan syndrome “chronic mucocutaneous candidiasis” is an impaired Th17 immune response. CMC patients suffer from a remarkable decrease in IL-17 and IL-22 producing leukocytes, while a specific stimulation with the disease-relevant yeast Candida albicans caused a massive production of IL-17 and IL-22 in healthy volunteers and even more in immune competent Candida-infected patients. The underlying mechanisms remains unknown, a mutation in a Th17-differentiating gene, however, seems unlikely based on the data provided in this thesis.

Secondly, we investigated another disease commonly associated with limited skin infections – atopic eczema (AE). Almost 100% of AE patients suffer from a disease-relevant skin colonisation with *Staphylococcus aureus*, most likely due to a diminished production of (IL-17 triggered) defensins. The current study demonstrates that IL-17 producing T cells infiltrate acute AE reactions, in particular the newly characterized subtype Th2/IL-17 cells. Interestingly, T cells challenged with their cognate allergen do not produce IL-17 *in vitro*; but IL-17 secretion can be triggered by *Staphylococcal*-derived enterotoxins (SEB) that are frequently present on AE skin. To analyse why IL-17 can be efficiently triggered in AE, but yet it´s main function (the induction of defensins in keratinocytes) is diminished, primary human keratinocytes from AE patients and healthy volunteers were stimulated with natural and

recombinant IL-17. It could be shown that the inefficient defensin induction is not due to an intrinsic defect in AE keratinocytes, but rather due to counteracting effects of Th2 cytokines like IL-4 and IL-13.

This manuscript provides insights in the importance of the skin microenvironment for the outcome of (T cell) mediated immune responses. Furthermore, it is of clinical relevance as 1.) the description of an impaired Th17 immune response in CMC patients could result in specific therapeutic approaches and 2.) it underlines the importance of early and consequent therapy of AE skin lesions to avoid insufficient and persistent triggering of the IL-17/HBD-2 axis in ongoing acute AE.

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9 Curriculum Vitae

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June 2006 – May 2007:	Intern at the Department of Dermatology and Allergology „Biederstein", TU Munich

Education:

Since October 2006:	PhD-Study course „Medical life science and technology", TU Munich
April 2006:	**University graduation,** **Grade: 1,83 (best 10%)**
October 2001 – April 2006:	Medical studies, „Klinikum Rechts der Isar", TU Munich
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1990 – 1999:	Theodor-Heuss-Gymnasium, Freiburg/ St. Georgen
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Doctoral thesis:

March 2007:	**Granting of a doctorate by the faculty of medicine, TU Munich,** **Grade: Summa cum laude (1,0; top grade)** „Mechanisms of allergic eczematous reactions" ZAUM – Center for Allergy and Environment, GSF/TUM PD C. Traidl-Hoffmann/ Prof. H. Behrendt

Temporary employments abroad:

January 2009:	Visiting scientist at the Imperial College London, UK
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October – December 2005:	Part of "PJ" (last year of medical studies), Concord Hospital, University of Sydney, Australia
July – August 2003:	Elective (surgery), Hopital Bethesda, Agou-Nyogbo, Togo

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June 2007 – May 2008:	Grant for a postdoktoral time by the BFS (Bayerische Forschungsstiftung) for project: „Analysis of the immunoregulatory functions of keratinocytes in delayed time hypersensitivity responses"
May 2007:	Grant of the DDG (Deutsche Dermatologische Gesellschaft) for project: „Comparison of effector phase of allergic eczematous reactions to pollen and Nickel by i*n situ* Topo-Proteom analysis"
June 2003:	Congress grant "Chiron project" by the EAACI (European Academy for Allergology and Clinical Immunology)

10 Appendix

Data shown in Figure 8:

PCR

IL17

CA	CMC		Healthy IC		IC Cand
MF	2,451	AR	6,766	DU	25,157
	0,081		60,969		
BC	6,821	SF	25,049		
	2,645	GP	10,149		
MS	1,396	SL	0,930		
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
2,68	1,13144	20,77	10,80923	25,16	0

PHA	CMC		Healthy IC		IC Cand
MF	0,006	AR	115,894	DU	222,290
	31,341		714,109		
BC	4,228	SF	195,361		
	14,757	GP	2,812		
	1,244	SL	5,566		
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
10,32	5,86278	206,75	131,8831	222,29	0

