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Differential regulation of T cell responses by pathogenic and apathogenic microorganisms

Julia Hiller

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Abbreviations

AhR	aryl hydrocarbon receptor
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS1	autoimmune polyendocrine type I syndrome
ASA	allogenic stimulation assay
ATP	adenosine 5'-triphosphat
Bcl-10	B cell lymphoma 10
BM	bone marrow
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphat
CARD9	caspase-recruitment domain family, member 9
CBP	histone acetyltransferases CREB-binding protein
CCL	C-Chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CDK	cyclic dependent kinase
CDKN	cyclic dependent kinase inhibitor
CFSE	carboxyfluorescein-Succinimidylester
Cfu	colony forming unit
CLR	C-type lectin receptor
CMC	chronic mucocutaneous candidiasis
Cpm	counts per minute
CREM	cyclic adenosine monophosphat response element modulator
CTL	cytotoxic T lymphocyte
CXCL	CX-chemokine-ligand
CYCS	cytochrome c
DC	dendritic cell
DC-SIGN	dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	deoxyribonucleic acid
DSS	dextran sulfan sodium
FACS	fluorescent associated cell sorter
DMSO	dimethylsulfoxid

DPBS	Dulbeccos modified eagle medium
DSS	dextran-sodium sulfat
EAE	experimental autoimmune encephalitis
EDTA	Ethylendiamintetraacetate
ELISA	enzyme linked immuno absorbent assay
FcγR	Fc γ chain receptor
FCS	fetal bovine serum
FGF	fibroplast growth factor
FGL2	fibrinogen-like protein 2
FICZ	6-formylindolo[3,2-β]carbazole
Fig	Figure
Foxp3	forkhead box P3
Gal-9	galectin-9
GAS	gamma activation sequence
GM-CSF	granulocyte macrophage stimulating factor
GPCR	G-protein coupled receptor
GVD	graft versus host disease
GZMB	granzyme B
H	hour
HBD2	human β-defensin 2
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HU-DC	human dendritic cell medium
IAP	inhibitors of apoptosis proteins
iCMC	isolated chronic mucocutaneous candidiasis
IBD	immune bowel disease
ICAM-1	intercellular adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IGF	insulin growth factor
IGFR	insulin growth factor receptor
IL	interleukin
IMS	immune modulatory strain
IRF1	interferon regulatory factor 1
ITIM	immune receptor tyrosine-based inhibitory motif

JAK	janus kinase
LAB	lactic acid bacteria
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MACS	magnetic cell sorting
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1
MHC	major histocompatibility complex
Min	minute
Ml	milliliter
MoDC	monocyte-derived dendritic cells
MR	mannose receptor
MRS	de Man, Rogosa and Sharpe
MS	multiples sclerosis
MSDM	Mendelian susceptibiliy to mycobacterial disease
N2	nitrogen
NAD^+	nicotinamide adenine dinucleotide
NFAT	transcription factor nuclear factor of activated T cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
$NiSO_4$	nickel(II) sulfate
NK cell	natural killer cell
NLRP	NACHT, LRR and PYD domains-containing protein
NOD	nuclear oligomerization domain
PAMP	pathogen-associated molecular pattern
PBMC	peropher blood mononuclear cells
PCR	polymerase chain reaction
PDCD1	programmed Cell Death 1
PDE	phosphodiesterase
PFA	para-formaldehyd
PGE2	prostaglandin2
PGN	peptidoglycan
PHA	phytohämagglutinin
PI	proliferation index
PKA	protein kinase A
PL	phospholipase
PLCγ2	phospholipase Cγ2
PMA	phorbol 12-myristate 13-acetate

PSA	polysaccharide A
RA	rheumatoid arthritis
RIPK2	receptor-interacting serine-threonine kinase 2
SAA	serum amyloid A
SAP	aspartyl proteinase
Ser	serine
SFB	segmented filamentous bacteria
SOCS	suppressor of cytokine signalling
SI	stimulation index
STAT	signal transducers and activators of transcription
Syk	spleen tyrosine kinase
TAP	transporter associated with antigen processing
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
TF	transcription factor
TGF-β	transforming growth factor β
Th	T helper cell
Thr	threonin
TLR	toll like receptor
TNF	tumor necrose factor
Th3	regulatory T cell type 3
Tr1	regulatory T cell type 1
Treg	$CD4^+CD25^+$ regulatory T cell
TSLP	thymic stromal lymphopoietin
Tyk	tyrosine kinase
U	Unit
UBD	ubiqutin like molecule
XAF1	XIAP associated factor 1
XIAP	x-linked inhibitor of apoptosis

1 Introduction

1.1 T cell populations

Human $CD4^+$ T cells were originally categorized into two main subsets, defined as Th1 and Th2 cells (1, 2). This Th1/Th2 paradigm dominated T cell immunology until the dichotomic view changed with the discovery of regulatory T cells, Th17 and Th22 cells (3) as demonstrated in figure 1.

Th1

Th1 cells play an important role in antiviral responses and mediate protection against intracellular pathogens by phagocyte activation and the production of opsonizing and complement-fixing antibodies (1, 4).

Th1 cells produce high amounts of the effector cytokine IFN-γ and are characterized by the expression of IL-12R, IL-18R, CXCR3 and CCR5. Naïve T cells differentiate in the presence of IL-12 to Th1 cells and binding of IL-12 to the IL-12R leads to STAT4 activation following expression of the Th1-specific transcription factor (TF) Tbet (1, 2, 5, 6).

Th2

Th2 cells are mainly involved in the protection against parasitic helminths and produce IL-4, IL-5, IL-13 and IL-9. The secretion of IL-4 and IL-13 leads to IgE class switching in B cells followed by mast cell and basophil activation whereas IL-5 regulates the function of eosinophils. IL-9 production by Th2 cells contributes to mast cell activation and mucin induction in epithelial cells (1, 2, 4-7). The interaction of IL-4 with its receptor on naïve T cells results in the differentiation of Th2 cells by STAT6 activation and induction of GATA3 transcription. IL-4 produced by Th2 cells amplifies in a paracrine manner STAT6 activation and Th2 development (1, 2, 5, 6). IL-4 and GATA3 in turn inhibit the generation of Th1 cells and regulatory T cells (Tregs) whereas IFN-γ and Tbet inhibit Th2 cells (8-10).

Th17

In 2003 the Th1/Th2 paradigm expanded by the discovery of Th17 cells that have been originally found to play a role in murine models of experimental autoimmune encephalomyelitis (EAE) with the identification of IL-23 (11) that is required for the differentiation of Th17 cells.

Human Th17 cells are characterized by the expression of the transcription factor RORC and production of the Th17 associated cytokines IL-17, IL-22, TNF-α and IL-6 (12-16). In addition to RORC numerous other transcription factors have been identified to be necessary for Th17 polarization and RORC upregulation. These include IRF4 and BATF that are upregulated in response to T cell receptor (TCR) stimulation and STAT3 induced by the Th17 differentiating cytokines IL-6, IL-21 and IL-23 (17-19). Further transcription factors that contribute to IL-17 expression in polarized T cells involve Runx, c-Maf and the aryl hydrocarbon receptor (AhR) (20-23). IL-21 produced by Th17 cells amplifies the frequency of Th17 cells while IL-23 terminally differentiates and stabilizes the phenotype of Th17 cells (16). Further characteristics of Th17 cells are the expression of the IL-23R and CD161 on the surface, as well as the expression of the chemokine receptors CCR4 and CCR6 which enable Th17 cells to migrate to site of infections (4, 24). Th17 cells are described to differentiate in the presence of IL-6, IL-1β, IL-21, IL-23 and TGF-β. Research on these Th17 polarizing cytokines has been a major focus over the last years: several groups proposed the combinations of IL-1β plus IL-6 (25) or IL-1β plus IL-23 (26) are required for IL-17 expression. TGF-β alone is known to induce Tregs (27) and its role in Th17 differentiation was controversial discussed as some studies revealed TGF-β not essential for Th17 polarization. In these studies naïve T cells were isolated from PBMCs by CD45RA expression and Th17 differentiation was performed in the presence of serum that contains platelets as a source of TGF-β (16). Three following independent reports proofed that TGF-β is required for the polarization of human Th17 cells as TGF-β is absolutely necessary for RORC induction (28-31).

Cytokines that are known to have inhibitory effects on Th17 development are IFN-γ, IL-4, IL-2, IL-25 and IL-27 (32-36). IL-27 originally described as Tbet- and Th1-inducing cytokine has more anti-inflammatory roles as it induces the generation of regulatory Tr1 cells together with TGF-β and suppresses STAT1 dependent Th17 responses (35-38). IL-4 and IFN-γ have also been indicated to interfere with Th17 development as

neutralizing antibodies to both cytokines have been described to increase IL-17 expression (12, 13). IL-2 has been reported to dampen Th17 activity in a STAT5 dependent manner (33) and IL-25 inhibits IL-17 production by promoting the polarization of Th2 cells (34). Moreover the AhR has also been supposed to be involved in Th17 polarization: Th17 cells express the the AhR (39) and AhR agonists have been shown to inhibit IL-17 in human whereas IL-22 was increased proposing environmental factors may influence Th17 cells (39-41). Another study by Kimura et al. revealed that Ahr interacts with Stat1 and Stat5 which negatively regulate the development of Th17 cells (42).

Th17 cells play a major role in protection against extracellular pathogens including fungi and autoimmune diseases in human such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disorders, but also psoriasis, contact dermatitis and atopic dermatitis (4, 43-45).

Treg

Regulatory T cells (Tregs) are important to maintain tolerance to self, commensal microbiota and control of autoimmune deviation (46-49).

Many different subsets of regulatory T cells have been reported including $CD4^+CD25^+Foxp3^+$ (47), $CD4^+$ Th3 (50, 51), $CD8^+$ Tr cells (51, 52), $CD8^+CD28^-$ (53, 54), $TCR^+CD4^-CD8^-$ Tr cells (55, 56) and NK Tr cells (57, 58). Naturally occurring $CD4^+CD25^+Foxp3^+$ and inducible Tregs including $Foxp3^+$ Tregs, Th3 and Tr1 cells are the most prominent Treg populations.

$CD4^+CD25^+Foxp3^+$ cells can be divided in two groups: naturally occurring, thymus-derived Tregs and adaptive or inducible Tregs. Naturally occurring Tregs originate in the thymus and constitute approximately 1-2 % of circulating $CD4^+$ T cells (59). They are characterized by a demethylated Foxp3 locus (60), the transcription factors Helios (61) and Foxp3 (46, 47). Tregs express the IL-2R α-chain CD25 (62), CTLA4 (63, 64) and GITR (65). Inducible Tregs derive from $CD4^+CD25^+Foxp3^-$ cells after TCR stimulation in the presence of TGF-β and IL-2 (27, 66). They express the same surface markers as natural Tregs but can be distinguished by methylation patterns of the Foxp3 promotor and the lack of Helios expression (67). In the last years additional Treg markers evolved such as CD127 (also known as IL-7Rα) that is low expressed in Tregs and facilitate the experimental purification of Tregs (67-69). Both Treg populations suppress through cell-cell dependent mechanisms and secretion of soluble factors including IL-10, IL-35 and TGF-β (66, 70). Tregs exert their suppressive function directly or indi-

rectly and cellular targets of Treg mediated suppression are $CD4^+$ and $CD8^+$ T cells, dendritic cells, macrophages, osteoblasts, mast cells, NK cells and NKT cells (71). Direct mechanisms of suppression include the production of the suppressive cytokines IL-10, IL-35 and TGF-β leading to cell cycle arrest in responder cells (66, 70), the secretion of granzyme A and B (72, 73) and surface molecules such as galectin-1/10 (74, 75) resulting in apoptosis and cell cycle arrest of target cells (71). It has also been reported that Tregs induce apoptosis via IL-2 consumption (76). Tregs inhibit indirectly by modulation of the function of antigen presenting cells (APCs) that involves the interaction of CTLA4 and CD80/CD86 (77) and the binding of LAG3 to MHC-II (78, 79) that results in an impaired maturation and suppressed costimulatory and antigen-presenting capacities of APCs (71). The expression of molecules such as CD39 (80, 81) or Nrp-1 (neurophilin) (82) on the surface of Tregs prolong Treg-APC interactions and modulate APC function by reducing the costimulatory capacity of APCs (71).

An interesting point is the reciprocal relationship between Tregs and Th17 cells. TGF-β induces Foxp3 expression that is required for generation of inducible Tregs (27). Addition of IL-6 to TGF-β suppresses Treg differentiation and induces Th17 cells (16). Both transcription factors Foxp3 and RORC antagonize each other's function (16, 83, 84). Increased levels of TGF-β and overexpression of Foxp3 can result in downregulation of Th17 genes followed by the conversion of Th17 cells to Tregs (83, 85) while IL-6 and IL-1β have been described to downmodulate Foxp3 expression resulting in enhanced Th17 gene expression (85, 86). T cells that coexpress Foxp3 and IL-17 have been described in mice and human as $Foxp3^+$ Tregs in human peripheral blood and tonsils are able to produce IL-17 after activation (87-90). Tregs have also been shown to be able of trans-differentiation to Th17 cells as Tregs differentiate into IL-17 producing cells after stimulated with allogeneic monocytes in the presence of rhIL-2/rhIL-15 (91). Osorio et al. reported that DCs stimulated with the *Candida*-cell wall compound curdlan are able to convert $Foxp3^+RORC^+$ Tregs into IL-17 producing Tregs (92). Lately glycolysis was found to be a key metabolic checkpoint to determine Treg or Th17 differentiation as deficiency of the TF hypoxia-inducible factor α (HIF-1α) leads to a decreased expression of glycolytic molecules resulting in Th17 inhibition and Treg induction (93). These data highlight the plasticity of Tregs and Th17 cells suggesting during a steady state TGF-β favors Treg development preventing autoimmunity while during infection IL-6 secretion by the innate immune system induces together with TGF-β the generation of proinflammatory Th17 cells (94). Tregs regulate Th1, Th2 and Th17 responses and con-

trol immune responses to pathogens and allergens (95-98). Their absence or dysfunction seems to play a role in various diseases including multiples sclerosis (MS), rheumatoid arthritis (RA), immune bowel disease (IBD), type 1 diabetes, psoriasis, systemic lupus erythematosus and allergy (67, 99).

Tr1

Tr1 cells were originally described in the murine system by Groux et al. showing that TCR transgenic $CD4^+$ T cells developed into an unique Treg population after repetitive antigenic stimulation in the presence of IL-10 and this Treg population secreted high levels of IL-10, low levels of IL-2 and no IL-4 (100). In the human system Tr1 clones isolated from skin challenged with $NiSO_4$ and peripheral blood of nickel-allergic patients inhibited IL-10 dependent the capacity of monocytes to stimulate nickel-specific Th1 responses in the presence of nickel (101).

Tr1 cells are characterized by the production of high amounts of IL-10, moderate amounts of TGF-β, and some IL-5 as well as IFN-γ, but no IL-2 or IL-4 in response to TCR activation (58, 100). Tr1 do not constitutively express Foxp3 but Foxp3 can be induced upon stimulation (58, 102, 103). Tr1 cells proliferate poorly after TCR stimulation but can be expanded ex vivo in the presence of IL-2 and IL-15 (58, 104). Tr1 cells upregulate normal levels of activation markers as CD25, CD40L, CD69, CTLA4 after TCR stimulation (58, 104).

Tr1 cells have been described to differentiate in humans in the presence of IFN-α and IL-10 (105). Moreover IL-27 also known to inhibit Th17 cells (35, 106) and $Foxp3^+$ Tregs has been identified as differentiation factor for Tr1 cells (38, 107, 108). IL-27 and the AhR are involved in Tr1 induction as IL-27 directly induces the TF c-Maf, which is crucial in Tr1 differentiation, followed by IL-21 induction that acts as an autocrine growth factor for Tr1 cells (109-111). The AhR and c-Maf act in synergy to induce IL-27-mediated Tr1 cell differentiation as IL-27 induces the AhR that associates with c-Maf and leads to transactivation of the IL-10 and IL-21 promotors (112). Costimulation of CD2 or CD46 crosslinking with CD3 have also been reported to induce the generation of a Tr1-like regulatory phenotype mediating suppression through IL-10 (113, 114). Moreover immature DCs have been shown to be able to induce IL-10 producing Tr1 cells (103, 115-117). Additionally to immature DCs also specific tolerogenic DCs have been implicated to promote Tr1 responses. Tolerogenic DCs can be induced in multiple ways by biological and pharmacological agents such as IL-10, TGF-β; certain

pathogens or allergens (*Lactobacillus reuteri*, *L. casei)* (118); tumor antigens (myeloma cells) (119) or endogenous proteins (heavy chain ferritin) (120). Finally DCs primed with immunosuppressive drugs as vitamin D3 and/or dexamethasone can promote anergic T cells (121, 122).

Tr1 cells suppress naïve and memory T cell responses mainly by IL-10 and TGF-β production in a direct cell-cell contact manner (100, 123-125). The immunosuppressive effects can be inhibited by neutralizing IL-10 antibodies suggesting that Tr1-mediated suppressive effects are mainly mediated by IL-10 (123).

Tr1 cells play an important role in maintaining self-tolerance and preventing autoimmunediseases such as skin disorders (Pemphigus vulgaris) (126), allergy (101, 127, 128), IBD (129-131) and cancer (58, 132, 133).

Th22

IL-22 was originally reported as a Th17-specific cytokine in mice and humans. Recently a new T cell subset of skin-homing, tissue-instructing Th22 cells has been described that promotes wound healing and epithelial integrity and is characterized by the production of IL-22 but no IL-17 or IFN-γ (39, 41, 134, 135). Moreover the production of fibroplast growth factors FGF1 and FGF5 has been identified in Th22 cells (134). Th22 cells express the platelet-derived growth factor (PDGF) receptor and the skin homing chemokine receptors CCR4, CCR6 and CCR10 on their surface showing epidermal homing characteristics (41, 134). Th22 cells express similar to Th17 cells the ligand-dependent TF AhR that binds to halogenated and non-halogenated olycyclic aromatic hydrocarbons such as the synthetic compound β-naphthoflavone and the prominent environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and also to the tryptophane metabolite 6-formylindolo(3,2-b)carbazole (FICZ) as an endogenous ligand of AhR (39, 136). The AhR plays a role in regulating IL-17 and IL-22 production as FICZ has been shown to inhibit IL-17 production while IL-22 was enhanced in human $CD4^+$ T cells suggesting an AhR regulated balance of Th22 and Th17 (39, 40). A key transcription factor for Th22 development has not been identified so far and the AhR has been shown to play an essential role in regulation of IL-22 production but it remains to be determined whether AhR is a master TF for Th22 cells.

IL-22 is recognized by the IL-22 receptor and its expression is restricted to tissue cells underlining the role of IL-22 as tissue instructing cytokine. IL-22 induces STAT3-dependent the secretion of antimicrobial peptides such as S100 proteins and defensins in

keratinocytes (137). Recently, investigation of Th22 supernatants on keratinocytes revealed that the Th22 key cytokines IL-22 and TNF-α lead synergistically to expression of complement factors such as C1s, C1r and CFB, TLR3 and TLR6 expression and antimicrobial chemokines CXCL9/10/11 in human keratinocytes representing Th22 cells as an important T cell subset for cutaneous immunity (134, 138).

The differentiation pathway of Th22 cells is still not fully clear as simple T cell receptor (TCR) activation results in IL-22 production. Moreover Th22 cells were proposed to differentiate from naïve T cells in the presence of plasmacytoid dendritic cells and TNF-α and IL-6 (41).

Th22 cells play an important role in tissue homeostasis and in particular IL-22 is involved in chronic, T cell-mediated diseases, such as skin diseases including psoriasis (139, 140), inflammatory bowel disease (141, 142), and rheumatoid arthritis (137, 143, 144).

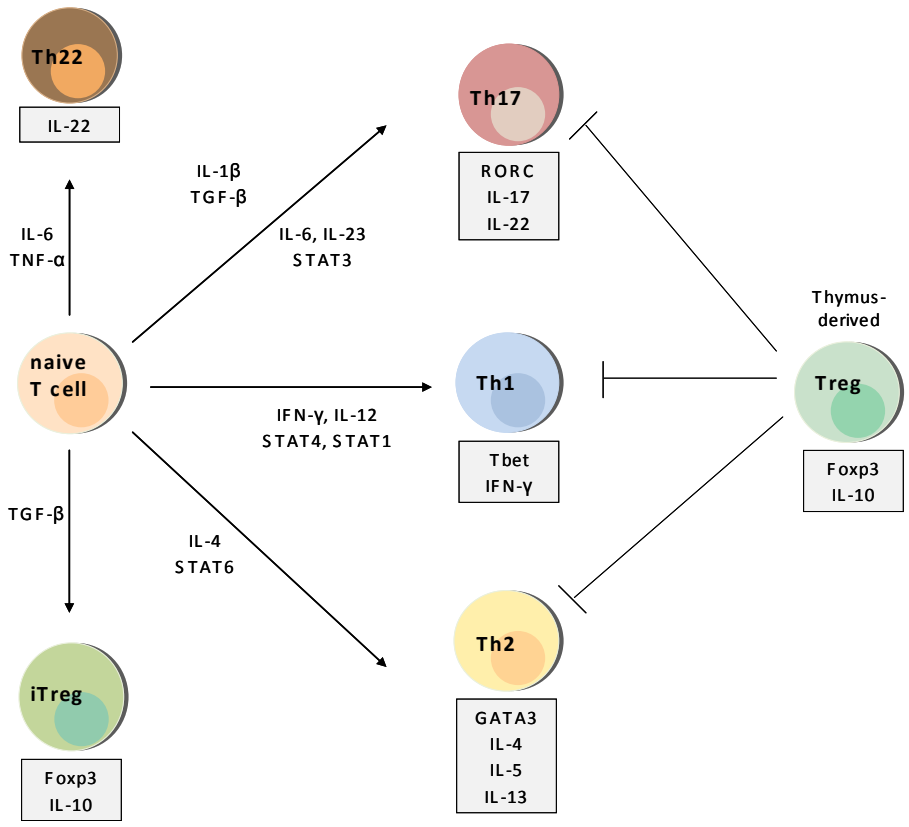


Figure 1: T cell subsets

1.2 Fungi

Fungi are ubiquitous heterotrophic eukaryotes morphologically classified into yeast and filamentous forms. Fungi are very efficient in adapting changing environment conditions resulting in symbiotic, commensal, latent or pathogenic relationships with plants, animals or humans (145-148). The human immunes system recognizes commensal or ubiquitous fungi followed by fine balanced pro- and anti-inflammatory immune responses that are important to ensure a stable host-fungus relationship (145). To avoid pathological consequences of an interrupted or imbalanced homeostasis between the host and the fungus two components of the immune response are important: resistance (limiting fungal growth) and tolerance (limiting host damage caused by the immune response) (145, 149). The increasing incidence of fungal diseases clearly indicates the importance of a stable host-fungus relationship. Table 1 shows common fungi and associated diseases (145, 149-151).

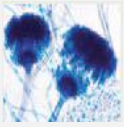
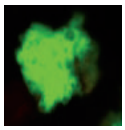
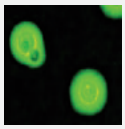
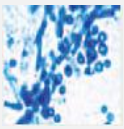
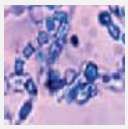
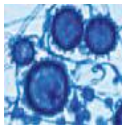
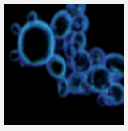
Organism	Habitat		Disease
Aspergillus spp.	Soil, decaying organic materials, indoor air environments		• Aspergilloma • Acute and chronic pneumonias • Cerebral aspergillosis • Allergy, ABPA, SAFS
Pneumocystis spp.	No known environmental habitat, person-to-person transmission		• Pneumnia • COPD
Cryptococcus spp.	Environment in association with decaying materials and trees		• Pneumonia • Meningitis • Disseminated disease
Candida spp.	Commensal of human gastrointestinal tract and vagina		• Disseminated infections • Mucocutaneous infections (oropharyngeal, skin and nail infections) • Vaginitis
Malassezia spp.	Commensal of human skin		• Cutaneous infections • Allergic atopic eczema
Blastomyces dermatitidis	Soil in association with decaying wood		• Acute and chronic pneumonias • Skin lesions • Disseminated disease
Coccidioides immitis	Alkaline soil		• Self-linited influenza-like syndrome • Pneumonia • Disseminated disease
Histoplasma capsulatum	Soil contaminated with bird or bat guano		• Self-linited influenza-like syndrome • Acute and chronic pneumonias • Disseminated disease
Paracoccidioides brasiliensis	Soil and digestive tract of some animals		• Assymptomatic • Acute and chronic pneumonias • Disseminated disease • Cutaneous lesions

Table 1: Major fungal pathogens, their habitats and associated diseases

ABPA=allergic bronchopulmonary aspergillosis, COPD=chronic obstructive pulmonary disease, SAFS=severe asthma with fungal sensitisation

Adapted from Romani et al. (145)

1.2.1 *Candida*

Candida are eukaryotic diploid sexual yeasts of the kingdom fungi. About 150 species are identified to date (152). *Candida* species reside as commensal organisms in the gut, oral cavity or vagina in approximately 50 % of the population (153). The heterogeneous members of *Candida* grow as yeast (blastospores), some members can also grow filamentous and form hyphae (pseudohyphae). *C.dubliniensis* and *C.albicans* are polymorph as these two *Candida* species are capable of forming true hyphae (152).

Candida becomes pathogenic when the homeostasis between the virulence of the microbe and the resistance of the host immune system is disturbed. 17 out of 150 known *Candida* species have been described to be responsible for mycosis in human with an increasing incidence caused by *C.tropicalis, C.lusitaniae* and *C.krusei* (152, 154). One of the most prevalent fungal pathogen in human responsible for yeast infections is *Candida albicans* (154, 155) (table 2) which will be further described in the next part of the thesis.

Candidosis	References	Period of observation	Region/ country	Number of strains	*C. albicans* (%)	*C. tropicalis* (%)	*C. parapsilosis* (%)	*C. glabrata* (%)
Oral candidosis	Gonzalez Gravina *et al.* (2007)	February–May 2003	Venezuela	43	42.3	12.8	14.9	2.1
	Martins *et al.* (2010a, b)	May 2005–2006	Portugal	53	79	4.8	6.5	4.8
	Luque *et al.* (2009)	-	Argentine	-	60.7	4.5	-	5.6
Candiduria	Kauffman *et al.* (2000)	-	USA	530	51.8	7.9	4.1	15.6
	Kobayashi *et al.* (2004)	-	Brazil	45	35.5	22.3	11.1	8.8
	Passos *et al.* (2005)	-	Brazil	43	70	4.6	4.6	7
	Binelli *et al.* (2006)	1999–2001	Brazil	23	52	43.5	-	17.3
	Chen *et al.* (2008)	June–August 2006	Australia	65	85.2	-	4.4	27.8
	Álvarez-Lerma *et al.* (2003)	1998–1999	Spain	389	68.4	36	0.5	8.2
	Dorko *et al.* (2002)	-	Slovakia	94	61.7	6.3	24.5	-
Candidemia	Hazen *et al.* (1986)	-	USA	126	21	38	12	3
	Chakrabarti *et al.* (2009)	-	India	-	26.3		-	10.5
	Colombo *et al.* (2007)	-	Brazil	282	38	48	23	9
	Costa-de-Oliveira *et al.* (2008)	During 2004	Portugal	-	35	-	26.5	-
	Bassetti *et al.* (2006)	1999–2003	Italy	182	40	9	23	15
	Miranda *et al.* (2009)	2004–2005	Brazil	-	42	33	16	2
	Tortorano *et al.* (2006)	1997–1999	Europe	473	53	7	14	14
	Trick *et al.* (2002)	During 1999	USA	-	59	10	11	12
	Pfaller *et al.* (2010)	2008–2009	Europe/ Asia/ American	1239	50	9.8	17.4	17.4

Table 2: Selected epidemiological studies about the distribution of *Candida* species isolates among various types of candidosis published from 2000 to 2010

Adapted from Silva et al. (156)

1.2.1.1 *Candida albicans*

1.2.1.1.1 Occurrence, taxonomy and morpholoy

Candida albicans is a diploid, ubiquitous and opportunistic yeast which is part of the human microbial flora of mucocutaneous, gastrointestinal and genitourinary areas by colonizing mucosal surfaces of 30-60 % of humans (157).

The cell wall composition of the fungus depends on morphotype, growth stage and environment of the fungal species and acts as an important aspect of biology and pathogenicity of *Candida albicans* as permeability of the barrier mediates interactions between the microorganism and the host. 80-90 % of the cell wall of *Candida albicans* consist of carbohydrates such as the three mayor polysaccharide components β-1,3- and β-1,6-glucans, N-acetyl-D-glucosamins (Chitin) and mannose polymers associated with proteins (mannoproteins). The β-glucans are with 50-60 % the main constituent of the wall (158-163).

Characteristic for *Candida albicans* is the morphotype switching between the yeast growth (blastospores) and the filamentous type of growth (hyphae and more frequently pseudohyphae). Pseudohyphae are built from yeast or hyphae by budding. The new growth form remains attached to the parent cell followed by elongation resulting in filamentous structures with constrictions at cell-cell junctions. True hyphae develop from yeast by "germ tube" projection followed by elongation and building of separate fungal units (156, 164, 165). This dimorphismus depends on pH and temperature conditions and contributes to virulence of *Candida albicans* providing a mechanism for evasion of the host defence mechanisms (166, 167).

In addition *Candida albicans* show different forms of phenotype switching named "white-opaque transition" that involves distinct colony morphologies distinguishable in colony size, shape and colour. In the "white" phenotype state cells form round, white, dome-shaped colonies, whereas in the "opaque" phenotype state cells build flat, grey and elongated colonies, demonstrated in figure 2 (168-170). The process of white-opaque transition is influenced by several factors as temperature (170), oxygen (171, 172), carbon dioxide (173), genotoxic and oxidative stress (169, 174). Virulence of *C.albicans* can attributed to "white-opaque" transition as studies with cutaneous infection modles described a higher virulence of opaque-cells in cutaneous models whereas white-cells were more virulent in systemic infection (175-177). In order to better understand how fungi interact with their environment numerous studies focus on the underly-

ing molecular mechanisms of phenotype switching by identifying genes differently expressed between the two stages.

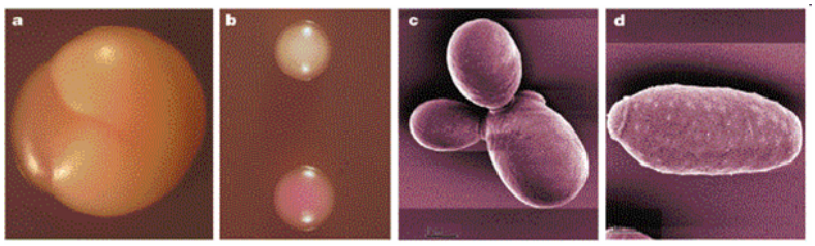


Figure 2: Colonies of *Candida albicans* in the white and opaque form

a) White colony with an opaque sector **b)** White (upper) and opaque (lower) colonies **c)** Scanning electron micrograph of white cells **d)** Scanning electron micrograph of opaque cells

Adapted from Johnson et al. (178)

1.2.1.1.2 Pathogenesis and virulence factors

Numerous virulence (ability to cause disease) factors have been attributed to the pathogenicity of *Candida albicans*: adhesins such as Ala1p, Als1p, Int1p, Mnt1p and Hwp1p (179-183) mediate the adherence of *Candida albicans* to host cells. Other virulence mechanisms are the secretion of degradative enzymes as aspartyl proteinases (SAP1-9) and phospholipases (PL) that disrupt mucosal barrier function facilitating invasion and colonization of host tissues (156, 184-186). Moreover morphology switching from the yeast form to the filamentous form, phenotype transition (187, 188) and biofilm formation (189, 190) contribute to virulence of *Candida albicans*. All these virulence-promoting mechanisms enable *Candida albicans* to invade and adapt different environmental conditions in the host (191).

Within the interplay between the host and its microbiome the host has to distinguish between the commensal and pathogenic states of opportunistic microbes as *Candida albicans*. In case of a changing environment, alterations in the microbiota or a disturbed immunity *Candida albicans* becomes pathogenic.

1.2.1.1.3 Immune recognition of *Candida albicans*

The antifungal immunity involves many cell types including neutrophils, monocytes, macrophages, natural killer (NK) cells, dendritic cells, T cells, non-MHC restricted T cells such as γδT cells, mucosal epithelial cells, stromal cells and keratinocytes that

provide a protective effect against *Candida*-infections (192, 193). The following sections of this thesis will focus on DC and T cell responses in *Candida*-infections.

1.2.1.1.3.1 Recognition of *Candida albicans* by the innate immune system (dendritic cells)

Innate immune cells including monocytes, macrophages, neutrophils and dendritic cells recognize fungi by opsonic or phagocytic mechanisms or by receptor mediated mechanisms. Pattern recognition receptors (PRR) including toll-like-receptors (TLR), C-type-lectin receptors (CLR) and galectin-family proteins sense pathogen-associated molecular patterns (PAMP) of the fungi (145, 194-196). The sugar polymers forming the candidal cell wall are the main source of fungal antigens. The uptake of the fungi by DCs induces maturation and secretion of a certain cytokine pattern that determines the ensuing differentiation of Th cell subtypes.

CLRs play an important role in immunity against fungi involving dectin-1/2, mincle, DC-SIGN, the mannose receptor, langerin and mannose-binding lectin (145, 197). Figure 3 gives an overview about receptor signalling involved in *Candida* recognition.

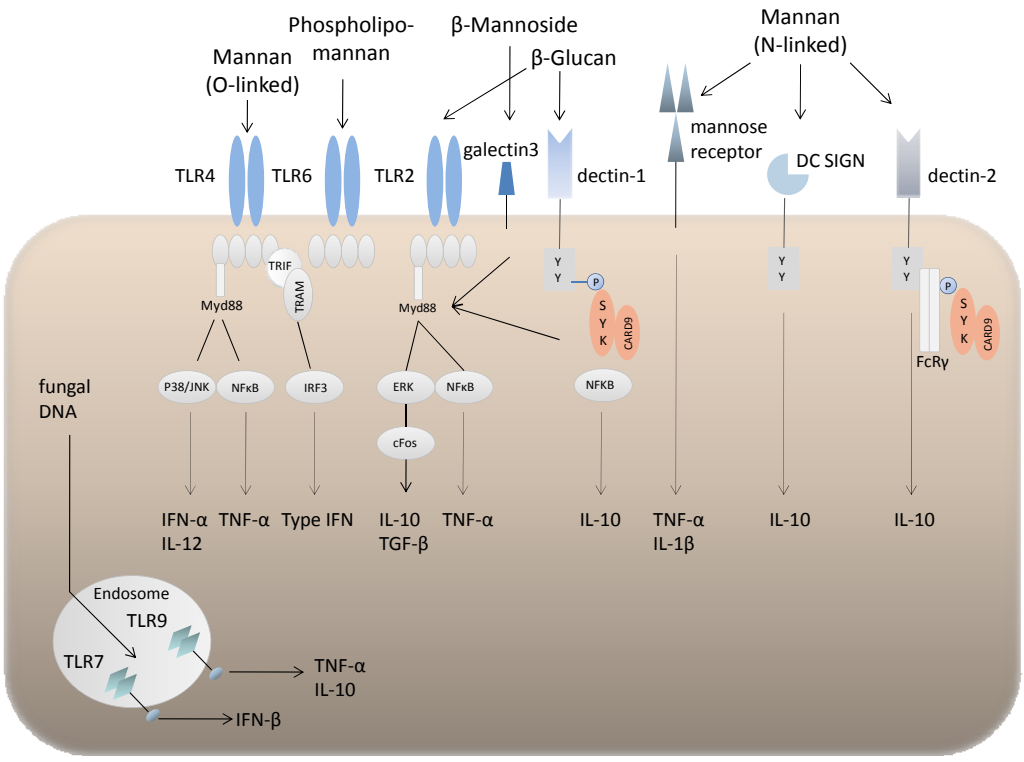


Figure 3: Receptor signalling on APCs in response to *Candida albicans*

Adapted from Veerdonk et al. (195)

One of the most important receptors for *Candida* recognition constitutes dectin-1 expressed on neutrophils, macrophages, dendritic cells, T cells, B cells and keratinocytes (198-202). Dectin-1 primarly recognizes β-glucans of the core *Candida* cell wall that are not expressed on the surface in the hyphal form of *Candida*. After switching of *Candida* to its yeast form β-glucans are exposed and get accessible for dectin-1 (203, 204). Dectin-1 signalling in response to fungal β-glucans induces phagocytosis, phospholipase A2, COX2, respiratory burst and the secretion of various cytokines including IL-10, IL-6, IL-2, TNF-α, IL-1β, IL-23 and little IL-12 and induction of Th1/Th17 cells (205, 206). Signalling pathways of dectin-1 are shown in figure 4.

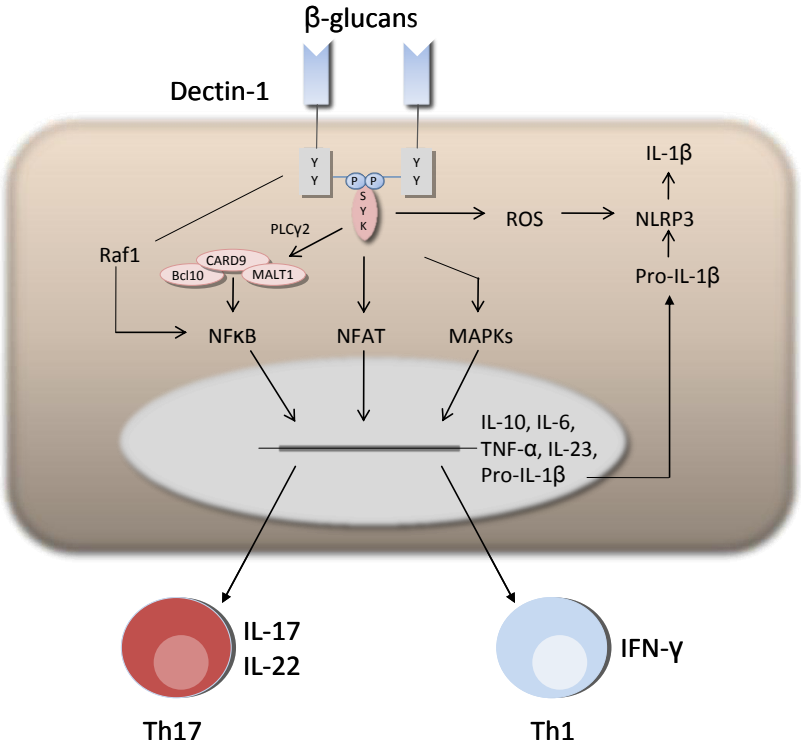


Figure 4: Dectin-1 signalling of DCs in response to *Candida*
Adapted from Osorio et al. and Drummond et al. (207, 208)

Ligation of dectin-1 by β-glucans leads to activation of two distinct signalling pathways, the spleen tyrosine kinase (syk)-caspase recruitment domain containing protein 9 (CARD9) and the Raf pathway, both ending in the activation of NFκB (209). Dectin-1 signals through the tyrosine kinase syk and the following engagement of the complex consisting of caspase recruitmet domaine 9 (CARD9), Bcl-10 and MALT1 inducing the canonical NFκB pathway by activating NFκB subunit p65 and cRel. Human and mouse

studies described an association between genetic failures of dectin-1 and CARD9 and an enhanced susceptibility to *Candida*-infections (210-212).

Dectin-1 ligation also results in the non-canonical NFκB RelB pathway through Raf1 signalling (213-216) (figure 5). The syk-independent Raf1 pathway integrates with the syk-pathway at the level of NFκB and controls the balance between p65 and RelB activity leading to IL-12p70, IL-23 and IL-1β expression (213). Dectin-1 activation leads to phosphorylation and activation of Raf1 resulting in the phosphorylation of p65 at Ser276. The histone acetyltransferases CREB-binding protein (CBP) binds to the phosphorylated Ser276 and acetylates p65 followed by an increased transcriptional activity of p65 and induction of IL-6 and IL-10 (209, 213, 217). The phosphorylated p65 also binds to RelB to form inactive dimers. The decreased transcriptional activity of RelB leads to an enhanced expression of IL-1β, IL-12p40 and IL-23 which play a role in the development of Th1 and Th17 cells (209, 213).

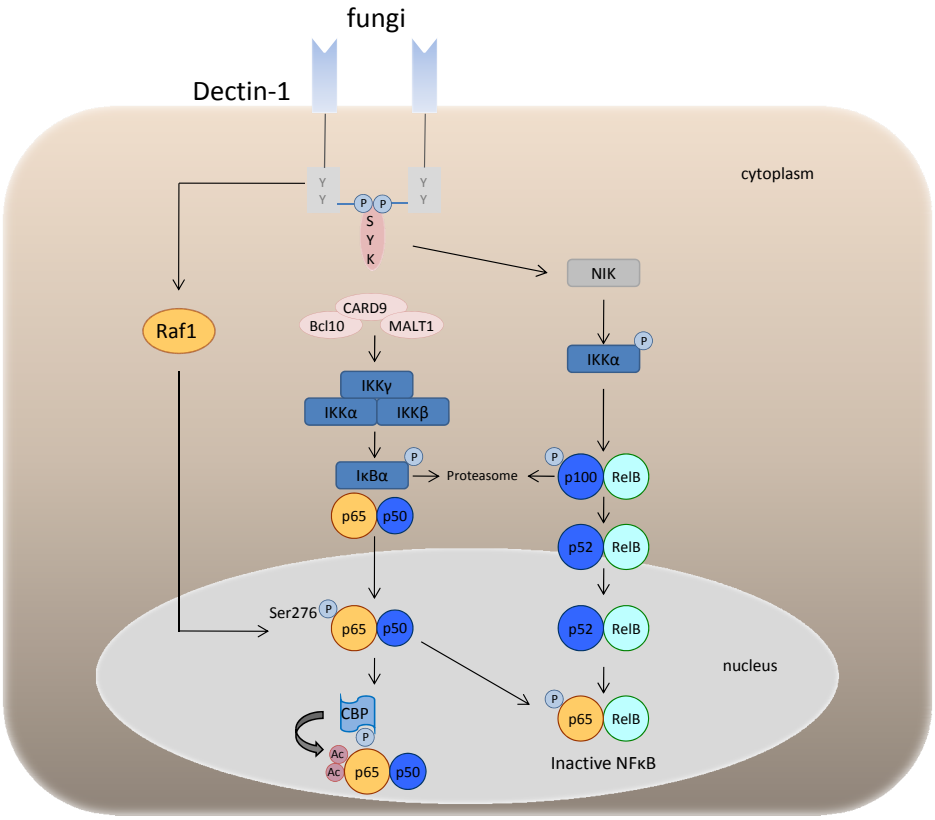


Figure 5: Dectin-1 signalling through Raf1 in response to fungi
Adapted from Geijtenbeek et al. (209)

Moreover dectin-1 activates the NLRP3 inflammsome in a ROS-dependent manner resulting in caspase-1 dependent IL-1β induction (figure 4) and NLRP3 deficient mice show an increased susceptibility to *Candida*-infections (218). Tassi et al. reported a syk-dependent activation of phospholipase chain γ2 ($PLC\gamma_2$) and NFAT downstream of dectin-1 and showed that $PLC\gamma_2$ deficient mice lack Th1/1h17 cells after glucan-stimulation (219).