PCR

IL22

CA	CMC		Healthy IC		IC Cand
MF	30,555	AR	695,384	DU	1409,674
	568,100		3476,291		
BC	39,397	SF	3019,303		
	465,725	GP	44,529		
MS		SL			
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
275,94	125,842	1808,88	757,1083	1409,67	0

PHA	CMC		Healthy IC		IC Cand
MF	134,986	AR	311,553	DU	1057,149
	130,690		1588,372		
BC	148,742	SF	1884,544		
	112,725	GP	11,340		
		SL			
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
131,79	6,64458	948,95	413,8909	1057,15	0

Data shown in Figure 9:

ELISA

IL-22

CA	CMC		Healthy IC		IC Cand
MF	0,00	SF	1996,15	DU	3413,71
	46,28		936,03		1876,37
MS	414,91	SG	2338,18	WH	3745,12
	50,86	CTH	1493,15	LK	1292,36
BC	133,61	SK	2922,18	SB	776,56
	16,43	DC217	3495,78	JS	1527,94
MG	2230,77	KE	1516,41	EM	1693,10
		Kö	1665,75		
		SL	291,89		
		XX	1991,16		
		AR	1796,47		
		GP	1431,09		
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
110,35	63,7549	1822,85	243,754	2046,45	418,58

PHA	CMC		Healthy IC		IC Cand
MF	10,10	SF	1651,55	DU	1018,41
	97,28		640,07		995,84
MS	26,59	SG	1932,86	WH	1480,84
	10,23	CTH	121,07	LK	901,16
BC	21,90	SK	1751,29	SB	343,99
	72,26	DC217	1170,71	JS	1121,72
MG	2213,74	KE	1006,33	EM	1412,59

		Kö	1334,38		
		SL	396,95		
		XX	1087,39		
		AR	1312,69		
		GP	706,60		
mean CMC	SEM	mean ctrl	SEM	mean C	SEM
49,83	14,842	1092,66	159,411	1039,22	141,922

Data shown in Figure 10:

anti-CD3 PCR

IL-17	CMC		Healthy IC		IC Cand
MF	0,293	SF		DU	10,429
MS	0,540	SG		WH	
		CTH		LK	
BC	1,640	SK		SB	
		DC217		JS	
MG		KE		EM	
		Kö			
		SL	1,811		
		XX	3,088		
		AR	26,785		
		GP	0,751		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
1,12	0,29261	8,11	3,604774	10,43	0

IL-22	CMC		Healthy IC		IC Cand
MF	27,221	SF		DU	211,224
MS		SG		WH	
		CTH		LK	
BC	54,317	SK		SB	
		DC217		JS	
MG		KE		EM	
		Kö			
		SL	450,00		
		XX	0,727		

		AR	444,180		
		GP	8,244		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
67,99	7,82186	225,79	73,77591	211,22	0

anti-CD3 ELISA

IL-17	CMC		Healthy IC		IC Cand
MF	16,22	SF		DU	174,459
	0,00				602,36
MS	91,53	SG		WH	
	0,00	CTH		LK	
BC	2,83	SK	186,145	SB	
		DC217	419,15	JS	
MG		KE	30,914	EM	
		Kö	231,33		
		SL	11,981		
		XX	311,52		
		AR	277,055		
		GP	923,15		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
38,34	16,0777	298,91	82,82284	388,41	114,3619

IL-22	CMC		Healthy IC		IC Cand
MF	5,97	SF		DU	403,807
	18,96				1.431,29
MS	31,11	SG		WH	
	10,356	CTH		LK	
BC	26,13	SK	666,730	SB	
		DC217	1.326,35	JS	
MG		KE	126,831	EM	
		Kö	573,18		
		SL	154,012		
		XX	1000,00		
		AR	972,827		
		GP	1.967,17		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
24,47	4,28559	848,39	176,9493	917,55	274,6069

Data shown in Figure 13:

PCR

IL-1β

CA	CMC		Healthy IC		IC Cand
MF	41,070	AR	8,196	DU	2,099
	691,379		6,409		
BC	6,612	SF	27,096		
	61,963	GP	0,920		
MS	4,248	SL	5,709		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
161,05	133,023	9,67	4,520588	2,10	0