Dectin-2 recognizes mannan structures of *Candida albicans* that results in Fc receptor γ-chain (FcRγ) dependent syk-CARD9 activation. This explains why dectin-$2^{-/-}$ mice are characterized by a higher susceptibility to *Candida albicans* (220, 221). *Candida albicans* can also be sensed by the FcRγ-associated Mincle receptor that mediates Syk-CARD9-dependent NFκB activation (222). The mannose receptor (MR) and DC-SIGN bind N-linked mannans of *Candida albicans* and induce antifungal Th17 responses (223, 224). Additionally DC-SIGN has been shown to induce acetylation of the NFκB subunit p65 in the presence of TLR-dependent NFκB activation leading to enhanced IL-10 responses and shifting inflammatory TLR responses to a more anti-inflammatory condition (195, 209, 217).

The Galectin-3 receptor gets activated by binding of *Candida*-derived β-mannosides and is essential for TLR2-dependent cytokine production in response to *Candida albicans* (225).

The TLR family includes 12 members recognizing microbial-derived structures as shown in table 3 (226). Fungal compounds of *Candida albicans* such as zymosan, phospholipomannan, O-linked mannans and fungal DNA are sensed by TLR2, TLR4, TLR6, TLR7 and TLR9 (table 3) (195, 227).

Species	**PAMPS**	**TLR**
Bacteria, mycobacteria	LPS	TLR4
	lipoproteins, LTA, PGN	TLR2/1, TLR2/6
	flagellin	TLR5
	DNA	TLR9
	RNA	TLR7

Viruses	DNA	TLR9
	RNA	TLR3, TLR7, TLR8
	structural protein	TLR2, TLR4
Fungus	**Zymosan, ß-glucan**	**TLR2, TLR6**
	Mannan	**TLR2, TLR4**
	DNA	**TLR9**
	RNA	**TLR7**
Parasites	tGPI-mutin (Trypanosoma)	TLR2
	glycoinositolphospholipids	TLR4
	DNA	TLR9
	hemozoin (Plasmodium)	TLR9
	profilin-like molecule (Toxoplasma gondii)	TLR11

Table 3: PAMP detection by TLRs

Candida-relevant TLRs are printed in bold. Adapted from Kawai et al. (226)

One of the first studies demonstrating the involvement of TLR2 in *Candida* defence described that monocytes produced decreased amounts of TNF-α and IL-1β after challenge with *Candida albicans* when TLR2 was blocked by antibodies (228). A further study reported that TLR2 deficient mice are characterized by a reduced TNF-α secretion and decreased neutrophil recruitment in response to *Candida* (229). However, other studies demonstrated that $TLR2^{-/-}$ mice are more resistant to *Candida*-infections in combination with a decreased Treg population and reduced amounts of IL-10 (230, 231). This immune-modulatory effect of TLR2 has been shown to be mediated by TLR2 dependent induction of immunosuppressive Tregs (231, 232) and is further supported by a study describing that TLR2 induces TLR2-/dectin-1-dependent tolerogenic DCs and Treg proliferation (233). Moreover TLR2 has been described to inhibit TLR4-mediated IL-12 production through c-fos (234). The anti-inflammatory role of TLR2 in *Candida*-infections was further confirmed by reports describing a collaborative recognition of fungal β-glucans by dectin-1 and TLR2 (235, 236) and this costimulation in response to the *Candida* cell wall compound zymosan can result in the development of a regulatory T cell type (233, 237). TLR4 plays a role in antifungal host response by binding O-linked mannans (238, 239) that is supported by the finding of an enhanced suscepti-

bility to disseminated candidiasis in TLR4 deficient mice followed by decreased chemokine production and impaired neutrophil recruitment (228). TLR4 is also known to be amplified by dectin-1 in a syk-dependent manner (240, 241).

TLR6 and TLR1 can mediate zymosan-induced signalling in combination with TLR2 only playing a limited role among the various PRRs involved in *Candida* recognition (242). Recently TLR7 has been described to get activated by *Candida* resulting in IFN-β production that subsequently drives IFNAR1-dependent STAT1 activation and IRF7 expression (227).

Taken together the huge variety of different PRRs activated by various specific fungal PAMPs in combination with distinct complex crosstalks between the PRRs underline the complexity of the innate immune response against fungi including *Candida albicans*.

1.2.1.1.3.2 Recognition of *Candida albicans* by the adaptive immune system (T cells)

The ligation of different PRRs on fungi-primed DCs induces distinct signalling pathways that lead to the development of different T cell responses as depicted in figure 6. These DC-fungi interactions shape a balanced adaptive antifungal response of $CD4^+$ effector T cells and Tregs followed by commensalisms or infection (145).

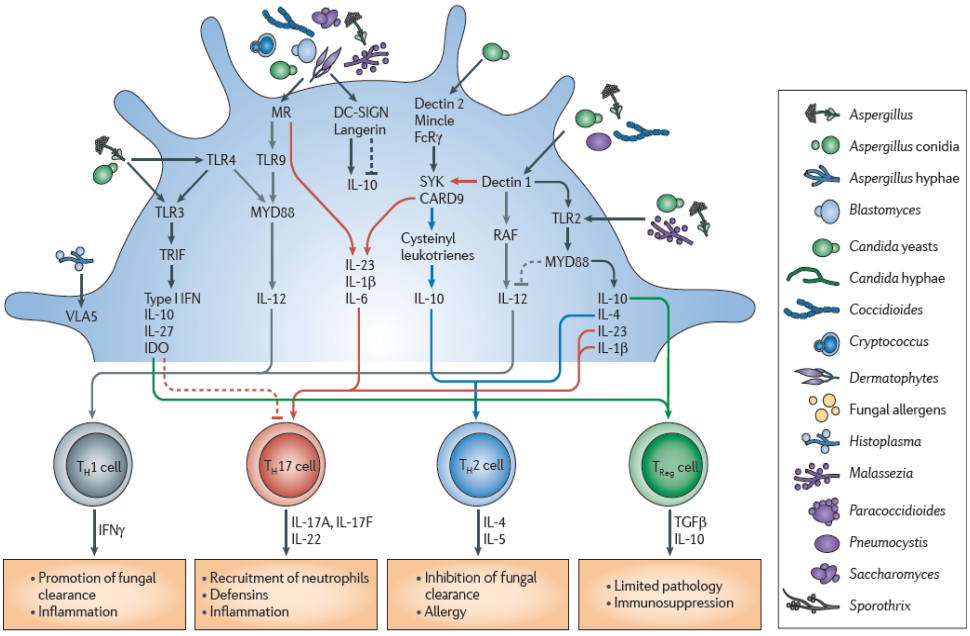


Figure 6: DC signalling in response to fungi and the following shape of T cell responses
Adapted from Romani et al. (145)

Th1 and Th2 cells

Early studies suggested a protective Th1 and non-protective Th2 response in fungus immunity (243) but the involvement of a biased Th2 cytokine production has been observed later on in patients with HIV infection or patients with chronic mucocutaneous candidiasis (CMC) proposing a more important role of Th2 cells in fungal infections (244-247). Th1 reactivity was found to be associated with disseminated or vaginal infections (248-250) and the requirement of Th1 cells in immunity to *Candida albicans* was further confirmed by several human studies (251, 252). Studies with defect IL-12 receptor function revealed that IL-12 production and IFN-γ-dependent IL-12 responsiveness are important mechanisms for the generation of an adequate Th1 response against *Candida* and underline the Th1-promoting role for IFN-γ in resistance to *Candida* (250, 253-255). In contrast, some Th2 cytokines, such as IL-4, IL-10, and TGF-β were shown to be necessary for the development and maintenance of a protective Th1 response in fungal infections (253, 254). C-type-lectins induce antifungal Th1 immune responses after fungi recognition (206, 209, 213). The importance of a functional Th1 response in *Candida*-infections could further confirmed because patients with CMC produced diminished amounts of Th1 cytokines (256-258).

Th17 cells

Th17 cells are involved in viral, bacterial, fungal and parasitic infections and play an important role in protection against extracellular pathogens, autoimmune disorders and allergy. (259). Table 4 demonstrates the findings of Th17-related Candidiasis during the last years. Th17 cells are essential in antifungal immune responses in mice and human (260-263) according to the production of IL-17 and IL-22.

Year	Candida model/ human disease	Primary finding	Th17 protective	Th17 pathogenic	References
2004	Systemic model	IL-17RA protective	√		Huang et al. [38]
2006	Oral model	IL-12p40^{KO} mice susceptible	√		Farah et al. [17]
2007	Systemic model	Dectin-1 directs protective Th17 response	√		Leibundgut-Landmann et al. [67]
2007	Gastric model	IL-23 and Th17-increase inflammation and infection		√	Zelante et al. [84]
2008	HIES	STAT3 mutations lead to Th17-deficiency, correlates with mucosal candidiasis	√		Ma et al. [81]; Milner et al. [82]; Al Khatib S et al. [87]; Renner ED et al. [78]; de Beaudoudrey et al. [77]
2008	CMC	Reduced production of IL-17 and IL-22	√		Foerster et al. [10]
2009	Oral model	IL-23^{KO} and IL-17RA^{KO} mice susceptible to disease; IL-12^{KO} mice resistant to disease	√		Conti et al. [16]
2009	Systemic model	Dectin-2 directs protective Th17-response	√		Robinson et al. [68]
2009	Human PBMCs *in vitro*	Mannose receptor induces IL-17 in response to *Candida*	√		Van de Veerdonk et al. [69]
2009	Oral model	NLRP3, TLR2, dectin-1, play roles in host defense against Candida	√		Hise et al. [18], Gross et al. [72]
2009	Familial recurrent Candidiasis	Dectin-1 mutations and defective IL-17 production linked to prevalence of mucocutaneous Candida infection	√		Ferwerda et al. [83]
2009	Familial recurrent Candidiasis	CARD9 mutations associated with defective Th17 response and susceptibility to CMC	√		Glocker et al. [70]
2010	APECED	Auto-antibodies against Th17- cytokines account for CMC susceptibility	√		Kisand et al. [5], Puel et al. [6]
2010	Systemic and Septic multiple organ failure models	IL-17RAKO mice susceptible to disseminated disease, IL-17 does not mediate pathogenic inflammation or organ failure	√		van de Veerdonk et al. [30]
2010	HIV	Selective depletion of Th17 subset during course of HIV infection	√		Prendergast et al. [88] El Hed et al. [89]

Table 4: Th17 cells and Candidiasis
Adapted from Conti et al. (192)

The importance of IL-17 and IL-22 is further underlined by the fact that CMC patients are characterized by IL-17 and IL-22 deficiency (257, 264) and can have mutations in the IL-17A and IL-17R genes (265, 266).

IL-17 has been shown to regulate the production of specific chemokine ligands of CXCR1/CXCR2 in epithelial cells, the induction of CXCL8 (IL-8), G-CSF and antimicrobial peptides leading to recruitment of neutrophils to mucosal sites (135, 139, 267-274). IL-17 and IL-22 induce synergistically the expression of the antimicrobial peptides β-defensin 2 and S100A9 and additively increase the expression of S100A7 and S100A8 that are important forming a barrier against microbial pathogens (273, 275-278).

The tissue-instructing cytokine IL-22 plays an essential role in controlling *Candida*-infections as it has been shown that IL-22 is necessary to defence *Candida* growth at mucosal sites when Th17 and Th1 cells are absent (279). IL-22 producing cells enrich at mucosal sites where consistent exposure to fungi takes place during infection and patients with chronic *Candida*-infections lack these cells while they are present in healthy subjects (257, 280). IL-22 together with TNF-α has been shown in a *in vitro* infection

model to effectively inhibit the growth of *Candida albicans* in keratinocytes underlining the important role for IL-22 in cutaneous immune responses (138). IL-22 is produced by NK cells, NK T cells, lymphoid tissue-inducer cells, Th1 and Th17 cells and leads STAT3-dependent to the induction of antimicrobial peptides such as S100 proteins and defensins in epithelial cells (135, 145, 281, 282). This role of IL-22 in defending *Candida*-infections raises by the fact that patients with the hyper-IgE syndrome (HIES) suffering from chronic *Candida*-infections show an impaired function of Th17 cells due to *STAT3* mutations. This STAT3 dependent Th17 defect suggests impaired IL-22 responses at epithelial sites in these patients (283, 284).

As described above stimulation of the C-type-lectin receptor dectin-1 on DCs and macrophages with purified β-glucans is followed by syk-CARD9 signalling. This pathway plays a mayor role in immunity against *Candida* leading to the induction of IL-17/IFN-γ producing T cells. It can be inhibited by the Raf and TRIF-type I IFN pathways (145, 206, 211). Observed genetic failures of the dectin-1/CARD9 and STAT3 pathways further highlight a role for Th17 cells in *Candida*-infections (151, 285). How disturbed 17 pathways can affect *Candida*-infections will be demonstrated and described later in this thesis.

Tregs

During *Candida*-infection Th17- and Th1-mediated inflammation eliminates the pathogen whereas Tregs have to restore homeostasis and prevent immunopathology.

Several studies described an inverse relationship of IFN-γ and IL-10 in patients that suffer from *Candida*-infections. High amounts of IL-10 that negatively affects IFN-γ production have been linked to susceptibility to *Candida* (246, 247, 286-288).

Tregs have been described to be involved in clearing *Candida*-infections in mice and human as they are responsible for long-lasting protection against fungi and negatively regulation of Th1 responses against *Candida* (231, 289). Of note, mouse $CD25^{+}Foxp3^{+}ROR\text{-}\gamma t^{+}$ Tregs can be converted into $Foxp3^{+}ROR\text{-}\gamma t^{+}$IL-17 producing cells after dectin-1 stimulation indicating that some $Foxp3^{+}$ T cells can produce IL-17 and play an unexpected pro-inflammatory role in some settings (92).

Moreover a potential role of the enzyme indoleamine 2,3-dioxygenase (IDO) in control of fungal infections has been discussed as IDO induced by IFN-γ is involved in induction of peripheral tolerance (290-292). IFN-γ dependent IDO induction at the host-

pathogen interface controls *Candida* morphology and leads to a Treg-dependent anti-fungal resistance (293).

1.2.2 Chronic mucocutaneous Candidiasis (CMC)

Candida infections include invasive or local manifestations. In the case of the rare invasive candidiasis bloodstream and major organs are affected and the clinical spectrum involves minimal fever to severe sepsis with multiorgan failure and is associated with an increased mortality (294-296). Common clinical pictures of local mucosal Candidiasis involve vulvovaginal candidiasis, oropharyngeal candidiasis, intertrigo or nappy rash. Predisposing factors for Candidiasis are the age of infancy or senility, diabetes mellitus, pregnancy, hormonal contraception or immune suppression as in the case of HIV and lymphoma (154).

These mucosal manifestations can also become chronic and can be associated with primary immune-defiencies as in the case of the chronic mucocutaneous candidiasis (CMC) that is described in the following part of the thesis (296).

1.2.2.1 Definition, heterogeneity and prevalence of CMC

Chronic mucocutaneous candidiasis (CMC) is a very rare and heterogeneous group of syndromes characterized be recurrent or chronic infections of skin, nails and mucosal tissues with *Candida* species, in particular *Candida albicans* as shown in figure 7 (297).

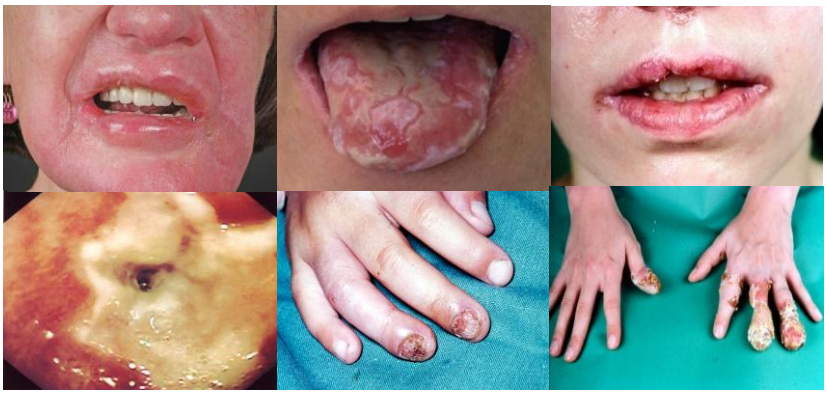


Figure 7: Clinical features of CMC

The term “CMC” was introduced in 1960 (298) and the first case of CMC was reported by Thorpe and Handley in 1929 (299) followed by further reports in the 1950s (300, 301). CMC occurs within the first weeks or month of life (302), the mucocutaneous

leasions are themselves do not cause death, but associations between candidiasis and intracranial aneurisms and squamous cell carcinoma have been described in several patients with isolated CMC (285, 303-306).

The heterogeneous and complex group of CMC syndromes can be classified according to distribution (local vs. generalized invasive candidiasis) and by the underlying pathomechanism (primary vs. secondary syndromes) as shown in figure 8. Patients with CMC rarely develop disseminated or systemic *Candida*-infections (297, 307). Secondary syndromes are usually the consequence of local or systemic immune suppression in response to infections, eg as in the case of AIDS (297, 308). Primary CMC syndromes include up to date the isolated CMC form, the autoimmune polyendocrine type I syndrome (APS1) and the hyper IgE syndrome (HIES). Patients with isolated CMC suffer from chronic *Candida*-infections and have no other severe infections or autoimmune diseases (285). Concerning the prevalence of CMC no data exist but noticeable, most reports describe more affected women than men (257, 309, 310).

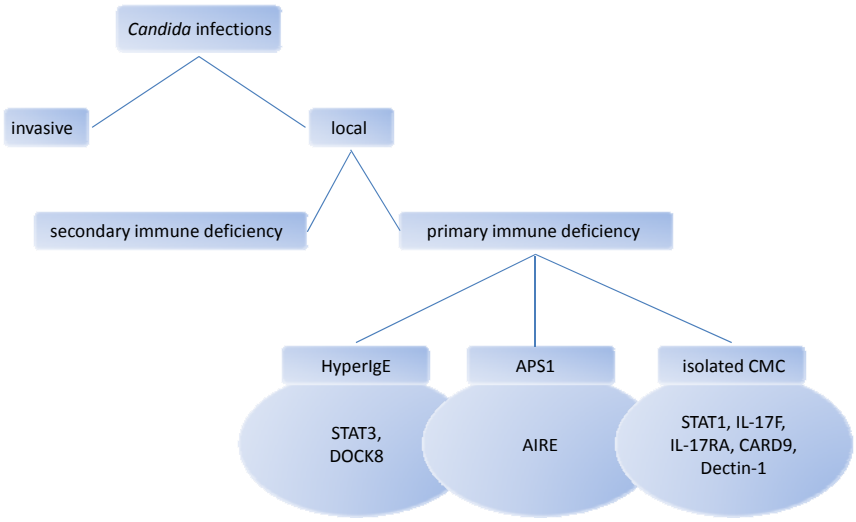


Figure 8: Classification of CMC syndromes

1.2.2.2 Genetic details of CMC inheritance

In 2009 in a family with recurrent vulvovaginal candidiasis or onchomycosis a Tyr238X polymorphism in the β-glucan receptor dectin-1 was identified. This defective form of dectin-1 resulted in an impaired cytokine production of IL-17, TNF-α and IL-6 by macrophages and monocytes after stimualtion with β-glucan or *Candida albicans*, but

normal killing of *Candida albicans* by neutrophils (212). Moreover susceptibility to CMC has been documented to be associated with homozygous mutations in the CARD9 gene and low proportions of IL-17 producing T cells analyzed in a consanguieneous Iranian family with systemic candidiasis and peripheral dermatophytosis in 2009 (210). In 2011 mutations in the IL-17A receptor and IL-17F have been identified in two case reports as new genetic etiologies of CMC. In one case Puel et al described an autosomal recessive mutation in the IL-17RA that binds IL-17A and IL-17F and is expressed in endothelial cells, T cells, B cells, fibroplasts, lung, myelomonocytic cells and bone narrow stromal cells. This nonsense homozygotic mutation results in a diminished expression of the IL-17RA in fibroblasts and peripheral blood cells followed by unresponsiveness to IL-17A and IL-17F (311). In a second case report an autosomal dominant mutation in the IL-17F gene with a partial loss of gene function was found in a family with CMC leading to an impaired binding capacity to the IL-17RA on fibroblasts (311).

1.2.2.3 Primary CMC associated syndromes

HIES

CMC can be a clinical feature of the hyper IgE syndrome (HIES). HIES was first reported in 1972 and is characterized by high levels of serum IgE, Staphylococcus aureus abscesses, severe atopic dermatitis and recurrent lung infections (312). Both the typical autosomal dominant (313) and autosomal recessive forms (314) of HIES have been described. HIES usually occurs very early in life and woman and men are equally affected (315). Concerning the genetic etiology a homozygous *Tyk2* mutation was identified in 2006 (316) followed by the finding of *STAT3* mutations in the autosomal-dominant form of HIES in 2007 (317, 318) and homozygous as well as compound heterozygous mutations of *DOCK8* in patients with autosomal recessive HIES in 2009 (319, 320). Related syndrome wit mild form of CMC has been described in a patient with Tyk2 deficiency (316).

APS1

CMC can also be associated with autoimmune polyendocrine type I syndrome (APS1), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) that is associated with mutations in the autoimmune regulator (*AIRE*) gene. APS1 is diagnosed if at least two of the following symptoms occur: adrenal insufficiency, hyperparathyroidism or chronic mucucutaneous candidiasis (321, 322). In 2006

high levels of neutralizing antibodies against IFN-α and IFN-ω were identified in APS1 patients. Today analysis of auto-antibodies against type I IFNs has been developed now a secure diagnostic method (285, 323, 324). In 1929 an association between APS1 and CMC was described the first time by Thorpe and about 70 %-100 % of APS1 patients suffer from CMC that is usually one of the first symptoms of APS1 to appear (285, 299, 325, 326). APS1 starts at early onset (1-20 years) and is very frequent among Finnish (326), Sardinians (327), Iranian Jews (328) and Norways (329), woman and men equally affected.