PHA	CMC		Healthy IC		IC Cand
MF	17,348	AR	6,704	DU	1,414
	155,776		3,498		
BC	3,568	SF	12,182		
	25,753	GP	0,273		
MS	2,351	SL	1,307		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
40,96	29,0348	4,79	2,150661	1,41	0

PCR

IL-6

CA	CMC		Kontrollen		Candidose
MF	135,298	AR	1,608	DU	58,283
	692,978		22,213		
BC	27,954	SF	72,505		
	167,730	GP	2,630		
MS	29,926	SL	16,186		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
210,78	123,733	23,03	12,98137	58,28	0

PHA	CMC		Healthy IC		IC Cand
MF	31,963	AR	0,478	DU	12,084

	67,806		5,979		
BC	7,210	SF	9,514		
	34,060	GP	0,290		
MS	6,596	SL	2,311		

mean CMC	SEM	mean ctrl	SEM	mean C	SEM
29,53	11,2152	3,71	1,773862	12,08	0

Data shown in Figure 17:

Dose response clone 3

pg/ml	IL-4	SEM	IL-17	SEM
5000 w/o Der	0	0	2	0
100	0	0	1124	218
1000	0	0	1313	146
5000	152	9,5	2798	298
10000	296	28,5	2998	258,5

IFN-γ	2798	298	152	3,5
IL-23	2642	177	355	8
IL-6	2767	127	254	2
IL-1β	2844	261	389	8,5

Der + SEB	2519	182	1100	17,5
SEB	2538	154,5	1150	36

Cytokine distribution in %

	Der p 1	Der+SEB		
IL-17	1,4543	10,4236		
IFN-γ	0	0		
IL-4	26,77	23,87		
IL-10	6,2189	6,9554		
IL-13	26,9582	29,1797		
IL-22	23,1152	16,0997		
TNF-α	15,4835	13,4717		

Dose response clone 96

pg/ml	IL-4	SEM	IL-17	SEM	IFN-γ	SEM
5000 w/o Der	0	0	0	0	0	0
100	2187	209	0	0	357	54
1000	2470	125	0	0	819	213
5000	3245	265	122	8	1675	278
10000	3326	50	236	6	1819	289
IFN-γ	3245	265	122	8	1675	278
IL-23	3113	73	255	2	1613	275
IL-6	3219	134	218	3	1358	226
IL-1β	3150	100	296	3	1625	281
Der + SEB	3391	81	950	15	2975	315
SEB	3208	82	975	23	3026	327

Cytokine distribution in %

	Der p 1	Der+SEB		
IL-17	0,4646	2,9196		
IFN-γ	6,3788	9,143		
IL-4	12,3578	10,4214		
IL-10	12,1826	11,9796		
IL-13	30,7644	31,3811		
IL-22	28,2231	25,9845		
TNF-α	9,6286	8,1708		

Data shown in Figure 18:

Dose response clone 3

pg/ml	IL-4	SEM	IL-17	SEM
5000 w/o Der	0	0	2	0
100	0	0	8	3
1000	0	0	122,5	35,5
5000	69	9,5	914	81
10000	128	28,5	1483	26,5
IFN-γ	783,5	10,5	89	5
IL-23	1434	75	145	15
IL-6	1406,5	79,5	110	12
IL-1β	1506	98	123	12
Der + SEB	2116,5	140,5	1240	26
SEB	2086	80,5	1162	54

Dose response clone 96

pg/ml	IL-4	SEM	IL-17	SEM	IFN-γ	SEM
5000 w/o Der	0	0	0	0	0	0
100	1187	109	0	0	357	54
1000	1470	125	0	0	719	213
5000	1745	165	92	8	1030	278
10000	1826	50	136	6	1275	289
IFN-γ	1745	165	92	8	1030	278
IL-23	1713	73	155	2	1113	275
IL-6	1799	134	135	3	1158	226
IL-1β	1850	100	156	3	1156	281
Der + SEB	2193	81	725	15	1975	215
SEB	2109	82	790	23	1896	227

Data shown in Figure 19:

Dose response clone 3

pg/ml	IL-4	SEM	IL-17	SEM
5000 w/o Der	0	0	2	0
100	0	0	8	3
1000	0	0	640	35,5
5000	0	0	1133	65
10000	39	12,1	1512	26,5
IFN-γ	1323	10,5	12	5
IL-23	1465	75	45	15
IL-6	1555	79,5	42	12
IL-1β	1613	98	55	12
Der + SEB	1876	140,5	675	26
SEB	1912	80,5	613	54

Dose response clone 96

pg/ml	IL-4	SEM	IL-17	SEM	IFN-γ	SEM
5000 w/o Der	0	0	0	0	0	0
100	536	109	0	0	0	0
1000	1639	125	0	0	0	0
5000	1799	165	0	0	635	178
10000	1913	50	25	6	875	189
IFN-γ	1799	155	0	0	635	178
IL-23	1639	73	46	2	1113	275
IL-6	1800	134	15	3	1158	226
IL-1β	1825	100	35	3	1156	281
Der + SEB	2295	81	375	45	1460	45
SEB	2170	82	476	63	1530	63

Data shown in Figure 20:

Dose response clone 3

pg/ml	IL-4	SEM	IL-17	SEM
1 μg/ml Der p 1				
0	0	0	0	0
500	0	0	610	78
5000	32	12	1310	218
5 μg/ml Der p 1				
0	0	0	0	0
500	0	0	1584	218
5000	124	24	3020	146

Data shown in Figure 21:

	cpm	SEM	cpm aMHC	SEM
Control	2148	134	1936	223
0,5μg/ml SEB	13124	301	5502	267
1μg/ml SEB	23138	245	6646	301
5μg/ml SEB	22691	413	8954	405
10μg/ml SEB	18119	656	7410	401
5μg/ml SEB	22783	289	6960	312
Der p 1 + SEB	23832	337	6476	322

	IL-17	SEM	aMHC IL-17	SEM
Control	0	0	0	0
0,5μg/ml SEB	287	110	0	0
1μg/ml SEB	1196	178	0	0
5μg/ml SEB	1830	101	34	22
10μg/ml SEB	2276	223	321	56
5μg/ml SEB	189	39	166	76
Der p 1 + SEB	1828	129	498	101

Data shown in Figure 22:

fold induction	HBD-2	SEM
Control	1,75	0,67
Ctrl AE	3,4933	1,7809
IFN-γ/TNF-α	337,3	7,6
IFN-γ/TNF-α AE	2050,73	1143,84
IFN/TNF/IL17	13064,5	1599,5
IFN/TNF/IL17 AE	13394,7	2268,59
IL4/TNF-α	74,95	0,95
IL4/TNF-α AE	290,3	150,063
IL4/TNF/IL17	3040,5	31,5
IL4/TNF/IL17 AE	5192	1656,29

pg/ml	HBD-2	SEM
Control	1,75	0,67
Ctrl AE	3,4933	1,7809
IFN-γ/TNF-α	337,3	7,6
IFN-γ/TNF-α AE	2050,73	1143,839
IFN/TNF/IL17	13064,5	1599,5
IFN/TNF/IL17 AE	13394,7	2268,5887
IL4/TNF-α	74,95	0,95
IL4/TNF-α AE	290,3	150,0629
IL4/TNF/IL17	3040,5	31,5
IL4/TNF/IL17 AE	5192	1656,2931

pg/ml	HBD-2	SEM
Control	0	6
Th1	0	33
Th1/17	589	560
Th1/17 a17	70	21
Th2	0	14
Th2/17	225	285
Th2/17 a17	70	15

Data shown in Figure 23:

	IL-4	IFN-γ	IL-10	IL-17
Control	0,43	2,74	13,87	0
Der p	0	15	17,14	5,58
Der p + SEB	0,76	14,57	20,34	12,13
HBD-2	PCR	SEM	ELISA	SEM
Control	1	0	43	8
Der p	14,75	2,5	727	28

Der p + SEB	28,74	3,5	1417	36

Cytokine profile of skin-derived clones:

Clone no	IFN-γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF-α	Subtype
1	0	153	1928	4576	0	145	676	Th2
2	3229	1	498	699	3704	1686	1561	Th1/17
3	0	598	237	2598	0	44	278	Th2
4	0	807	3670	7380	0	8776	1459	Th2
5	21	1	480	947	3405	18	694	Th17
6	0	245	237	8666	0	27	385	Th2
7	4068	80	1167	1456	903	60	1851	Th1/17
8	0	112	304	22905	0	102	1062	Th2
9	5	255	4933	15538	0	194	1101	Th2
10	2577	6	1189	2777	1137	86	985	Th1/17
11	28	0	3157	174	4551	7499	3558	Th17
12	0	470	6008	18312	0	9835	352	Th2
13	12468 4325	0 0	273 74	1456	0 0	21 0	2746 5237	Th1
14	0	110	533	11738	0	2325	1140	Th2
15	1507	3	3358	0	0	18	975	Th1
16	0	1	1663	53	240	7332	2062	Th22
17	1444	1	1545	0	2799	2413	2515	Th1/17
18	0	44	132	1774	0	63	132	Th2
19	0 0	895 2017	487 515	3745	0 0	18 0	161 1083	Th2
20	0	44	2585	15437	0	2562	1258	Th22
21	0	1	1367	174	0	158	222	
22	0	1	329	249	191	207	246	Th17
23	2683	1	766	510	0	50	1419	Th1
24	150 0	357 858	810 568	1228	4255 2526	83 0	1348 1886	Th2/17
25	1	1	2192	1257	2824	816	946	Th17
26	3681	6	1172	1003	0	31	2385	Th1
27	0 0	1 1	526 2214	947	4596 8306	865 377	532 5432	Th17
29	0 0	221 2282	326 923	25817	0 0	1044 229	1348 9357	Th2
30	0 0	22 168	169 475	11672	16 0	9574 11909	1160 6527	Th22
31	188 201	0 0	837 1052	0	5095 8016	63 0	3478 12268	Th17
32	0	163	3042	15135	0	7922	1278	Th2

33	2127	0	515	0	2004	34	1052	Th1/17
34	10	1211	2238	14498	0	3638	1520	Th2
35	150	6	674	9575	1691	946	3858	Th17
No	IFN-γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF-α	Subtype
36	429	3	477	100	58	138	1551	Th1/17
37	0	35	1349	6901	0	513	676	Th2
38	0	9	677	3745	52	8557	1378	Th22
40	0	9	2448	484	0	5586	1004	Th22
41	0	11	849	4669	0	4622	1120	Th22
42	1665	1	1522	0	7	3638	1062	Th1
43	0	1174	5553	26409	0	9463	1130	Th2
44	326	6	1150	1031	0	138	956	Th1
45	0	1	1398	618	1112	4859	1935	Th17
46	0 0	1088 1576	237 296	14297	0 0	24 0	176 488	Th2
47	208	0	1251	0	555	1911	2935	Th1/17
48	8222	0	1340	0	0	14	2461	Th1
49	96	0	415	0	2498	3162	1592	Th17
50	7678	2	5088	0	0	60	4932	Th1
51	842	0	349	0	550	275	1248	Th1/17
52	0	285	140	3929	0	27	154	Th2
53	0	785	874	5323	0	728	1298	Th2
54	0	325	621	13663	0	1507	558	Th2
56	406 97	63 734	10523 2486	2598	0 6604	106 0	1883 8088	Tr1
57	293	1	837	726	0	1064	2768	Th1
58	746	0	1308	0	0	21	851	Th1
59	0	525	2553	17530	0	409	1428	Th2
60	0	312	857	12997	0	4827	748	Th2
61	0	503	432	6552	0	917	730	Th2
62	0	578	6177	9347	0	10121	2019	Th2
63	0	652	1176	3441	0	366	411	Th2
64	0	154	1043	12036	301	5087	1923	Th2/17
65	0	59	355	18890	0	2230	1705	Th2
66	2701	24	5783	3654	0	2979	2362	Th1
67	0	0	3330	4886	0	285	302	Tr1
68	0 0	362 2588	8519 5412	5668	195 180	168 0	870 4808	Th2/17
69	0	123	112	2360	0	0	103	Th2
70	0	883	3392	27596	0	1135	1726	Th2
71	0	3	4518	1745	227	7867	411	Th22
72	0	1998	2916	40930	0	7938	814	Th2
73	13658	0	1467	53	0	24	2019	Th1