AIRE, the gene mutated in APS1 patients, is primarly expressed in thymic medullary epithelial cells and regulates the expression of tissue-restricted antigens (325, 330) followed by elimination of autoreactive T cells escaping the negative selection in the thymus. An extrathymic role of AIRE in antigen-presentation or tolerance induction has been discussed as some reports described AIRE expression in lymph nodes, spleen, monocytes and DCs (331-335). In a human monocytic cell line AIRE has been recently shown to form a transient complex with the dectin-1 receptor pathway which is required for immune defence against *Candida* species (336). $AIRE^{-/-}$ mice showed an increased T cell activation capacity *in vitro* by enhanced VCAM-1 expression (337). It has also been speculated that AIRE is involved in the selection of Tregs that is still not fully clear and the primary function of AIRE seem to be the regulation of negative selection in the thymus (330, 335). Up to now more than 60 mutations are known (338, 339) leading to loss of tolerance and following destruction of endocrine tissues (322, 340).

The spectrum of clinical manifestations of APS1 is very broad: autoimmune destruction of endocrine glands results in Addison's disease, hypothyroidism, hypoparathyreodism and insulin-dependent diabetes mellitus. As tissue-specific nonendocrinological autoimmune diseases have been described hepatic and biliary disease, pernicious anemia, alopecia areata, vitiligo and keratopathy. Other ectodermal manifestations reported in APS1 are dental enamel hypoplasia, nail dystrophy and calcification of the tympanic membranes. Cutaneous manifestations include Sjögren's syndrome, cutaneous vasculitis and systemic lupus erythematosus (321, 326, 328, 341-345). Chronic inflammations in the mouth can lead to squamous cell carcinoma (322, 326, 342-344).

In 2010 neutralizing antibodies against IL-17A/F and/or IL-22 were identified in the plasma of almost 200 patients (265, 266). Because of the finding of auto-antibodies in APS1 or thymoma patients without CMC or APS1 patients before the onset of CMC let

the authors speculate that auto-antibodies cause CMC rather than result from CMC (285).

1.2.2.4 Immunological pathogenesis of CMC

Intact mucosal and skin barriers, an adequate innate and adaptive immunity in response to recognition of the fungus are important antifungal defence mechanisms of the host. Concerning function of the innate immunity in CMC contradicting reports exist (346). Defective activation and migration of phagocytic cells including neutrophil granulocytes, macrophages or monocytes has been reported in CMC (307, 347-349). Some studies showed decreased numbers or functionally defective NK cells (258, 350). However, other reports described a normal migration of neutrophils in CMC patients (288, 351).

Humoral immunity seem to be normal in CMC as serum concentrations of immunglobulins and titres of specific antibodies against *Candida* species are normal (245, 297, 307). As a small group of CMC patients have been described to suffer from lung disease associated with IgG subclass defiency that further highlights the complexity of CMC syndromes (352). Compelling evidence suggests that T cell mediated immunity plays a major role in *Candida*-infections. The proliferation capacity of T cells in response to *Candida* antigen is controversial discussed. There exist data of a reduced (352) or normal (247) proliferation to both *Candida* and mitogen, whereas others describe a *Candida*-specific enhanced and normal proliferative capacity after mitogen stimulation (258, 288, 297).

An adequate Th1 response have been described to be important in defending *Candida*-infections as IFN-γ-deficient mice show an enhanced susceptibility to *Candida*-infections (243, 255, 353-355). In contrast, decreased levels of IL-10 are associated with an enhanced *Candida*-resistance (356). Numerous studies report a deregulated cytokine secretion of T cells with decreased amounts of type 1 cytokines (246, 288, 357, 358) including IFN-γ and IL-12 and an enhanced production of IL-10 and IL-4 (247). To date the most striking defect in CMC relies on IL-17/IL-22 deficiency. Thus Th17-associated cytokines are essential in defending *Candida*-infections in mice and human (192, 260-262). CMC patients show a drastically reduced production of the Th17 cytokines IL-17A, IL-17F and IL-22 (257, 264, 266, 288). It is not fully clear whether the Th17-related pathogenesis of CMC results from a direct T cell defect or aberrant T cell differentiation capacity of innate immune cells. A normal distribution of pattern recog-

nition receptors has been described on dendritic cells (DCs) (310), while maturation and secretion of Th17 associated cytokines was diminished in response to *Candida* in CMC patients (309, 359) indicating defects on the APC level. In contrast, HIES patients showed an impaired Th17 differentiation due to *STAT3* mutations pointing to T cell specific defects (283, 284).

1.2.2.5 Diagnosis, Therapy and Resistance of CMC

The diagnosis of CMC is made clinically and by *in vitro* isolation and cultivation of *Candida* from smear tests. In subgroups with known underlying genetic failures CMC can be confirmed by an additionally analysis of mutations (297).

The standard therapy of *Candida*-infections consists in azole antimycotics including fluconazole that inhibit ergosterol synthesis and is recommended as the first line systemic drug (360). In CMC often resistance to fluconazole occurs and can be substituted by itraconazole, voriconazole or posaconazole (361, 362). Another class of antifungal agents are echinocandins (363) including caspofungin (364), micafungin (365) and anidulafungin that act against glucan-synthesis and cell wall formation of *Candida*. A third class of drugs are polyenes ivolving the ergosterol-binding amphotericin B which is considered as third-line agent due to its intravenously administration and side effects (297, 366).

1.3 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) compromise gram-positive bacteria with a low G+C content, belong to the hylum *Firmicutes* and are members of the *Clostridium-Bacillus* subdivision of gram-positive eubacteria. LAB are nonsporing, catalase-negative organisms that are aerotolerant, fastidious, acid-tolerant, and strictly fermentative and produce lactic acid as a major end product of sugar fermentation. The natural habitat ranges from dairy, meat and plant fermentations to the gastrointestinal tract of humans and animals (367-372).

In gram-positive bacteria such as LAB the cell wall consists of several characteristic structures: a thick and multilayered wall of peptidoglycan, decorated with proteins, teichoid acids and polysaccharides as demonstrated in figure 9. These cell wall structures determine species- and strain-specific properties including adapting to changing host environment and mediating multiple immunological effects of LAB (373). LAB are the most important probiotic microorganisms that are typically associated with the human gastrointestinal tract and are considered to entertain beneficial health promoting interactions with the host.

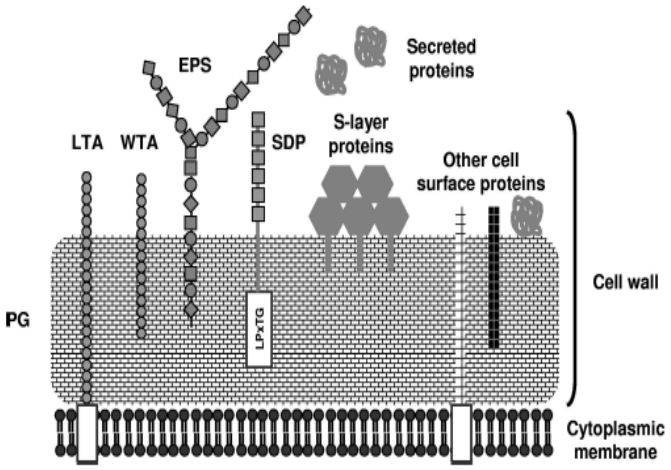


Figure 9: The cell surface composition of LAB

PG=petidoglycan, LTA=lipteichoicacid, WTA=wall teichoic acid, EPS=exopolysaccharides, SDP=sortase-dependent protein. Adapted from Lebeer et al. (373)

1.3.1 Probiotics

The term probiotics was originally introduced in 1970 (374) and redefined by Fuller as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (375). Up to date a broadly accepted definition of probiotics was formulated by the World Health Organization that declares probiotics as "live organisms which when administered in adequate amounts confer a health benefit on the host" (376). A huge number of microbial species and genera is considered as probiotics (table 5) and are claimed to have beneficial effects on the host (table 6) (370, 377).

***Lactobacillus* species**	***Bifidobacterium* species**	**Other lactic acid bacteria**	**Nonlactic acid bacteria**
L. acidophilus L. amylovorus L. casei L. crispatus L. delbrueckii sub spec. bulgaricus L. gallinarum L. gasseri L. johnsonii L. paracesei L. plantarum L. reuteri L. rhamnosus	B. adolescentis B. animalis B. bifidum B. breve B. infantis B. lactis B. longum	Enterococcus faecalis Enterococcus faecium Lactococcus lactis Leuconstoc mesenteroides Pediococcus acidilactici Sporolactobacillus inulinus Streptococcus thermophilus	Bacillus cereus var. toyoi Escherichia coli strain nissle Propionibacterium freudenreichii Saccharomyces cerevisiae Saccharomyces boulardii

Table 5: Microorganisms considered as probiotics

Adapted from Holzapfel et al. (370)

Enhanced barrier effects of gut mucosa	Modification of the gut microflora
	Adherence to intestinal mucosa → adherence and activation of pathogens ↓
	Modification of dietary proteins by intestinal flora
	Modification of tumor-related bacterial enzyme activity
	Influence on mucosal permeability
Immune modulation	Increased activity of dendritic cells, Th1-/Treg-induction
 Effects on inflammatory bowel disease, colon cancer, diarrhoe, food allergy, lactose intolerance …	

Table 6: Effects of probiotics

Adapted from Holzapfel et al. and Salminen et al. (377, 378)

Probiotics are thought to benefit the human microbial ecosystem. A comprehensive microbial analysis of 27 distinct sites of the human body including the oral cavity, gut and skin revealed the presence of 22 bacterial phyla with most sequences related to the four phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes (379, 380). Each habitat of the body harboured a characteristic microbiota that was stable between individuals and over time. The human intestine is the most important habitat with an extremely high density of bacterial microflora. The human intestine harbors a complex and diverse microbial community, referred to as gut microflora or microbiota. The human gut microbiota is estimated to be composed of approximately 1100 prevalent species, with approximately 160 such species per individual. The human microbiome representing the collective genomes of the gut microbiota is estimated to contain 150-fold more genes than our own host genomes (381-386).

Sterile at birth the body gets colonized during birth with microorganisms of the mother and immediate environment followed by subsequent development of the microbiota by genetic, diet and environment of the host. It has been shown that the composition of the microbiota can differ between infants that are fed with either breast milk or formula

(380, 387, 388). Environmental microbial exposure and appropriate gastrointestinal microbial colonization in early infancy are essential for immune response maturation and allergic disease development (389, 390). Several studies reported that early events in microbial gastrointestinal tract colonization during infancy influence the outcome of allergies and asthma later in life (391-396).

An altered or less diverse microbiota composition has been associated with various disorders such as autoimmune and allergic diseases, colorectal cancer, metabolic diseases, bacterial infections, IBD, type 1 diabetes, asthma and obesity (397-399). Such an imbalance of the microbiota can be influenced by intrinsic factors (genetic inheritance) or extrinsic factors (lifestyle, nutrition, hygiene and microbial compounds) (397). The use of probiotics to restore and maintain the microbiota by influencing immune responses has been received more and more attention as a possible strategy to prevent such diseases.

1.3.2 The influence of LAB on the immune system

In recent years the human microbiota has been under intensive investigation. Growing interest for probiotic bacteria exist as they influence the composition or metabolic activity of the human microflora and modulate the immune system of the host (400). LABs as probiotics influence the host in multiple ways including the intestinal environment, epithelial and mucosal barriers and the mucosal immune system. They affect various cell types involved in innate and adaptive immune responses such as epithelial cells, dendritic cells, monocytes, macrophages, B cells, T cells and NK cells (401).

1.3.2.1 Recognition of LAB by the innate immune system (dendritic cells)

The effects of LAB on DC modulation are important as LAB-triggered DCs mediate different outcomes of T cell polarization.

Immature DCs take the antigen up, mature and migrate from peripheral tissues to secondary lymphoid organs where present the antigen to naive T cells and induce adaptive immune responses (402, 403). During maturation DCs upregulate the major histocompatibility complex (MHC) molecules for antigen presentation and costimulatory molecules such as CD80, CD83, CD86 and CD40 for an adequate T cell stimulation (404). LAB are known to differently induce activation and maturation of DCs by upregulation of maturation markers CD80, CD83, CD86 and CD40 (405-409).

LAB induce in DCs specific cytokine pattern and among these cytokines the most important are IL-10 and IL-12 as they control the balance of the immune system: IL-12 produced by DCs drives Th1 responses that induce cell-mediated immunity whereas IL-10 is an anti-inflammatory key molecule for Treg induction mediating tolerance (410-413). The ability of LAB to induce IL-10 and IL-12 is highly strain-dependent and the effect of LAB on DC function is often assessed by the ratio of IL-10/IL-12 production that allows determining pro-inflammatory or anti-inflammatory capacities of LAB. Christensen et al. described LAB have distinct capacities to modulate DC function with respect to IL-12 and IL-10 production by testing various lactobacilli srains (414). Other studies adressing the heterogeneity of LAB showed that the probiotic mixture VSL#3 exhibits high levels of IL-10 and decresead amounts of IL-12 in lamina propria and systemic DCs (415, 416). These studies were in line with data of *L.casei* and *L.rhamnosus* which are able to prime IL-10 secreting DCs demonstrating anti-inflammatory effects of LAB (118, 408, 417). In contrast, enhanced amounts of IL-12 were observed in human DCs stimulated with *L.paracasei*, *L.reuteri*, *L.gasseri*, *L. johnsonii*, *Acinetobacter Iwofii* or *L.lactis* describing an pro-iflammatory activity of LAB (408, 418-420).

The ability of probiotic LAB to modulate strain-specific the function of DCs can be related to different structures expressed on the surface of these bacteria. LAB can also secrete active proteins or peptides that are able to promote immune responses (421-423). PRRs play an essential role in recognizing such structures. Several PPRs are involved in LAB-mediated immunemodulation of DCs including scavenger receptors, C-type lectins, nucleotide binding oligomerization domain (NOD) and TLRs (424-426). Compounds of the bacterial cell wall such as the two major bacterial cell wall compounds peptidoglycan in the case of gram-positive bacteria and LPS in the case of gram negative bacteria are important ligands for PRR activation on DCs. TLR2 is known to bind peptidoglycans, lipoteichoic acid (LTA), bacteial lipoprotein and LPS (427) and TLR6 couples to fatty acid chains present on LTA (428, 429). NOD1 and NOD2 receptors play also an important role in LAB recognition as they recognize intracellularly peptidoglycan-derived muramyl dipeptides of the bacterial cell wall (430). *L.casei* has been shown to upregulate TLR2 expression in mouse DCs and macrophages (431). Foligne et al. demonstrated the transfer of *L.rhamnosus*-triggered DCs from Myd88-, TLR2- and NOD2-deficient mice into mice with TNBS induced colitis did not affect the severity of colitis indicating the involvment of TLR2 and NOD2 (432). Further evidence for the role in LAB recognition has come from a study describing the effect of

L.plantarum containing LTA on stimulated PBMCs is TLR2-dependent (433) further supporting that the impact of LAB on APCs seems to be predominately mediated by the involvement of TLR2 and NOD2. The content of LTA of the bacterial cell wall in particular its chain length or degree of D-alanine (D-Ala) substituation of different LAB species has an influence on their stimulating activity (434-436). The D-Ala mutant of *L.plantarum* has been shown to result in enhanced amounts of IL-10 and mediates protective effects in a colitis mouse model compared to the wild type (428). Another receptor on DCs associated with LAB is the calcium-dependent C-type lectin DC-SIGN as it was described to induce Tregs by *Lactobacilli*. How LAB interact with DC-SIGN is currently unknown but some pathogens bind to DC-SIGN activating the NFκB subunit p65 followed by IL-10 induction (118, 217, 373). Moreover it has been shown that LAB such as *L.bulgaricus* OLL1181 is able to activate the intestinal AhR pathway resulting in amelioration of DSS-induced colitis in mice proposing the involvement of LAB recognition (437).

1.3.2.2 Recognition of LAB by the adaptive immune system (T cells)

The human microbiota shapes T cell responses within the gastrointestinal and systemic immune system. The T cell compartment including, Th1, Th17 or Tregs influences the behaviour of different bacterial species as commensals, mutualistic or oportunistic (438). For an adequate homeostasis between harmful and harmless microorganisms controlled immune responses are required that implies different T cell responses of different subsets in the host.

LAB differ in their ability to modulated DC responses and subsequent T cell induction. Several LAB strains have been shown to modulate the Th1/Th2 balance toward Th2 dominance indirectly through stimulation of monocytes, macrophages or DCs (439). IL-12 induction by *L.rhamnosus* GG leads to inhibition of Th2 cytokine secretion in PBMCs of allergic patients (440). Certain Bifidostrains are also able to prevent Th2 cytokine production by monocytes via IL-10 secretion (407). *L.casei* Shirota has been described to facilitate the generation of Th1 cells through IL-12 induction in macrophages (441). Moreover DCs primed with LAB such as *L.reuteri* skewed $CD4^+$ T cells to Th1 differentiation characterized by IFN-γ production but no IL-4 or IL-13 (408). It was also shown that *A.Iwofii* mediates LPS-dependent Th1-polarizing effects (419) supporting that probiotic bacteria have the potential to induce pro-inflammatory Th1 responses.

Th17 development is specifically affected by commensal bacteria as germ-free mice are deficient of Th17 cells (442) and is proposed to be regulated by $CD11b^+$ DCs and macrophages in the lamina propia (443). Another mechanism of intestinal Th17 polarization in the lamina propria that has been suggested is the production of adenosine 5'-triphophosphat (ATP) by commensal bacteria inducing the secretion of Th17-instructing cytokines by lamina propria cells (444). Segmented filamentous bacteria (SFB) have shown to induce intestinal Th17 responses by serum amyloid A (SAA) production and protect from pathogenic bacteria such as *C.rodentium* and *E.coli* demonstrating a proinflammtory Th17 response that is protective (442, 445). In contrast, the commensal *B.fragilis* has been identified to promote Treg differentiation (446, 447). Therefore a new model for coevolution of adaptive immunity with the microbiota has been proposed as the modulation of Tregs and Th17 cells by commensal microorganisms (*B.fragilis*) and pathobionts (SFB) appears to shape the immune system in the gut depending on balanced Treg-Th17 proportions (448).

Much attention has been paid to the abilities of probiotic bacteria to promote Treg responses. Induction of Treg is important to maintain or restore a homeostatic environment of the host in response to infection. Several studies reported the generation of Tregs by the intestinal microbiota: Commensal bacteria as *Bifidobacterium infantis* or *Faecalibacterium prausnitzii* have been described to drive the development of IL-10 producing Tregs in the gut (449, 450). Mucosal Tregs have been reported to be continously generated through retinoic-acid primed DCs and endigous clostridia species have been described to be potent inducers of mucosal and systemic Tregs protecting animals from colitis (451). Single bacterial components such as polysaccharide A (PSA) from *B.fragilis* are known to promote $Foxp3^+$ Treg and reduce Th17 cells. These Tregs prevented experimental colitis in mice indicating PSA as a beneficial bacterial molecule suppressing inflammation-drived host pathology (446-448, 452, 453). *L.paracasei* was described to induce the generation of a Treg-like population producing IL-10 and TGF-β in combination with a low proliferative capacity (454). LAB also induce Treg in the context of intestinal inflammation as the probioic mix VSL#3 decreases intestinal inflammation attributed to IL-10 producing TGF-β positive Tregs (455). Smits et al. reported that LAB such as *L. reuteri* ASM20016 or *L.casei* NIZO B255 are capable of priming IL-10 producing T cells that exert suppressive effects on peripheral $CD4^+$ T cells (118). A probiotic mixture of five LAB strains was identified inducing $CD4^+Foxp3^+$ Tregs from $CD4^+CD25^-$ cells which increased suppressor activity of natu-

rally occurring $CD4^+CD25^+$ $Foxp3^+$ Tregs and suppressed inflammatory bowel disease, atopic dermatitis and rheumatoid arthritis in mice (456). More evidence for the role of probiotics in Treg induction was shown by describing dairy supplementation of *L.rhamnosus* GG and *B.lactis* strains resulted in induction of TGF-β producing Tregs which lead to suppression of allergic symptoms in a murine asthma model (457). Foligne et al. reported that *L.salivarius* Ls33 and *L.rhamnosus* Lr32 led to tolerogenic DCs and Tregs in an IL-10-independent but TLR2- and NOD2-dependent manner (432). *L.rhamnosus*-exposed DCs have been demonstrated to induce peripheral hyporesponsiveness in $CD4^+$ T cells characterized by decreased T cell proliferation and cytokine production (458). Similar results were found by Baba et al. showing different commensal bacterial strains convert naive T cells into hyporesponsive T cells with low or no IFN-γ, IL-10 and IL-17 production but displayed suppressive functions (405). Probiotic bacteria have also shown to be capable of Treg induction through stimulation of intraepithelial cells as certain *Lactobacillus* strains stimulate Caco-2 cells to produce TGF-β and thymic stromal lymphopoietin (TSLP) followed by the generation of tolerogenic DCs which in turn promoted TGF-β secreting Tregs (409). T cell induction by probiotic LAB is of growing interest concerning their therapeutical effects in experimental inflammatory bowel disease, atopic dermatitis, rheumatoid arthritis, allergy and asthma. Some LAB strains inducing larger amounts of pro-inflammatory amounts of IFN-γ are recommended to influence Th2-skewed immuneresponses such as allergy (459, 460), while LAB strains with anti-inflammatory capacities (high IL-10/IL-12 ratio and Treg induction) may be useful to treat and lower Th1-skewed responses as seen in IBD (461-466).

1.3.3 LAB and *Candida*

Fungi in particular *Candida* species colonizing several mucosal sites of the human as commensal increasingly cause *Candida*-infections. The number of patients suffering from local Candidiasis increased (467). Specific antifungal prophylaxis would be one suitable strategy to limit pathogenic fungal colonization and *Candida*-related disorders. Recent interest focused on the use of probiotics for the treatment of *Candida* as they could provide an additional innovative approach by colonizing the human flora. Mice models showed that fungi interactions with *L.rhamnosus* resulted in reduced fungal enteric colonization and systemic fungal infection (468, 469). Several mechanisms of *L.rhamnosus* to diminished *Candida* colonization were proposed: exclusion of *Candida*

by competition, enhancement of the mucosal barrier effect, increased IgA response, inhibition of *Candida*-adhesion and modulation of the immune response. An important aspect of probiotics is the treatment of vulvovaginal Candidiasis as *in vitro* studies reported the prevention of *Candida* adherence and growth on the vaginal epithelium by LAB (470-472). A clinical trial could confirm the positive effect of LAB showing that orally or intravaginally administered *L.rhamnosus* leads to colonization and/or inhibition of *Candida albicans* colonization and infection of the vagina (473, 474). Moreover it has been demonstrated that the addition of *L. casei* to *Candida*-infected malnourished mice normalized the immune response against *Candida albicans* followed by efficient recruitment and activation of phagocytes, as well as effective release of pro-inflammatory cytokines further supporting different LAB strains could have an effect in *Candida*-infections (475).