	5065	0	636		0	0	9468	Th1
74	1039	0	2987	174	1696	18	1460	Th1/17
75	250	0	1658	0	4861	451	2924	Th17
	1206	0	883		9044	0	7463	
No	IFN-γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF-α	Subtype
76	0	991	2516	28541	22	624	585	Th2
77	0	689	2076	18312	0	6997	1308	Th2
78	2076	1	1541	0	0	8	1882	Th1
79	0	481	2319	20668	0	60	453	Th2
80	3643	486	7186	12333	0	4664	5780	Th0
81	667	1	507	516	3579	5624	3972	Th17
84	748	2	1189	1043	0	1012	1449	Th1
85	1337	1263	5420	11160	0	1047	1736	Th0
86	0	955	1113	17936	0	1751	1839	Th2
87	0	132	1069	10093	228	173	1663	Th2/17
88	0	33	3016	19740	0	10439	1571	Th22
	0	0	1081		0	18784	8284	
89	0	1436	3111	23241	0	7000	702	Th2
90	0	1295	294	21136	0	746	1018	Th2
91	0	1084	4933	18317	173	7000	1540	Th2/17
	0	1498	4907		0	3944	6065	
92	0	544	62	2937	0	10079	739	Th2
93	0	1	655	0	0	14	351	Th2
94	18	3	5244	408	2194	7295	1066	Th17
96	0	2	1316	226	0	2378	241	Th2
97	0	6	1457	1214	0	3048	748	Th22
99	0	913	2696	6555	0	3592	181	Th2
100	0	91	846	4016	0	1328	914	Th2
101	0	1	405	31	4024	6147	1746	Th17
	742	0	838		9560	11215	13002	
102	0	180	810	20472	0	2923	1037	Th2
103	687	7	810	570	0	24	319	Th1
104	3162	1	409	303	1798	104	2039	Th1/17
105	10060	1	971	355	0	24	2768	Th1
	2998	7	272		5	0	9280	
106	2473	1	165	735	0	4	867	Th1
107	1763	1	708	902	4051	28	640	Th1/17
108	647	1650	6636	10393	0	153	578	Th0
109	0	7	2469	1737	1453	7898	1143	Th17
110	0	145	1382	7170	0	9177	1591	Th22
111	0	0	2061	150	3262	87	160	Th17
112	0	1589	336	19392	162	14	319	Th2/17
113	0	1	3360	101	4496	1162	1591	Th17
114	0	920	1972	14640	0	3637	1162	Th2

115	2026	2	3415	382	35	205	3356	Th1
116	0	1176	1672	9331	0	1217	1829	Th2
117	0	86	419	1767	0	860	535	Th2
118	0	1458	4844	13688	0	1616	1369	Th2
No	IFN-γ	IL-4	IL-10	IL-13	IL-17	IL-22	TNF-α	Subtype
119	0	826	1972	13959	0	4043	248	Th2
120	0	5	3153	0	0	8910	475	Th22
121	0	546	1448	13688	0	662	587	Th2
122	0	11	3956	382	0	499	335	Tr1
123	0	2413	996	22222	656	8002	729	Th2/17
	0	2585	611		525	575	1210	
124	0	1	175	0	735	11	72	Th17
125	0	428	1515	8870	0	14	152	Th2
	0	2032	531		0	0	1142	
126	7646	6	7522	5243	0	1626	3831	Th1
127	0	1635	380	21941	0	329	272	Th2
	0	3432	481		0	0	1184	
129	0	0	618	0	0	1158	311	Th22
130	5658	10	53	0	984	1505	475	Th1/17
	4562	186	296		14	0	6951	
131	3446	1	830	77	11	101	1339	Th1
132	0	1207	759	13010	0	14	47	Th2
133	0	467	2987	15357	0	4906	702	Th2
134	9350	2	1035	0	4169	9021	7233	Th1/17
	9928	1	668		7329	18745	22612	
137	0	832	1157	2603	0	17	72	Th2
139	0	2465	4124	24334	270	1002	631	Th2/17
	0	3010	1119		837	0	2660	
140	0	2130	521	16454	0	2001	241	Th2
141	0	1570	383	20472	0	73	287	Th2
	0	2962	2025		0	0	1116	
142	0	667	1122	101	0	52	47	Th2
143	0	1363	7569	12167	0	3117	711	Th2