More and more dairy products containing probiotics claim potential beneficial effects on human health. Because of the ability of probiotic bacteria to inhibit the growth of pathogens and to modulate human immune responses, these bacteria could also provide new strategies to prevent and treat fungal diseases.

2 Aim of the study

The impact of various microorganisms on the complex human microbiome is an expanding field of scientific research. Fungi such as *Candida* species or bacteria such as lactic acid bacteria play an important role in regulation of host mechanisms including the immune system. The immune system of the host has to discriminate between commensal microbes and pathogens - tolerance or infection and this is mainly mediated by different T cell responses.

In case of the fungal species *Candida* which is one the most common fungal pathogen of humans the incidence of fungal infections increased dramatically over the years. In particular patients with chronic mucocutaneous candidiasis (CMC) suffering from chronic *Candida*-infections are an example of imbalanced immune mechanisms including deregulated T cell responses. Successful therapy strategies for this disease are still not found as the cause of CMC is still not fully clear due to the complexity and heterogeneity of this disease. It is known that CMC patients show a defect of Th17 cells and in some cases inherited genetic failures could be identified. But the complexicity and existence of different CMC-subgroups complicates finding of the association between the immunological and molecular defects followed by an adequate therapy that is equally applicable for all CMC patients. Moreover only few data exist whether the Th17-related pathogenesis of CMC results from a direct T cell defect or aberrant T cell differentiation capacity of innate immune cells.

Aim of the study is to investigate the function of T cells in response to *Candida* with respect to Th17 cells in patients with isolated CMC and patients with the CMC-associated APS1 compared to healthy subjects with no *Candida*-infection. Because DCs activated by antigens such as *Candida* promote the polarization of naïve T cells including Th17 cells, T cell responses induced by DCs stimulated with *Candida* compounds will be characterized. Moreover the capacity of CMC patients to develop Th17 cells will be examined and due to the fact that Th17 cells play an important role in anticandidal skin immunity, possible associations between Th17 cells and the skin will be analyzed in human keratinocytes.

Another point is the ability of LAB strains to mediate strain-specific effects on the immune system of the host stimulating the release of certain cytokines and induction of

T cell responses. LAB that are widely used in the food industry are of special interest in view of their application as probiotics.

Aim of this study is to investigate the immune modulatory capacity of the bacterial immune-modulatory strains (IMS) 1 and 2 on DCs and the ensuing T cell responses of healthy non-atopic volunteers. Most strain-specific effects of probiotic bacteria are assessed by determing their ability to induce the immune regulating cytokines IL-10 and IL-12 concerning their capacity to induce Tregs or Th1 cells. Therefore the IL-10/IL-12 ratio of both strains will be examined in human DCs. In order to assess the potency of the two bacterial strains to promote T cell responses, in particular Tregs, naïve T cells will be stimulated with IMS1/IMS2 primed DCs and phenotypically characterized due to their cytokine pattern. Finally T cells instructed by these bacteria will be analyzed for their functional capacity in a suppression assay. It is of special interest if the different LAB that will be tested in this study show possible beneficial properties concerning the treatment of different diseases such as *Candida*-infections, atopic eczema or allergy and production of health promoting food and drinks with these LAB as additives.

The results of this study could contribute to a better understanding how different microorganisms influence the human immune system: on the one hand how fungi species such as *Candida albians* can lead to deregulated T cell responses as a pathogen, on the other hand how apathogenic lactic acid bacteria can induce tolerance in the host.

3 Material and Methods

3.1 Material

3.1.1 Reagents

Reagent	**Supplier**
2,2'-azino-bis-3-ethylbenzthia-zoline-6-sulfonic acid	Sigma-Aldrich, Munich
10x D-PBS w/o Ca/Mg	Gibco/Invitrogen, Paisley, Scotland
ABTS	Roche Diagnostics, Mannheim
AIM V medium	Gibco /Invitrogen, Paisley, Scotland
Albumin from bovine serum (BSA)	Sigma-Aldrich, München
Aqua ad injectabilia	Laboratori Diaco Biomedicali, Trieste, Italy
autoMACS rinsing solution	Miltenyi Biotech, Bergisch Gladbach
autoMACS running buffer	Miltenyi Biotech, Bergisch Gladbach,
Candida albicans	Allergopharma, Reinbek
$CD4^+$ T cell isolation kit II (human)	Miltenyi Biotech, Bergisch Gladbach,
$CD14^+$ micro-beads (human)	Miltenyi Biotech, Bergisch Gladbach
CFSE	Molecular Probes, Leiden, Niederlande
Citrat-monohydrate	Merck, Darmstadt
Citric acid (0,1 M)	Merck, Darmstadt
Curdlan	Wako Chemicals, USA
DEPC treated water (pyrogen free)	Invitrogen, Paisley, Scotland
DermaLife K Cell Culture Medium	Lifeline Cell Technology, USA
DermaLife K Cell Culture Medium Compo-nents	Lifeline Cell Technology, USA
DermaLife Calcium-free basal medium	Lifeline Cell Technology, USA
Dimethylsulfoxid (DMSO)	Merck, Darmstadt
DNA Minikit	Quiagen, Hilden
D-PBS w/o Mg/Ca	Gibco /Invitrogen, Paisley, Scotland
D-PBS + Mg/Ca	Gibco /Invitrogen, Paisley, Scotland
EDTA (0,5 M, pH 8,0)	Gibco/Invitrogen, Paisley, Scotland
Ethanol absolute	Merck, Darmstadt
Foxp3 Staining Set (human)	Miltenyi Biotech, Bergisch Gladbach

Gentamycin	Gibco/Invitrogen, Paisley, Scotland
Glycerol	Sigma, München
H_2O_2	Sigma-Aldrich, München
H_2SO_4	Merck, Darmstadt,
Heparin-Natrium 250.000U	Ratiopharm, Ulm
Human serum	Gibco /Invitrogen, Paisley, Scotland
Intracellular staining kit	BD Biosciences, Heidelberg
L-Glutamin	Gibco /Invitrogen, Paisley, Scotland
Lymphoprep	FreseniusKabiNorge AS, Oslo, Norway
2-Mercapto-Ethanol	Sigma, München
MRS Agar	Merck, Darmstadt
MRS Bouillon	Merck, Darmstadt
Naïve T cell isolation kit (human)	Miltenyi Biotech, Bergisch Gladbach
NaN3 (sodium azide)	Merck, Darmstadt
Na-Pyruvat	Gibco /Invitrogen, Paisley, Scotland
"Non-essential Amino Acids"	Gibco /Invitrogen, Paisley, Scotland
Penicillin-Streptomycin	Gibco /Invitrogen, Paisley, Scotland
Perfusor syringes	Braun, Melsungen
PGE_2 (10^{-2}M)	Cayman Chemical, Tallinn, Estonia
PHA (Lectin)	Sigma, München
2-Propanol	Merck, Darmstadt
Propidiumiodide (PI)	Sigma, München
rh GM-CMSF ($5x10^5$)	ImmunoTools, Friesoythe
rh IFNγ (10^6 U/ml)	R&D Systems, Wiesbaden
rh IL-4 ($5x10^5$)	ImmunoTools, Friesoythe
rh IL-1ß	Miltenyi Biotech, Bergisch Gladbach
rh IL-2	Proleukin, Novartis, Basel, Switzerland
rh IL-6	Promocell, Heidelberg
rh IL-12	Promocell, Heidelberg
rh IL-23	eBioscience, Alasdar Stewart, UK
rh IL-17	R&D GE Healthcare UK limited, Wiesbaden
rh IL-22	R&D GE Healthcare UK limited, Wiesbaden
rh TGFß (100U/ml)	Promocell, GmbH
rh TNF-α	R&D GE Healthcare UK limited, Wiesbaden
RPMI 1640 + L-Glutamin	Gibco /Invitrogen, Paisley, Scotland

Sodium pyruvat	Gibco /Invitrogen, Paisley, Scotland
Streptavidin-horseradish peroxidase	R&D GE Healthcare UK limited, Wiesbaden
Tetramethylbenzidin (TMB)	Fluka, (Sigma-Aldrich) München
Trypanblue 0.4% solution	Gibco /Invitrogen, Paisley, Scotland
Trypsin 0.05% EDTA	Sigma, München
Tween 20 detergent	Calbiochem, San Diego
Ultra pure LPS	Invivogen, San Diego
Zymosan (S.cervisiae)	Sigma, München

3.1.2 Antibodies and ELISA kits

Antibody/ELISA-Kit	**Supplier**
Anti-CCR7 PE	BD Biosciences, Heidelberg
Anti-CD3 (anti-human)	BD Biosciences, Heidelberg
Anti-CD28 (anti-human)	BD Biosciences, Heidelberg
Anti-CD1a PE	eBioscience, Alasdar Stewart, UK
Anti-CD4 FITC	BD Biosciences, Heidelberg BD Biosciences,
Anti-CD14 FITC	eBioscience, Alasdar Stewart, UK
Anti-CD25 PE	BD Biosciences, Heidelberg
Anti-CD40 FITC	BD Biosciences, Heidelberg
Anti-CD80 FITC	BD Biosciences, Heidelberg
Anti-CD83 PE	eBioscience, Alasdar Stewart, UK
Anti-CD86 APC	BD Biosciences, Heidelberg
Anti-Foxp3-APC	Miltenyi Biotech, Bergisch Gladbach
Anti-IFN-γ	eBioscience, Alasdar Stewart, UK
Anti-IL-4 (rat anti-human)	eBioscience, Alasdar Stewart, UK
Anti-IL-12 (mouse anti-human)	eBioscience, Alasdar Stewart, UK
Anti-HLA-DR APC	eBioscience, Alasdar Stewart, UK
ELISA kit (IL-12p70, IL-6, IL-23)	eBioscience, Alasdar Stewart, UK
ELISA kit (IL-1ß, IL-10)	BD Biosciences, Heidelberg
ELISA kit (IL-17, IL-22, IFN-γ)	R&D Systems, Wiesbaden
ELISA kit human β-Defensin	Phoenix Pharmaceuticals, USA
Mouse IgG1 FITC	BD Biosciences, Heidelberg
Mouse IgG1 PE	BD Biosciences, Heidelberg
Mouse IgG1 APC	BD Biosciences, Heidelberg
Nuclear Extract Isolation kit	Active Motif, Belgium

STAT1 TransAM	Active Motif, Belgium
STAT3 TransAM	Active Motif, Belgium

3.1.3 Materials

Material	**Supplier**
AnaeroGen packages	Oxoid Ltd., Basingstoke, Hampshire, England
Clustertubes 1.2ml	Abgene, Surrey, UK
Cryotubes 1.8ml	Nunc, Roskilde, Denmark
EDTA-Monovettes	Sarstedt, Nümbrecht
Falcon tubes 15ml	Becton Dickinson, NJ, USA
Falcon tubes 50ml	Becton Dickinson, NJ, USA
Heatsealing paper	Perkin Elmer, Rodgau-Rüdesheim
Maxi sorp plates (96 well)	Nunc, Roskilde, Denmark
MeltiLex TMA (β-counter)	Perkin Elmer, Rodgau-Rüdesheim
Printed Filtermat (β-counter)	Perkin Elmer, Rodgau-Rüdesheim
Petri Dish	Greiner bio-one, Frickenhausen
Pipette tips	Sarstedt Eppendorf, Newton, USA
Pipettes (1, 5, 10, and 25 ml)	Greiner bio-one, Frickenhausen
Printed Filtermat (β-counter)	Perkin Elmer, Rodgau-Rüdesheim
Serum monovettes	Sarstedt, Nümbrecht
Sterile filter (0.22; 0.45 µm)	Millipore express, Cork, Ireland
Sterile filter device (250ml, 500ml)	Millipore, Billerica, USA
Tissue culture flask (175 cm^2, 650ml)	Greiner bio-one, Frickenhausen
Tissue culture plates (96 well; 24 well, 6well) flat/U-bottom	Sarstedt, Nümbrecht
Uvette 50–2000 microliter; 220-1600 nm	Eppendorf, Hamburg

3.1.4 Media

All media and buffers were sterile filtered and stored at 4°C.

Medium	
HU-DC medium	50 ml FCS (Fetal Bovine Serum)
	2,5 ml Gentamycin (10M)
	5 ml L-Glutamin (200mM)
	450 ml RPMI (+L-Glutamin)
Proliferation medium	5 ml L-Glutamin (200mM)
	25 ml Human serum

	500 µl 2-Mercaptoethanol
	5,6 ml Sodium-Pyruvate
	5,6 ml Non-essential Amino Acids
	5 ml Penicillin-Streptamycin
	450 ml RPMI (+L-Glutamin)

3.1.5 Instruments

Instrument	**Supplier**
AutoMACSpro	Miltenyi biotec, Bergisch Gladbach
Bio Photometer	Eppendorf, Hamburg
Centrifuge	Thermo scientific, Schwerte
Centrifuge Megafuge 1.0R	Heraeus, Hanau
ELISA-Reader Mrx tc revelation	Thermo Labsystems, Chantilly, VA, USA
FACSCalibur	Becton Dickinson Heidelberg
Incubator	Heraeus, Hanau
Incubator for bacteria	Thermoshake Gerhardt, Königswinter
Micro scale	MC1 Research, Sartorius, Göttingen
Microscope Axiovert	Zeiss, Jena
Multichannel Pipettes	Eppendorf, Hamburg,
Pipettes with disposable tips	Eppendorf, Hamburg,
Precision balance	Kern 770, Witten
Shaker Titramax 100	Heidolph, Schwabach
Water quench Typ1003	GFL, Burgwedel

3.2 Methods

3.2.1 Patients and healthy volunteers

For *Candida*-based studies 12 CMC patients (5 with isolated CMC, 7 with APS1) were enrolled in the study. Blood samples of three patients with isolated CMC were kindly provided by the Department of Dermatology Tübingen and samples of APS1 patients by the Department of Pediatrics-Neonatal Intensive Care, V. Fazzi Regional Hospital in Italy. Controls were healthy volunteers, matched for sex and age, with no *Candida*-infection and volunteers with a current *Candida*-infection (Candidiasis), diagnosed by laboratory testing with common diagnostic methods in the Department of Dermatology Munich.

For LAB-based experiments healthy non-atopic volunteers were included in the study (total serum IgE levels lower 100kU).

3.2.2 Isolation of peripheral blood monocytes (PBMCs)

Peripheral blood monocytes (PBMCs) from CMC patients, patients with a current *Candida* infection and healthy volunteers were isolated from peripheral blood by density gradient centrifugation. 25 ml heparinized blood was diluted in 25 ml D-PBS w/o $Ca2^{+}$/$Mg2^{+}$ and 25 ml of this dilution was layered carefully over 10 ml lymphoprep and centrifuged (15 min, 2000 rpm, without brake). Because of the density of the lymphoprep (1.077 g/ml) erythrocytes and granulocytes get collected at the bottom of the column while PBMCs (lymphocytes, monocytes und thrombocytes) get build the interphase between lymphoprep and plasma. The interphase containing PBMCs was removed, washed three times with 5 mM EDTA in D-PBS w/o and counted in trypane-blue. For the candida-based experiments PBMCs of CMC patients and controls were cryopreserved in FCS and DMSO in liquid nitrogen (N_2) to obtain storage of the rare samples for further experiments because of the complexity and rareness of the disease.

3.2.2.1 Stimulation of peripheral blood monocytes (PBMCs) with *Candida albicans*

PBMCs of CMC patients and healthy controls were stimulated with *Candida albicans* (50 µg/ml), PHA (1 %) or anti-CD3/anti-CD28 (1 µg/ml) for 48-72 h. Afterwards, supernatants were investigated for secretion of cytokines IL-17A, IL-22, IL-10, IL-6,

IL-1β, IL-23, IFN-γ and CCL20 by ELISA according to the manufacturer's protocol. Cytokines were also analyzed by intracellular staining (Intraellular staining kit BD) by flow cytometry. After 72 h proliferation was examined by using the ^{3}H-thymidine assay adding 2Ci/ml ^{3}H-thymidine to the culture for further six hours. During each cell cycle cells incorporate ^{3}H-thymidine into its DNA. Afterwards cells were harvested on a glassfiber filter where intact DNA gets collected on the filter whereas small DNA fragments pass trough. The amount of radioactive DNA harvested on the filter correlates with the proliferation rate of the cells during incubation. After the filter was dried, the filter was melted on a scintillator sheet and radioactivity was analyzed by a scintillation β-counter. The measured radioactivity in counts per minute (cpm) corresponds to the number of cells or number of cell divisions in the well during incubation. Untreated cells served as a control.

3.2.2.2 DNA Isolation from PBMCs or whole blood and whole exome sequencing analysis/Sanger sequencing

Three iCMC patients and some of their healthy relatives were tested for STAT1 mutations using whole exome sequencing in cooperation with Jeau Laurent Casanova, St.Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, The Rockefeller University New York. For STAT1 exome sequencing DNA from PBMCs or whole blood of iCMC patients and healthy relatives was isolated using the QIAamp DNA Blood Mini Kit from Qiagen.

All centrifugation steps were carried out at room temperature (15–25°C) and all samples had to be equilibrated to room temperature. 20 µl QIAGEN Protease and 4 µl RNase A stock solution were added to 5x10^{6} PBMCs in 200 µl D-PBS or added to 200 µl whole blood in a 1.5 ml microcentrifuge tube. After further adding of 200 µl Buffer AL the sample got vortexed for 15 s and incubated at 56 °C for 10 min. The sample got briefly centrifuged to remove drops from the inside of the lid and 200 µl ethanol (96–100 %) were added to the sample. After mixing the sample by pulse-vortexing for 15 s and a short centrifugation step the sample was then applied to the QIAamp Mini spin column (in a provided 2 ml collection tube). The QIAamp Mini spin column was placed in a clean 2 ml collection tube after centrifugation at 8000 rpm for 1 min the and the tube containing the filtrate was discarded. 500 µl Buffer AW1 was added into the spin column and centrifuged at 8000 rpm for 1 min. Again, the QIAamp Mini spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was

discarded. After adding 500 µl Buffer AW2 and centrifugation at full speed at 14,000 rpm for 3 min the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. Finally 70 µl Buffer AE or distilled water was added into the spin column, incubated at room temperature (15–25 °C) for 1 min and then centrifuged at 8000 rpm for 1 min.

The DNA-content was determined at the NanoDrop and 5 µg DNA was sent to Prof. Casanova for exome sequencing analysis. Remaining DNA samples were stored at -20 °C for long-term storage.

In this cooperative study probands of five multiplex kindreds with iCMC were analyzed using whole exome sequencing where selectively protein-coding regions in the human genomic DNA are investigated. Identified mutations were then sequenced by the Sanger sequencing method. In chain terminator sequencing (Sanger sequencing), an oligonucleotide primer is annealed to a single-stranded DNA template and extended using a DNA polymerase in the presence of four deoxyribonucleoside triphosphates (dNTPs). In the reaction included is also one of four dideoxyribonucleoside triphosphates (ddNTPs) which terminate elongation when incorporated into the growing DNA chain. The obtained DNA fragments after completion of the sequencing reactions are then size-separated by electrophoresis on a high-resolution denaturing polyacrylamide gel to obtain the DNA sequence (476-478).

3.2.2.3 Stimulation with IL-6/IFN-γ and following signal transducer and activator of transcription3/1 (STAT3/STAT1) analysis

To investigate STAT3 activation in PBMCs of CMC patients and healthy controls PBMCs were stimulated with the STAT3-activating cytokine rhIL-6 and with IFN-γ for the analysis of STAT1 activation followed by isolation of nuclear extracts and analysis of these extracts in the STAT3 or STAT1 TransAM. PBMCs of CMC patients and healthy controls were stimulated with 20 ng/ml rhIL-6 (STAT3 activation) or 100 U/ml IFN-γ (STAT1 activation) for one hour and lysed with the Nuclear extract kit (Actife Motiv) for analyzing the lysates in the STAT3 or STAT1 TransAM (Active Motif). For the isolation of nuclear extracts with the Nuclear extract kit buffers were prepared as indicated in the kit manual, cells of each stimulation condition were collected in pre-cooled tubes in the presence of 3-5 ml phosphatase Inhibitor diluted in ice-cold D-PBS w/o to limit further protein modifications (expression, proteolysis, dephosphorylation, etc.). After washing cells twice with 3-5 ml D-PBS w/o with phosphatase inhibitor cells

were resuspended in 500 µl hypotonic buffer to swell the cell membrane and make it fragile. Then cells were transferred in cold tubes and incubated for 15 min on ice followed by the addition of detergent that causes the leakage of the cytoplasmic proteins into the supernatant. A further centrifugation step at 4°C (14000 rpm, 30sec) allowed the collection of the supernatant that contains the cytoplasmic fraction; the lysed nuclei in the pellet were resuspended in 50 µl lysis buffer and were vortexed every 5 min for 30 min. In this step nuclear proteins get solubilised in the lysis buffer in the presence of the protease inhibitor cocktail. Finally nuclear extracts were obtained from the supernatant by centrifugation at 4 °C (14000 rpm, 10 min). The protein concentration of the cell extract of each sample was then determined by the usage of Coomassie reagent and absorbance was measured at 595 nm on the photospectrometer.

Afterwards the translocation and DNA-binding capacity of STAT3 or STAT1 in the nuclear extracts was investigated using the STAT3 or STAT1 TransAM, an enzyme-linked immunosorbent assay (ELISA)-based assay. The TransAM comprises a 96-well plate on which oligonucleotides containing a STAT3 or STAT1 consensus binding site have been immobilized. Buffers were prepared as indicated in the kit manual. Adding 30 µl complete binding buffer and 2-20 µg nuclear extract of each sample diluted in lysis buffer allows STAT3/STAT1 contained in the nuclear extracts to bind specifically to its oligonucleotides coated on the plate. After incubation at room temperature on a shaking platform (100 rpm) wells were washed three times with 200 µl washing buffer and 100 µl STAT3/STAT1-antibody 1:500 diluted in antibody binding buffer were added per well so the active form of bound STAT3/STAT1 gets detected by its specific antibody. The plate was incubated at room temperature for one hour without agitation followed by a three times washing step. 100 µl horseradish peroxidase (HRP)-conjugated secondary antibody 1:1000 diluted in antibody binding buffer was added for one hour without shaking. After washing the plate four times 100 µl developing solution per well was added following 100 µl stop solution and absorbance at 450 nm was measured on the photospectrometer.

3.2.3 Generation of human monocyte-derived dendritic cells (MoDC)

The monocyte-derived dendritic cells (MoDC) were generated from $CD14^+$ monocytes from peripheral blood of CMC patients and healthy volunteers. First PBMCs were isolated by density gradient centrifugation as described in point 3.3.2 and $CD14^+$ monocytes were purified by magnetic cell sorting (MACS) with bead-coupled antibodies

against the surface molecule CD14. 30 µl anti-CD14 microbeads and 130 µl MACS buffer per 10^8 PBMCs were incubated for 15 min at 4 °C and separated by positive selection through a magnetic column. $CD14^+$ monocytes were then counted, washed and cultured in complete HU-DC medium for five days (37 °C, 5 % CO_2) in the presence of 50 U/ml rhGM-CSF and rhIL-4. Cultures were fed after two or three days by removing half of the medium and adding back the same amount of complete medium containing 50 U/ml fresh rhGM-CSF and rhIL-4. On day five immature MoDC were harvested, resuspended in complete HU-DC and counted. Maturation markers (CD80, CD83, CD86, HLA-DR) and CD1a were characterised using flow cytometry.

3.2.3.1 Stimulation of monocyte-derived dendritic cells (MoDC) with *Candida albicans* and its cell wall compounds

To investigate the impact of *Candida* and its cell wall structures on MoDC, on day five of DC culture, immature characterized MoDC as described in point 3.3.3. were seeded into 96-well flat bottom plates at a density of $1x10^5$ cells/ml and stimulated with 300 µg/ml Curdlan, 100 µg/ml Zymosan or LPS (100 ng/ml). Untreated MoDC served as control. After 48 h incubation at 37 °C and 5 % CO_2, cytokines IL-23, IL-1β, IL-10, TNF-α, IL-6 and IL-12p70 were analysed in cell-free supernatant by ELISA according to the manufacturer's protocol. MoDC were washed twice with D-PBS w/o, resuspended in proliferation medium and counted. MoDC at $0.1x10^6$ cells/ml per well were added to the naïve $CD4^+CD45^+$ T cells in the *Candida*-based allogenic stimulation assay (ASA) described in point 3.3.4.1.

3.2.3.2 Stimulation of monocyte-derived dendritic cells (MoDC) with lactic acid bacteria (LAB) strains

To analyse the impact of LAB on DCs, on day five of DC generation, the immature and characterized MoDC as described in point 3.3.3 were stimulated with different concentrations (10^7-10^5 cfu) of the bacterial strains IMS1 and IMS2. For patent reasons the bacterial strains are blinded as immunmodulatory strain (IMS) 1 and 2. The bacterial concentrations of 10^7-10^5 cfu were prepared from re-thawed stocks of the bacterial strains by serial dilution in D-PBS w/o as described in point 3.3.8. Immature MoDC were resuspended at $6x10^6$ cells/ml in complete HU-DC medium, plated in 96-well flat bottom plates and treated with 10^7-10^5 cfu bacterial isolates in a total of 200 µl per well. To obtain a positive control for DC stimulation 100ng/ml LPS alone was added to MoDC and pure HU-DC medium was used as negative control. Following 24 h incuba-

tion at 37 °C and 5 % CO_2, the culture supernatants were analyzed for cytokines IL-12p70, IL-10, IL-1β, IL-6 and IL-23 by ELISA according to the manufacturer's protocol.

Additionally immature MoDC were stimulated on day five of generation with the bacterial isolates for following co-incubation with naïve T cells in order to analyze the T cell response induced by bacteria-matured DCs. Therefore immature MoDC were plated in a 24-well plate at $6x10^5$ cells/ml per well and treated with the different bacterial isolates in the concentrations of 10^7-10^5 cfu. In order to obtain a positive control for Th1 polarization MoDC were stimulated with 100 ng/ml LPS plus 10^6 U/ml IFN-γ and 100 ng/ml LPS plus 10^{-2} M PGE_2 were added to MoDC to get a positive control for Th2 differentiation. Pure HU-DC medium was used as both negative control and for Treg polarisation, addition of 100 ng/ml LPS alone to MoDC was used as both positive control for DC stimulation. After 24 h incubation at 37 °C and 5 % CO_2, MoDC were washed twice with D-PBS w/o, resuspended in proliferation medium and counted. MoDC at $0.1x10^6$ cells/ml per well were added to the naïve $CD4^+CD45^+$ T cells in the bacteria-based allogenic stimulation assay described in point 3.3.4.2.

3.2.4 Coculture of DC - T cells in the allogenic stimulation assay (ASA)

To investigate the T cell responses induced by *Candida*- or LAB-stimulated MoDC, freshly isolated or N_2-re-thawed allogenic naïve and memory T cells were co-incubated with the differently stimulated MoDC at a DC: T cell ratio of 1:10 in proliferation medium.

3.2.4.1 *Candida*-based allogenic stimulation assay (ASA) with naïve and memory T cells

For the C*andida*-based allogenic stimulation assay MoDC stimulated as described in point 3.3.3.1 were used as antigen presenting cells. After 48 h incubation stimulated MoDC were washed twice in D-PBS w/o, resuspended in proliferation medium, counted and re-plated in 96 flat-bottom well plates at a density of 1 x 10^4 MoDC per well.

Allogenic naïve and memory T cells for the co-culture were either generated from fresh or re-thawed PBMCs from N_2 of CMC patients and healthy controls by MACS using the naïve $CD4^+CD45RA^+$ T cell isolation kit from Miltenyi. PBMCs were resuspended in 20 µl MACS running buffer and 10 µl biotin-coctail per 10^7 cells and incubated for 15 min at 4° C. After a second labelling step with 20 µl beads per 10^7 PBMCs for

15 min at 4 °C, naïve T cells were isolated by magnetically separation. The negatively separated fraction of naïve $CD4^+CD45RA^+$ T cells was used as naïve T cells and the other positively separated T cell fraction was used as memory T cells in the co-culture. Both T cell fractions were counted, washed and resuspended in proliferation medium at a density of $1x10^6$/ml. $1x10^5$ of both naïve $CD4^+CD45RA^+$ and memory T cells were added to MoDC in 96-well flat bottom plates for co-incubation at a DC:T cell ratio at 1:10 for five to six days. MoDC and T cells of CMC patients were co-incubated in different donor conditions: Healthy MoDC were co-cultured with healthy naïve T cells, MoDC of CMC patients with healthy T cells, healthy MoDC with CMC T cells and CMC MoDC with CMC T cells. On day five to six cytokines IL-17A, IL-22, IL-10 and IFN-γ were measured both in the cell-free supernatant by ELISA according to the manufacturer's instructions and by intracellular staining (intracellular staining kit BD) by flow cytometry. Furhermore the proliferation capacity was analysed using the ^3H-thymidine assay.

3.2.4.2 Lactic acid bacteria-based ASA with naïve T cells

For the LAB-based allogenic stimulation assay MoDC stimulated as described in point 3.3.3.2 were used as antigen presenting cells.

After 48h incubation stimulated MoDC were washed twice in D-PBS w/o, resuspended in proliferation medium, counted and re-plated in 96 flat-bottom well plates at a density of 1×10^4 MoDC per well. Allogenic naïve T cells for the co-culture were generated from fresh PBMC of healthy, non-atopic volunteers by negative MACS separation using the naïve $CD4^+CD45RA^+$ T cell isolation kit from Miltenyi as described in point 3.3.4.1. Isolated naïve $CD4^+CD45RA^+$ T cells were counted, washed and resuspended in proliferation medium at a density of $1x10^6$/ml. $1x10^5$ naïve $CD4^+CD45RA^+$ T cells were plated in 96-well flat bottom plates for co-incubation with MoDC at a DC:T cell ratio at 1:10 in a final volume of 200 µl for seven days. As a positive control for Th1 polarization naive T cells were primed with LPS plus IFN-γ stimulated MoDC and 5 µg/ml anti-IL-4 and 250 ng/ml rhIL-12 was added. To obtain a positive control for Treg polarization 5 ng/ml rhTGF-β, 200 U/ml rhIL-2, 5 µg/ml anti-IL-12 and 1 µg/ml anti-IFN-γ were added to medium-/LPS-primed T cells. T cells with medium alone and MoDC with medium or LPS alone were used as controls as well. 20 U/ml rhIL-2 was added to the cells on the third day of co-culture. On day seven of coculture supernatants were analyzed for cytokines IL-10 and IFN-γ by both ELISA and intracellular staining

(intracellular staining kit BD) by flow cytometry. Additionally proliferation capacity was measured on day seven by thymidine incorporation using the ^{3}H-thymidine assay and cells from the ASA were also restimulated with anti-CD3/anti-CD28 (1 µg/ml). After 48h of restimulation the expression of surface markers CD4, CD25 and the transcription factor Foxp3 was investigated by intracellular staining (Foxp3 staining kit eBioscience) by flow cytometry.

3.2.4.2.1 Suppression assay of T cells induced in the LAB-based ASA

The suppression assay represents a method to analyze the function of effector cells to suppress proliferation of responder cells. Suppression of proliferation can be investigated by using the ^{3}H-thymidine incorporation assay as described in point 3.3.2.1. where proliferation is measured over a period of time or by staining the responder cells with the fluorescent dye CFSE (Carboxyfluorescein succinimidyl ester) that allows to monitor the migration behaviour of single cells. During each cell cycle CFSE molecules are equally distributed to dividing daughter cells by halving the fluorescence intensity. More cell division cycles result in a decreasing signal of fluorescence intensity that allows detecting the numbers of cell divison by flow cytometry.

Differentiated T cells from the ASA were harvested on day seven as shown in figure 10 and cultured with CFSE-labelled responder $CD4^{+}$ T cells in proliferation medium for five days.

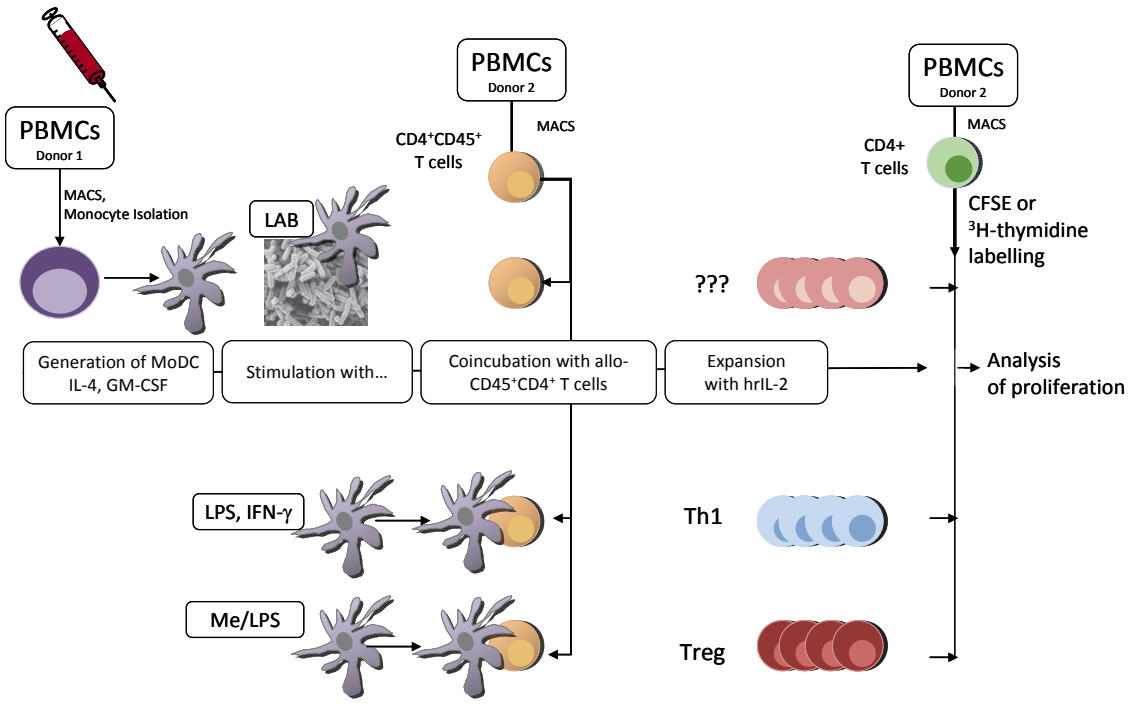


Figure 10: Methodical scheme of the bacteria-based suppression assay

$CD4^+$ T cells of the same healthy T cell donor from the ASA were obtained by depletion of $CD14^+$ cells and following positive separation of $CD4^+$ T cells using the $CD4^+$ T cell isolation kit from Miltenyi. Afterwards $CD4^+$ T cells were stained with 0.5 µM CFSE in D-PBS w/o in the dark under agitation four 4 min. Afterwards the reaction was stopped with cold D-PBS+10 % FCS, cells were washed two times with cold D-PBS w/o and plated in anti-CD3 coated 96-well flat bottom plates in proliferation medium at density of $1x10^6$/ml in the presence of 1 µg/ml soluble anti-CD28 and rhIL-2 (20 U/ml). $1x10^5$ effector T cells from the ASA were added at a effector T cell:responder T cell ratio of 1:1 in a final volume of 200 µl. CFSE-labelled $CD4^+$ T cells alone served as negative control. To CFSE-labelled $CD4^+$ T cells coincubated with generated regulatory T cells from the ASA 1 µg/ml soluble anti-CD28 and 100 U/ml rhIL-2 was added. $CD4^+CD25^+Foxp3^+CD127^-$ cells were isolated from the same T cell donor by using the Treg isolation kit from Miltenyi. These real Tregs were also coultured with CFSE-labelled $CD4^+$ responder T cells in the presence of 1 µg/ml soluble anti-CD28 and 100 U/ml IL-2. Five days later cells were harvested and proliferation was analyzed by flow cytometry.

3.2.5 Analysis of T cell differentiation

Naïve $CD4^+CD45RA^+$ T cells of CMC patients and healthy controls were purified from fresh or re-thawed PBMCs from N_2 by using the naïve T cell isolation kit (Miltenyi Biotec) as described in point 3.3.4.1. For the experiments of Th17 differentiation naïve T cells were stimulated for seven days in serum free AIM V medium with anti-CD3 and anti-CD28 (1 µg/ml) in the presence of following reagents: IL-6 (30 ng/ml), IL-23 (30ng/ml), IL-1β (20 ng/ml), anti-IL-4 (5 µg/ml) and anti-IFN-γ (1 µg/ml) for Th17 induction. For experiments with polarization of different T cell lines naïve T cells were also incubated five to six days in serum free AIM V medium with anti-CD3 and anti-CD28 (1 µg/ml) in the presence of following reagents: IL-12 (250 ng/ml) and anti-IL-4 (5 µg/ml) for Th1 differentiation, IL-6 (30 ng/ml), IL-23 (30 ng/ml), IL-1β (20 ng/ml), TGF-β (3 ng/ml), anti-IL-4 (5 µg/ml) and anti- IFN-γ (1 µg/ml) for Th17 induction. The supernatant was screened for the cytokines IL-17A, IFN-γ and IL-22 by ELISA according the manufacturer's instructions and cytokines were also intracellular stained using the intracellular staining kit (BD) for analysis by flow cytometry.

3.2.6 Gene expression analysis using Agilent Whole Human Genome Oligo Microarrays (one color) by Miltenyi

Untouched T cells of iCMC patients and healthy controls were stimulated with anti-CD3 and anti-CD28 for 0 and 6 hours and cell pellets were sent to Miltenyi for gene expression analysis using Agilent Whole Human Genome Oligo Microarrays (one color).

Untouched T cells were generated from re-thawed PBMCs from N_2 of iCMC patients and healthy controls by MACS using the naïve Pan T cell isolation kit from Miltenyi. Pan T cells are isolated by depletion of non-T cells (negative selection) and non-T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads as secondary labeling reagent.

PBMCs were resuspended in 40 µl MACS running buffer and 10 µl biotin-coctail per 10^7 cells and incubated for 10 min at 4 °C. After a second labelling step with 30 µl running buffer and 20 µl beads per 10^7 PBMCs for 15 min at 4 °C, untouched T cells were washed and isolated by magnetically separation using the AutoMACS. The Pan T cell fraction was counted, washed and resuspended in proliferation medium at a density of

1-3x10^6/ml. According to the obtained number of available cells Pan T cells were plated into in anti-CD3 coated 96-well at a density of 3x10^5 cells per well in a total volume of 200µl or into anti-CD3 coated 24-well flat bottom plates at a density of 5x10^5 per well in a total volume of 1.5 ml in the presence of 1 µg/ml soluble anti-CD28 and rhIL-2 (20 U/ml). Unstimulated T cells served as untreated control and after 6 h of stimulation with anti-CD3/CD28 T cells were centrifuged at 4 °C (1200 rpm, 10 min) and cell pellets were freezed in N_2 for 1 min and stored at -80° C.

Samples were sent to Miltenyi to analyze gene expression of the different cell samples. RNA was isolated using standard RNA extraction protocols (Nucleo Spin RNAII). Quality and integrity of RNA samples was checked with the Agilent 2100 Bioanalyzer platform (Agilent Technologies). 0.2-0.5 µg of each total RNA sample was amplified and Cy3 labeled using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) following the manufacturer's protocol. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit and Agilent Whole Human Genome Oligo Microarrays 4x44K (Agilent Technologies). Fluorescence signals of the hybridized arrays were detected using Agilent's Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software was used to read out and process the microarray image files, and differential gene expression Feature Extraction Software–derived output data files were further analyzed using the Gene Spring data analysis system (GeneSpring GX Software).

3.2.7 Analysis of cytokine secretion by enzyme-linked immunosorbent assay (ELISA) and flow cytometry

3.2.7.1 Flow cytometry

For intracellular staining of cytokines and transcription factors cells were fixed with paraformaldehyde to "fix" the cells/proteins followed by permeabilisation of cell membranes to allow the access of detecting antibodies to intracellular proteins.

For intracellular staining of cytokines cells were stained according to the instructions of the intracellular staining kit from BD. Cultured cells were stimulated with PMA (40 ng/ml) and ionomycin (1 µg/ml) for six hours and examined for intracellular cytokine accumulation. To prevent cytokine secretion, the stimulation was performed in the presence of Monensin (1:1000) (from the beginning) and Brefeldin A (1:1000) was

added for the final four hours. Addition of monensin and Brefeldin A to cell activation cultures blocks intracellular transport processes resulting in the accumulation of most cytokine proteins in the endoplasmic reticulum and enhancing cytokine staining signal. Cells were transferred in a 96well round bottom plate and fixed with 100 µl Cytofix/Cytoperm fixation/permeabilization solution for 20 min at 4 °C. After washing two times cells with 200 µl Perm/Wash staining buffer, fixed/permeabilisized cells were stained in 50 µl Perm/Wash staining buffer with human fluorochrome-conjugated anti-cytokine antibodies or isotype-matched control antibodies at 4 °C for 30 min in the dark. Finally cells were washed twice with 200 µl Perm/Wash staining buffer and resuspended in FACS-buffer. Acquisition and analysis was done using a FACS Calibur.

For intracellular staining of transcription factors the Foxp3 staining kit from eBioscience was used. Cultured cells were transferred in a 96well round bottom plate and stained for surface markers with human fluorochrome-conjugated anti-cytokine antibodies or isotype-matched control antibodies in MACS buffer at 4 °C for 15 min. After staining of surface markers the cell were washed with 200 µl MACS buffer and fixed with 200 µl Fixation/Permeabilisation solution for 30 min at 4 °C. The cell were washed twice, once in 200 µl MACS buffer and once in 200 µl Fixation/Permeabilisation buffer followed by adding 4 µl FcR-blocking antibody and the cell pellet was stained for transcription factors for 30 min at 4 °C. Afterwards cells were washed twice with 200 µl Fixation/Permeabilisation buffer, resuspended in FACS buffer and analysed by flow cytometry using FACS Calibur.

3.2.7.2 ELISA

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

3.2.8 Preparation of bacterial strains

The strains IMS1 and IMS2 were kindly provided by Univ. Prof. Dr.-Ing. Dr.-Ing. habil. W. Back, Department for Technology of Brewery I, TUM Weihenstephan.

Frozen stocks of IMS1 and IMS2 were inoculated in deMan, Rogosa, and Sharpe (MRS) medium and grown overnight at 48 °C (IMS1) and 37 °C (IMS2). To obtain mid-log cultures both strains were grown further four to six hours until an optical density of 1.0 (600 nm) was reached. For stationary phase cultures bacterial strains were

incubated for further 24 hours. Mid-log- and stationary phase-bacteria were harvested by centrifugation and washed three times with D-PBS w/o before stored at -80 °C as stock concentrations until they were used for stimulation experiments. To determine the number of bacteria in the culture by colony forming units (cfu) a serial dilution (1:10) was made of 500 µl of each strain culture and 100 µl of each dilution were plated on MRS agar plates for 48 hours in anaerobic jars. Agar plates of IMS1 were incubated at 48 °C, agar plates of IMS1 at 37 °C. For the use of bacteria in the experimental setups the stock concentrations at -80 °C were re-thawed and concentrations of 10^7-10^5 cfu were prepared by serial dilution in D-PBS w/o or HU-DC medium.

3.2.9 Isolation and culture of keratinocytes

Primary human keratinocytes of iCMC patients and healthy controls were isolated from the epidermis by suction blister as described earlier by Traidl et al. (479) and stored in liquid N_2. Experiments were performed in sterile 6-well culture plates with second-passage keratinocytes grown in keratinocyte growth medium (Lifeline) to 80 %-90 % confluence after seeding. At day five keratinocytes were washed five times with D-PBS+Ca/Mg and stimulated with rhIL-17 (50 ng/ml), rhIL-22 (50 ng/ml) and TNF-α (50 ng/ml) in different combinations in a total volume of two ml per well in Lifeline medium without hydrocortisone and incubated at 37 °C and 5 % CO_2. After 72 hours human β-Defensin 2 was measured in the cell-free supernatant by ELISA according to the manufacturer's instructions. Keratinocytes were detached with 0.05 % trypsin and 0.02 % EDTA and counted 1:2 in trypaneblue.

3.2.10 Statistical analysis

Data are presented as mean ± SEM and statistically analysed using the Mann Whitney Test, and $p \leq 0.05$ was considered significant.

4 Results

4.1 Characterization of CMC patients and controls

Characterization of patients with isolated CMC

The clinical features of five patients with isolated chronic mucucutaneous candidiasis (iCMC) are summarized in table 7. Two of the iCMC patients attended regularly the Department of Dermatology, Munich. Three iCMC patients were recruited for the study in cooperation with the Department of Dermatology Tübingen.

patient	CB	MF	MR	HC	HH
Year of birth	1973	1961	1963	1987	1959
Sex	F	F	F	F	F
Clinical symptoms	Chronic oral, esophageal, vaginal and cutaneous candidiasis, paronychia with nail dystrophy	Chronic oral, esophageal, vaginal and cutaneous candidiasis, paronychia with nail dystrophy	Chronic oral and cutaneous candidiasis	Chronic oral candidiasis, paronychia with nail dystrophy	Chronic oral and cutaneous candidiasis
Onset at age	early (4 years)	early (birth)	early (3. month)	early (4. month)	unknown
Endocrinology	normal	normal	autoimmunethyreoditis	normal	normal
Immunology	Squamous cell carcinoma, ANA+	Squamous cell carcinoma, ANA+	normal	normal	normal
Standard laboratory markers	CRP constantly elevated (3.5 mg/dl)	normal	no data	normal	no data
electrophoresis	Albumin 56.9% γ-globulin 22.0%	Albumin 55.9% γ -globulin 20.4%	no data	no data	no data
Candida-Abs serum	IgG <40 U/ml IgM <60 U/ml	IgG 170 U/ml IgM <60 U/ml	no Candida-antibodies	no Candida-antibodies	no data
Immunglobulins serum	normal	IgG 163.5 mg/l IgA 94 mg/l	IgG1 1344 mg/l	normal	no data
Phenotyping of Tcells	Lymphopenia	Lymphopenia	normal	normal	no data

Table 7: Clinical characterization of patients with isolated CMC

ANA= antinuclear antibody, CRP=C-reactive protein

Characterization of patients with APS1

Blood samples of patients with the autosomal-recessive polyglandular autoimmune syndrome type 1 (APS1) matching the criteria for CMC were provided by the Department of Pediatrics-Neonatal Intensive Care, V. Fazzi Regional Hospital in Italy. Genetic and endocrine features have been previously characterized (480-482). Characteristics of these patients including type of mutation and phenotype of the disease are listed in table 8.

patient	Sex	year of birth	AIRE mutation	phenotype (age at onset)
MA	F	1963	W78R homozygous	CMC (6), HPT (7), POF (23), A, EH
ML	M	1966	W78R homozygous	CMC (1), HPT (2), AD (13), HT (42), A, EH
BdL	F	1987	W78R homozygous	CMC (<1), HPT (1), AD (10), POF (18), A, EH, TC
GG	M	1985	W78R/Q358X	CMC (<1), HPT (1), AD (13), HT (10), AIH, (13), A, V, EH, TC
NM	M	1985	Q358X/W78R	CMC (<1), HPT (<1), AD (4), HT (11), EH
NG	M	1990	Q358X/W78R	CMC (7), HPT (8), AD (7), EH
NP	F	1994	V22_D23 del/W78R	CMC (<1), HPT (2), POF (15), HT (8)
QG	M	1978	C311fsX376 homoz.	CMC (13), HPT (6), A
CC	F	1993	P539L homozygous	CMC (<1), HPT (1), AD (5), POF (16)

Table 8: Clinical characterization of patients with APS1

S: sex; CMC: chronic/recurrent mucocutaneous candidiasis; HPT: hypoparathyroidism; AD: Addison disease; POF: premature ovarian failure; HT: hypothyroidism; AG: atrophic gastritis; AIH: autoimmune hepatitis; M. malabsorption; K: keratitis; A: alopecia; V: vitiligo; EH: dental enamel hypoplasia; TC: tympanic membrane calcifications

Characterization of patients with current *Candida*-infection (Candidiasis) and healthy controls

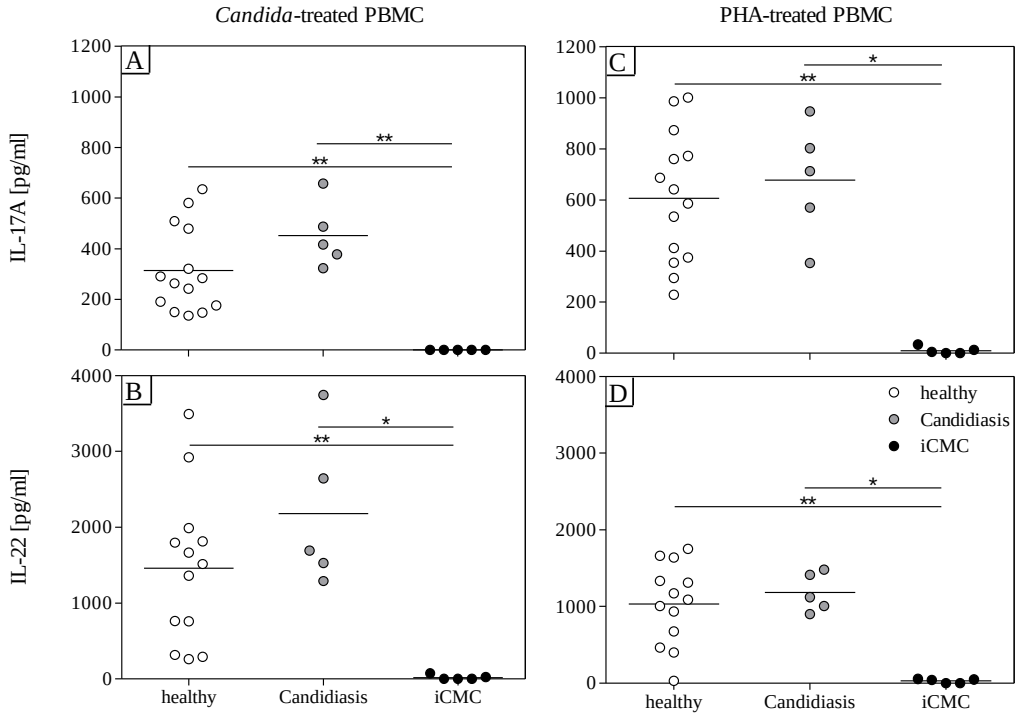
Patients with a current *Candida*-infection (Candidiasis) enrolled in this study attended the Department of Dermatology, Munich. *Candida*-infections of skin and nail biopsies or samples from any affected body area including mouth, vagina and others were identified by laboratory testing with common diagnostic methods. Volunteers with no *Candida*-infection were included in this study as healthy controls for the CMC experiments. Number of patients and controls may vary in this study because of the limitation of blood samples available.

For bacteria experiments non-allergic, non-atopic healthy volunteers were controls were screened for total IgE and for specific IgE against common allergens as described before (483). Individuals with total IgE lower 100 kU and no specific IgE were defined as healthy controls.

4.2 Analysis of the PBMC function of CMC patients

4.2.1 PBMCs of CMC patients show a distinct cytokine profile in response to *Candida*

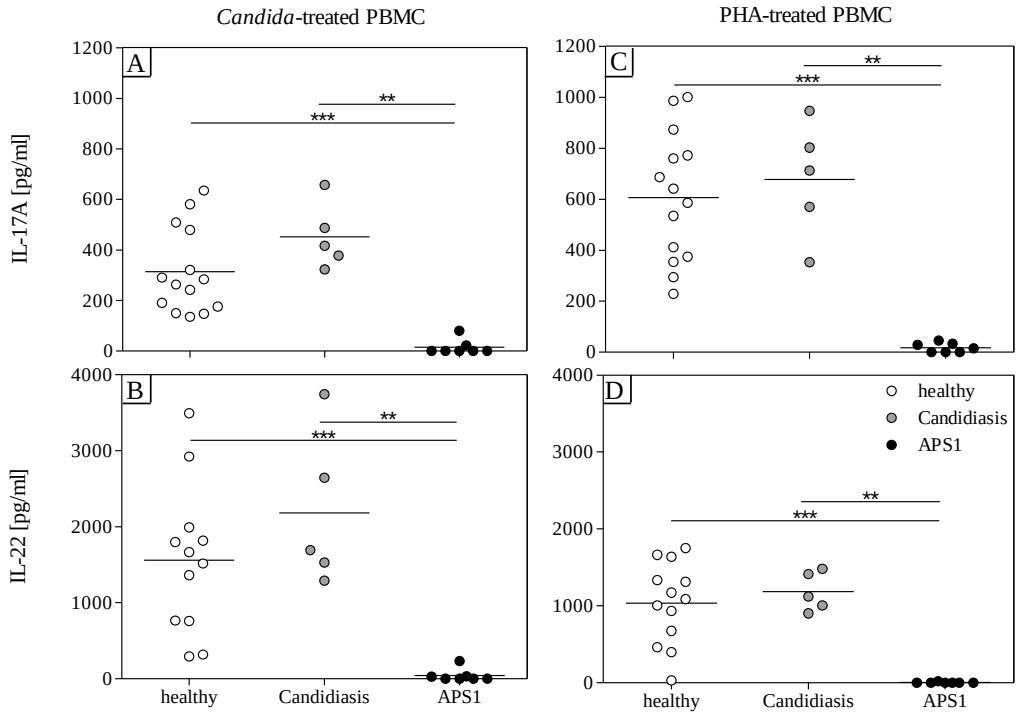
PBMCs of patients with isolated CMC (iCMC) (n=5), patients with a current *Candida*-infection (Candidiasis) (n=5) and healthy controls (n=14) were stimulated with *Candida albicans* or PHA and the supernatant was analyzed for cytokine production (figure 11). PBMCs of patients with a current *Candida*-infection produced higher amounts of IL-17A and IL-22 in response to *Candida* and PHA compared to healthy controls (Fig.11, A-D). In contrast, PBMCs of iCMC patients significantly released no IL-17A and IL-22 after stimulation with *Candida* or PHA (Fig. 11, A-D) compared to *Candida*-infected patients and healthy controls. The same defect of IL-17/IL-22 production in response to *Candida* could be observed for APS1 patients (n=7, Fig.12). PBMCs of APS1 patients secreted significantly reduced levels of IL-17A and IL-22 after stimulation with *Candida* (Fig.12, A-B) and PHA (Fig.12, C-D) compared to *Candida*-infected patients and healthy controls.



	Candida		PHA	
compared condition	IL-17A	IL-22	IL-17A	IL-22
iCMC vs. healthy	0.0014	0.0016	0.0014	0.0042
iCMC vs. Candidiasis	0.0112	0.0112	0.0119	0.0119
Candidasis vs. healthy	0.0710	0.2782	0.6770	0.6934

Figure 11: PBMCs of iCMC patients are characterized by reduced IL-17/IL-22 production in response to *Candida albicans*

PBMCs of iCMC patients (black circles), patients with a current *Candida*-infection (grey circles) and healthy controls (white circles) were stimulated with 50 µg/ml *Candida albicans* (A-B) or 1 % PHA (C-D) for 48 hours. Supernatant was analyzed for IL-17A (A,C) and IL-22 (B,D) secretion by ELISA. Results of PBMC stimulation of 5 iCMC patients, 5 patients with a current *Candida*-infection and 14 healthy controls are depicted as mean. *, $p<0.05$, **, $p<0.01$, Mann Whitney test. The analyzed p values are shown in the table



	Candida		PHA	
compared condition	IL-17A	IL-22	IL-17A	IL-22
APS1 vs. healthy	0.0003	0.0004	0.0003	0.0003
APS1 vs. Candidiasis	0.0042	0.0050	0.0054	0.0032
Candidasis vs. healthy	0.0710	0.3703	0.6770	0.6934

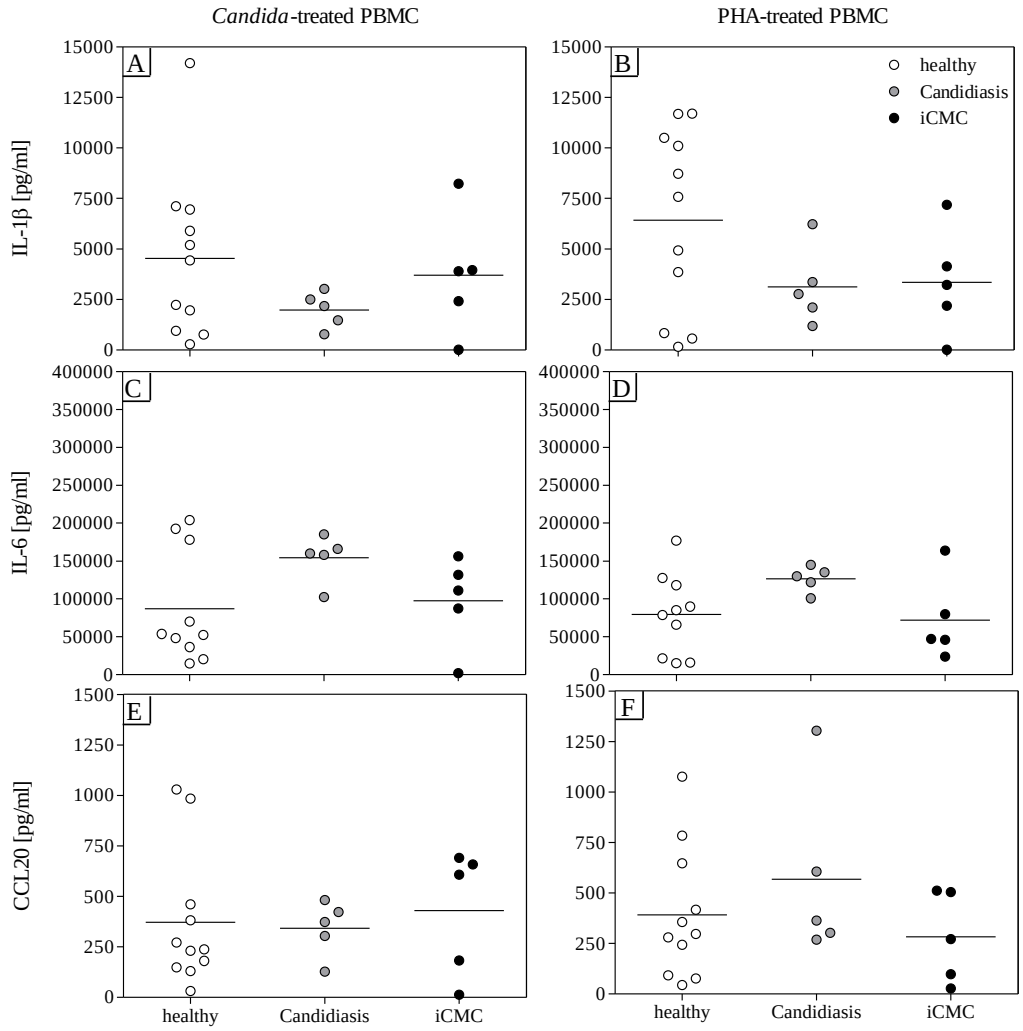
Figure 12: PBMCs of APS1 patients are characterized by reduced IL-17/IL-22 production in response to *Candida albicans*

PBMCs of APS1 patients (black circles), patients with a current *Candida*-infection (grey circles) and healthy controls (white circles) were stimulated with 50 µg/ml *Candida albicans* (A-B) or 1 % PHA (C-D) for 48 hours. Supernatant was analyzed for IL-17A (A,C) and IL-22 (B,D) secretion by ELISA. Results of PBMC stimulation of 7 APS1 patients, 5 patients with a current *Candida*-infection and 14 healthy controls are depicted as mean. *, $p<0.05$, **, $p<0.01$, ***, $p<0.1$, Mann Whitney test. The analyzed p values are shown in the table

The PBMC supernatant of iCMC patients was also screened for the Th17-differentiating cytokines IL-1β and IL-6 and the Th17-associated cytokine CCL20 (figure 13). PBMCs of iCMC and *Candida*-infected patients produced less IL-1β in response to *Candida* or PHA compared to healthy controls (Fig.13, A-B).

IL-6 secretion of PBMCs of iCMC was slightly decreased after stimulation with *Candida* compared to patients with a current *Candida*-infection whereas no significant differences between iCMC patients and healthy controls were detectable (Fig.13, C-D).

Concerning CCL20 secretion no significant differences could be observed in PBMCs of iCMC patients compared to the control groups (Fig.13, E-F).



	Candida			PHA		
compared condition	IL-1β	IL-6	CCL20	IL-1β	IL-6	CCL20
iCMC vs. healthy	0.6854	0.7679	0.7340	0.1848	0.8058	0.6504
iCMC vs. Candidiasis	0.2555	0.0556	0.5476	0.8870	0.0665	0.3095
Candidasis vs. healthy	1.932	0.2065	0.6504	0.1467	0.0782	0.3648

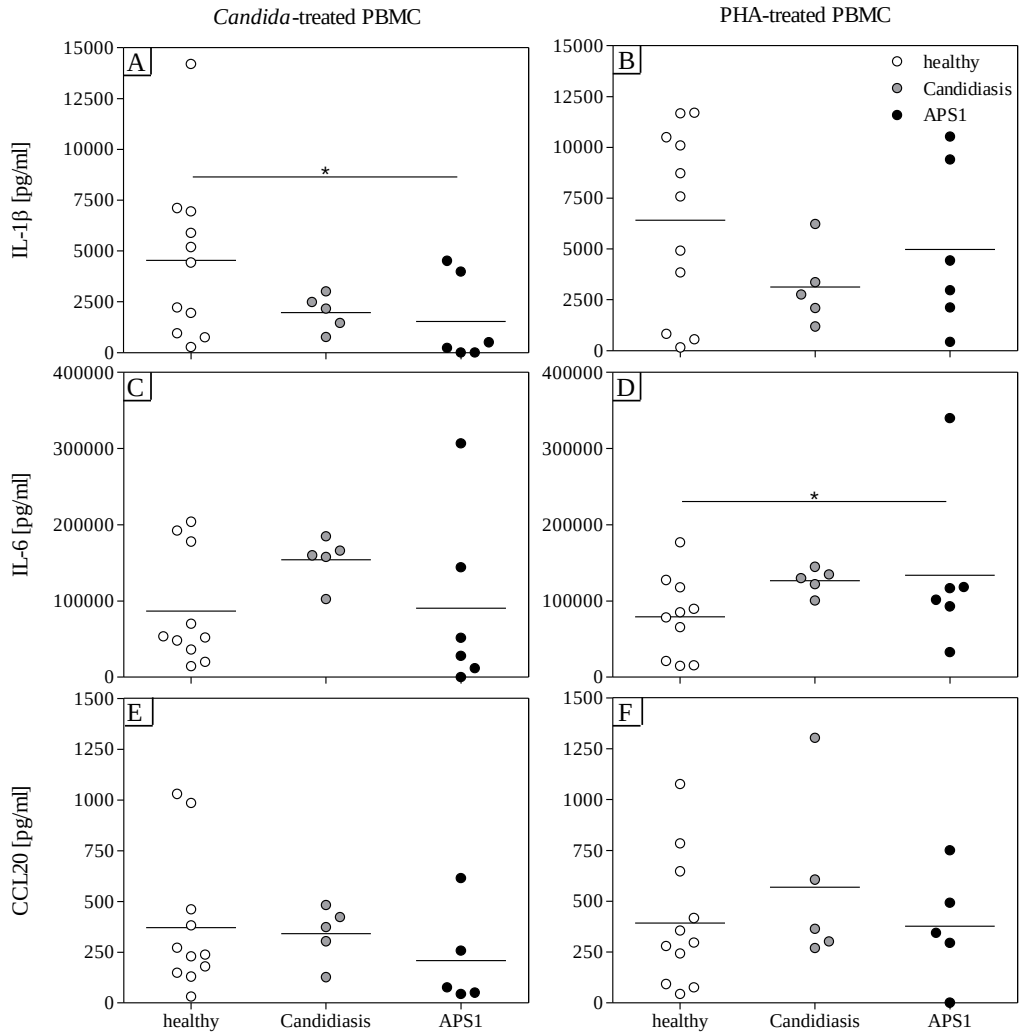
Figure 13: PBMCs of iCMC patients show a distinct profile of Th17-associated cytokines in response to *Candida albicans*

PBMCs from iCMC patients (n=5, black circles), patients with a current *Candida*-infection (n=5, grey circles) and healthy controls (n=11 in total, white circles) were stimulated with *Candida albicans* (50 µg/ml) or PHA (1 %) for 48 hours. Supernatant was analyzed for secretion of IL-1β (A-B), IL-6 (C-D) and CCL20 (E-F) by ELISA. Results are shown as mean, *, $p<0.05$, Mann Whitney test. The analyzed p values are shown in the table

Figure 14 demonstrates the cytokine release of PBMCs of APS1 patients. IL-1β secretion of PBMCs of APS1 patients was significantly diminished in comparison to PBMCs of healthy controls (Fig.14, A-B). IL-1β production of *Candida*-/PHA-primed PBMCs of APS1 patients and patients with a current *Candida*-infection was comparable (Fig.14, A-B).

PBMCs of APS1 patients produced slightly decreased amounts of IL-6 compared to patients with a current *Candida*-infection (Fig.14, C-D). No significant differences between CMC patients and healthy controls could be observed (Fig.14, C-D).

CCL20 secretion was comparable between all three analyzed groups, any significant differences could be detected for CCL20 production (Fig.14, E-F).

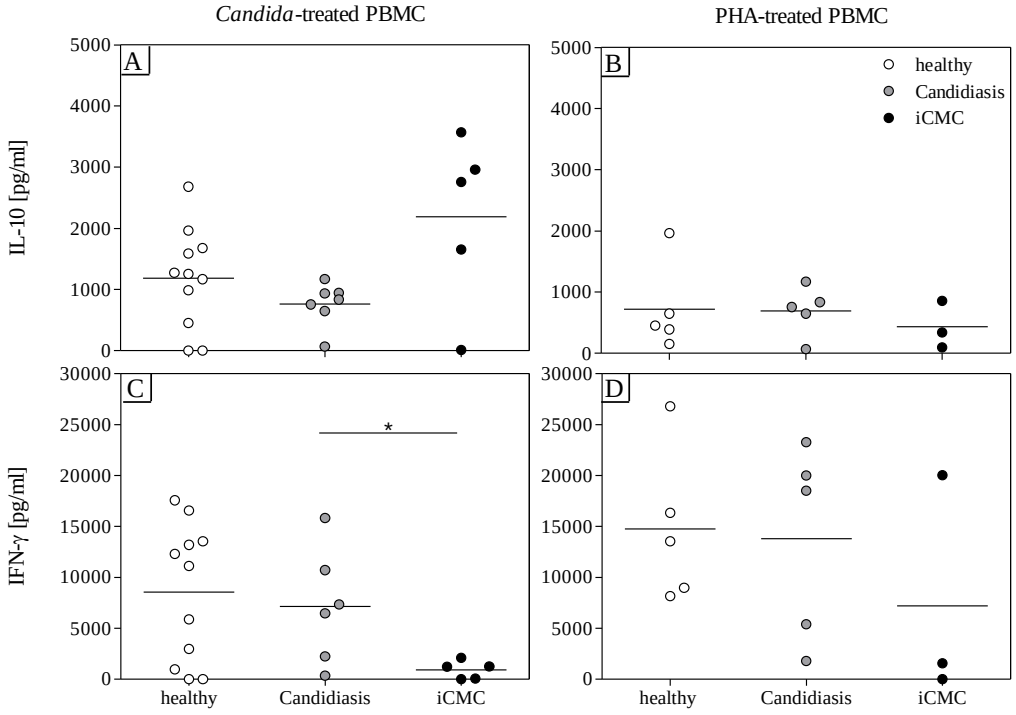


	Candida			PHA		
compared condition	IL-1β	IL-6	CCL20	IL-1β	IL-6	CCL20
APS1 vs. healthy	0.0393	0.4923	0.7340	0.5804	0.2198	0.6504
APS1 vs. Candidiasis	0.4103	0.1255	0.5476	0.5368	0.2468	0.3095
Candidasis vs. healthy	0.3648	0.2065	0.6504	0.2573	0.0400	0.3648

Figure 14: PBMCs of APS1 patients show a distinct profile of Th17-associated cytokines in response to *Candida albicans*

PBMCs from APS1 patients (n=6, black circles), patients with a current *Candida*-infection (n=5, grey circles) and healthy controls (n=11 in total, white circles) were stimulated with *Candida albicans* (50 µg/ml) or PHA (1 %) for 48 hours. Supernatant was analyzed for secretion of IL-1β (A-B), IL-6 (C-D) and CCL20 (E-F) by ELISA. Results are shown as mean, *, $p<0.05$, Mann Whitney test. The analyzed p values are shown in the table

Moreover the production of the effector cytokines IFN-γ and IL-10 was investigated in *Candida*-/PHA-treated PBMCs as presented in figure 15. The n number differs between the different analyzed cytokines due to the limited availability of culture supernatant. Strikingly PBMCs of iCMC patients were characterized by significantly increased levels of IL-10 and decreased amounts of IFN-γ after stimulation with *Candida* compared to the control groups (Fig.15, A-D).

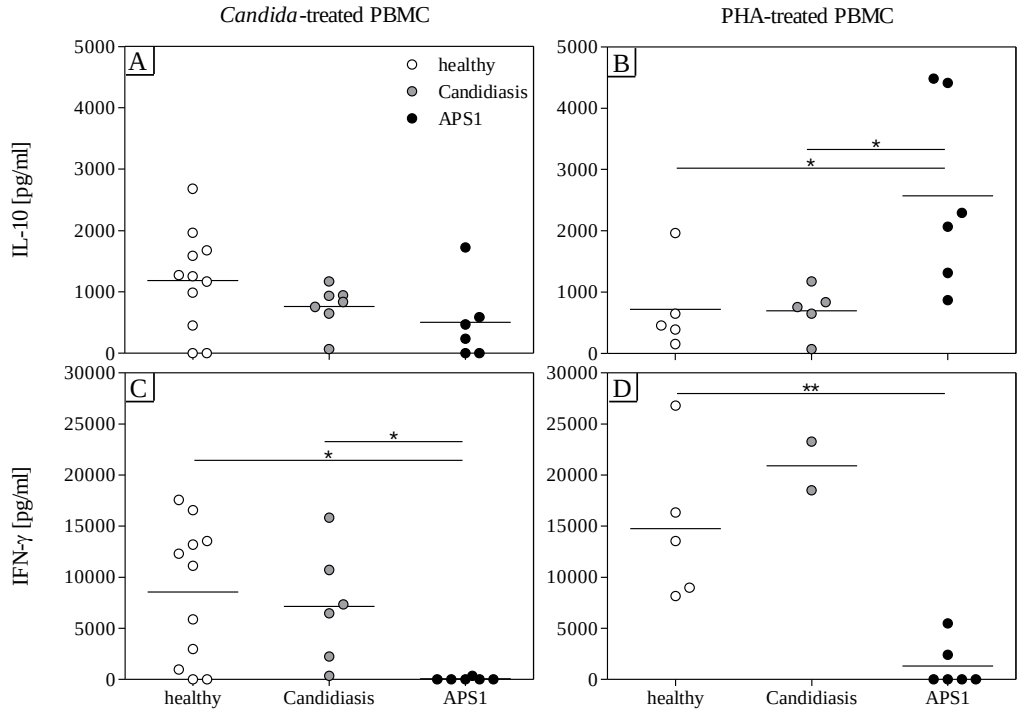


	Candida		PHA	
compared condition	IL-10	IFN-γ	IL-10	IFN-γ
iCMC vs. healthy	0.0893	0.0883	0.5714	0.3929
iCMC vs. Candidiasis	0.1061	0.0303	0.7857	0.3929
Candidasis vs. healthy	0.1471	0.7249	0.6905	1.0000

Figure 15: PBMCs of iCMC patients show a distinct profile of effector cytokines in response to *Candida albicans*

PBMCs from iCMC patients (n=5, black circles), patients with a current *Candida*-infection (n=7, grey circles) and healthy controls (n=11 in total, white circles) were stimulated with 50 µg/ml *Candida albicans* or 1 % PHA for 48 hours. Supernatant was analyzed for secretion of IL-10 (A-B) and IFN-γ (C-D) by ELISA. Results are depicted as mean. *, $p<0.05$, Mann Whitney test. The analyzed p values are shown in the table

The effect of enhanced IL-10 production in response to *Candida* could not be observed for PBMCs of APS1 patients (figure 16). Here, PBMCs of APS1 patients secreted the lowest amounts of IL-10 in comparison to both control groups (Fig.16, A). In contrast, after PHA-stimulation of PBMCs the highest level of IL-10 could be observed in PBMCs of APS1 patients (Fig.16, B). These PBMCs of APS1 patients secreted significantly reduced levels of IFN-γ after *Candida*- and PHA-stimulation compared to *Candida*-infected patients and healthy controls (Fig.16, C-D)



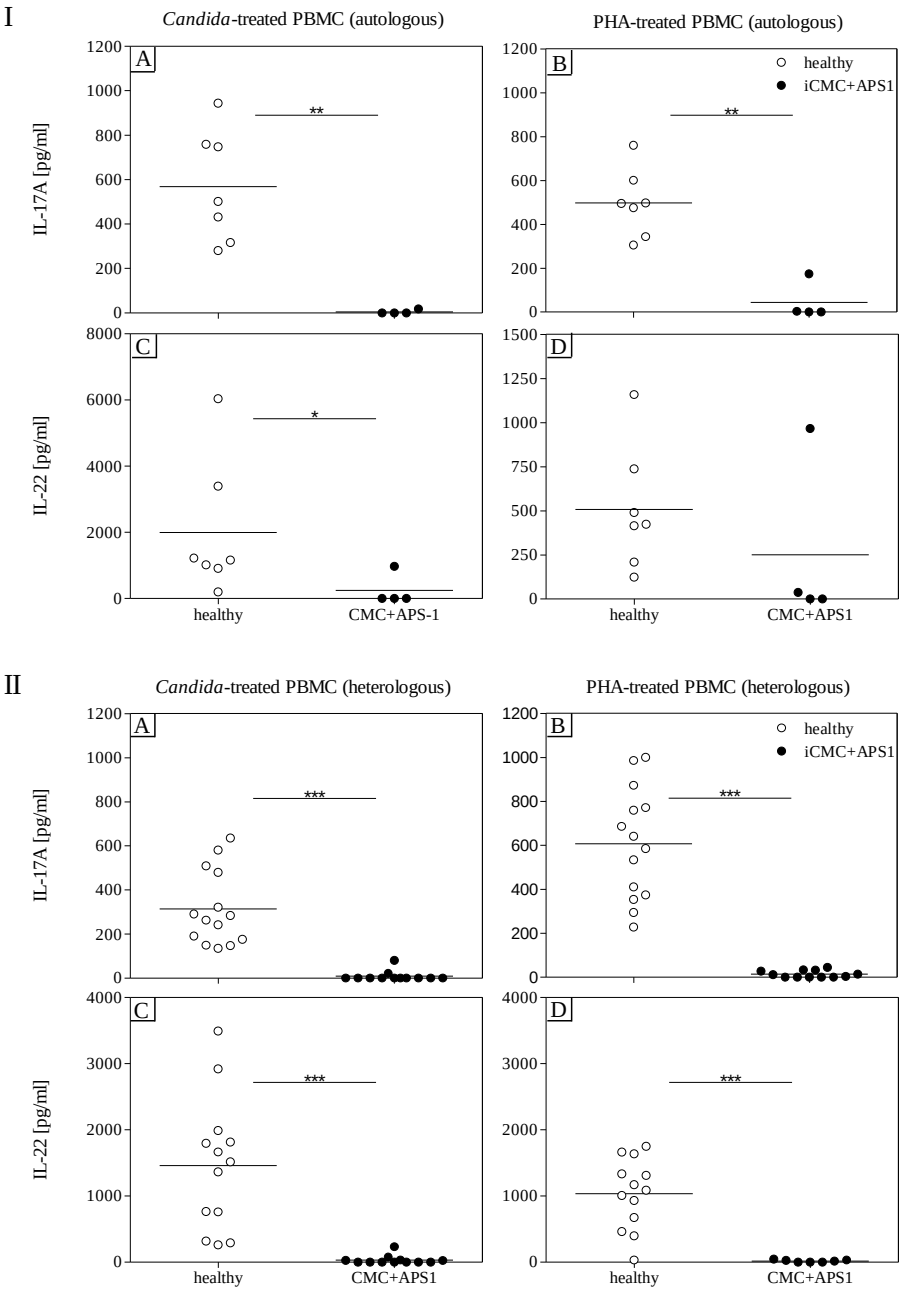
	Candida		PHA	
compared condition	IL-10	IFN-γ	IL-10	IFN-γ
APS1 vs. healthy	0.0981	0.0118	0.0362	0.0067
APS1 vs. Candidiasis	0.3714	0.0055	0.0273	1.0000
Candidasis vs. healthy	0.2185	0.7249	0.9469	1.0000

Figure 16: PBMCs of APS1 patients show a distinct profile of effector cytokines in response to *Candida albicans*

PBMCs from APS1 patients (n=6, black circles), patients with a current *Candida*-infection (n=7, grey circles) and healthy controls (n=11 in total, white circles) were stimulated with *Candida albicans* (50 µg/ml) or PHA (1 %) for 48 hours. Supernatant was analyzed for secretion of IL-10 (A-B) and IFN-γ (C-D) by ELISA. Results are shown as mean. *, $p<0.05$, **, $p<0.01$, Mann Whitney test. The analyzed p values are shown in the table

4.2.2 PBMCs of CMC patients produce no IL-17 and IL-22 in the auto- and heterologues system

Lately neutralizing autoantibodies have been described to be responsible for the observed reduced IL-17/IL-22 responses in APS1 patients (265, 266). To investigate whether autoantibodies play a possible role in IL-17/IL-22 deficiency in CMC patients included in this study, PBMCs of CMC patients (mixed group of iCMC and APS1 patients) and healthy controls were stimulated with *Candida albicans* or PHA in the presence of auto- or heterologous serum. In figure 17 a comparison of these cytokine results of stimulated PBMCs analyzed in an experimental setup with and without autologous serum is depicted. PBMCs of CMC patients produced signficantly no IL-17A and IL-22 in response to *Candida albicans* or PHA with autologous serum compared to healthy controls (Fig.17, I A-D). This effect was reproducible under heterologous serum conditions: PBMCs of CMC patients are also characterized by significantly reduced amounts of IL-17A and IL-22 after priming with *Candida* and PHA (Fig.17, II A-D).



	Candida		PHA	
compared condition of CMC vs. healthy	IL-17A	IL-22	IL-17A	IL-22
autologous system	0.0061	0.042	0.0061	0.1636
heterologous system	0.0001	0.0001	0.0001	0.0006

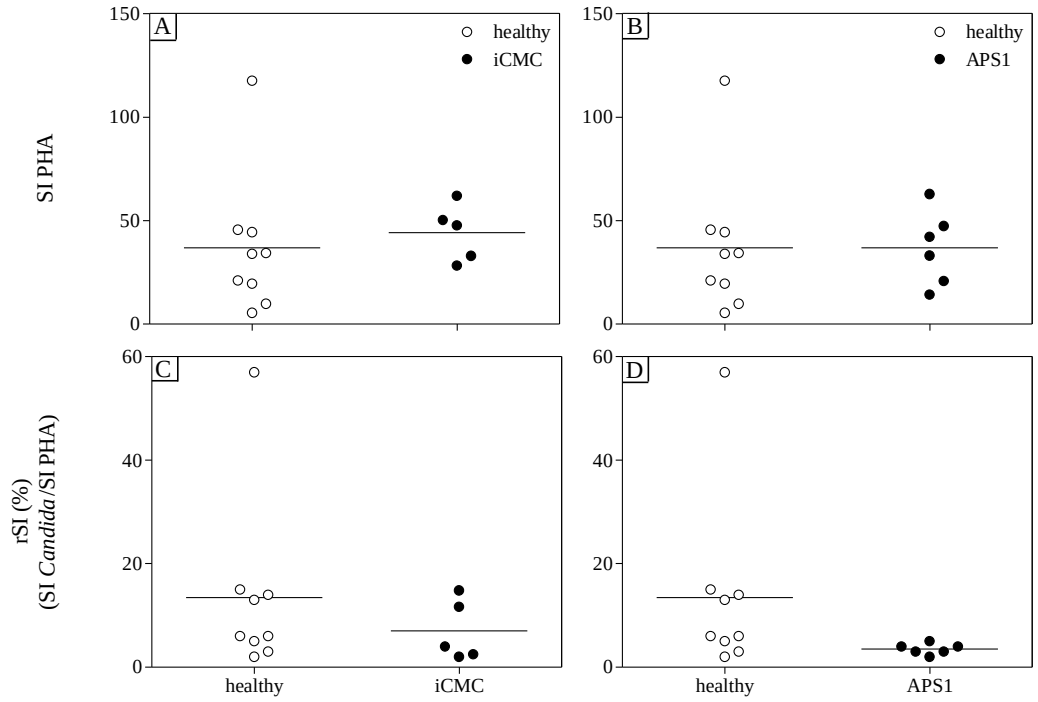
Figure 17: PBMCs of CMC patients show a decreased production of IL-17/IL-22 in response to *Candida albicans* in an experimental setup with and without autologous serum

PBMCs from CMC patients and healthy controls were stimulated with *Candida albicans* (50 µg/ml) or PHA (1 %) in autologous serum (5 %) or heterologous human Serum (5 %) for 72 hours. Supernatant was analyzed for secretion of IL-17A and IL-22 by ELISA **I)** Results of PBMC stimulation in autologous serum of 4 CMC patients (3 with APS-1) and 7 healthy controls are given as mean. *, $p<0.05$, **, $p<0.01$, Mann Whitney test **II)** Results of PBMC treat-

ment in heterologous serum of 12 CMC patients (6 with APS-1) and 14 healthy controls in total are depicted as mean. ***, p<0.001, Mann Whitney test. The determined p values are demonstrated in the table

4.2.3 PBMCs of CMC patients show an impaired proliferation capacity

To examine if the observed impaired IL-17A/IL-22 production of PBMCs is associated with an altered proliferation capacity, PBMCs of CMC patients were stimulated with *Candida albicans* or PHA and proliferation was analyzed by ^{3}H-thymidine incorporation assay. Figure 18 shows the results of proliferation related to the medium control (=stimulation index to PHA (SI PHA)) of iCMC and APS1 patients. PBMCs of both groups iCMC and APS1 patients were characterized by a normal proliferation capacity in response to PHA compared to healthy controls (Fig.18, A-B). PBMC proliferation in response to PHA was concerned as maximum proliferation capacity. In order to allow a more confident comparison of the tested individuals the proliferation to the specific antigen *Candida* was regarded relative to this maximum proliferation capacity (=relative stimulation index (rSI)). Therefore the relative stimulation index was determined as a ratio of proliferation to PHA and the response to *Candida* (Fig.18, C-D). PBMCs of both iCMC patients and APS1 patients showed a slightly decreased proliferative response to *Candida* in comparison to healthy controls (Fig.18, C-D).



compared condition	SI PHA	rSI
iCMC vs. healthy	0.2398	0.3856
APS1 vs. healthy	0.6889	0.0504

Figure 18: PBMCs of CMC patients show a decreased proliferation capacity in response to *Candida albicans*

PBMCs from CMC patients (black circles) and healthy controls (white circles) were stimulated with *Candida albicans* (50 µg/ml) or PHA (1 %) for 48 hours. Proliferation in cpm was measured by ^{3}H thymidine incorporation assay for 11 CMC patients (5 with iCMC, 6 with APS1 and 9 healthy controls. The stimulation index (SI) to PHA (A-B) and the relative stimulation index (SI *Candida*/SI PHA) (C-D) was determined. Results are shown as mean. P values analyzed by Mann Whitney test are listed in the table

4.2.4 PBMCs of CMC patients are characterized by an impaired expression of surface markers compared to healthy controls

PBMCs of CMC patients have been described to have an altered expression of surface markers (484). In order to analyze if surface molecules are differently expressed on PBMCs of CMC patients, PBMCs of one iCMC patient, two APS1 patients and healthy controls were stained for the surface markers CD4, CD8, CD45RA, CD4RO, CD161, CD127, CD3, CD19, CD56 and CD14 and mean fluorescence (MFI) was analyzed by

flow cytometry (figure 19). Because results of staining were similar between iCMC patients and APS1 patients the data of both types of CMC patients were analyzed together as one group. PBMCs of CMC patients were characterized by a not significant diminished expression of all tested surface markers compared to healthy PBMCs except for a comparable expression of the B cell marker CD56 and the barely detectable expression of the monocyte marker CD14. However, a clear trend could be oserved, but a higher patient collective is needed in order to reach significances.

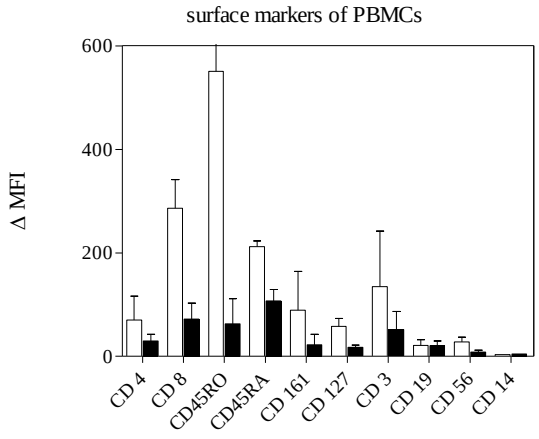


Figure 19: PBMCs of CMC patients are characterized by an impaired expression of surface markers

PBMCs of 3 healthy controls (white bars) and 3 CMC patients (1 iCMC patient, 2 APS1 patients, black bars) were stained for surface markers. MFI was analyzed by flow cytometry. Results are expressed as mean MFI ± SEM

4.2.5 PBMCs of CMC patients show a gain-of-function *STAT1* mutation

Isolated DNA from three patients with isolated CMC and their healthy relatives was analyzed in cooperation with Jean Laurent Casanova, St.Giles Laboratory of Human Genetics and Infectious Diseases, Rockefeller Branch, The Rockefeller University New York, for mutation analysis using whole exome sequencing. Heterozygous germline mutations in *STAT1* were found in 47 patients from 20 kindreds with isolated CMC. Sequencing of the corresponding coding region of *STAT1* revealed that all three of our tested iCMC patients were hetereozygous for a *STAT1* missense mutation carrying the *STAT1* mutant alleles Q271P, R274W and N357D which affected the coiled-coil domain of STAT1 and were gain-of-function (Fig. 20II). None of these mutations were

found in any of the tested healthy relatives of patient A. The relatives of patient B and C couldn't be analyzed so far. Figure 20 shows the pedigrees of the analyzed iCMC patients (I), the STAT1α isoform with its known pathogenic mutations (II) and the three-dimensional structure of phosphorylated STAT1 in complex with the DNA.

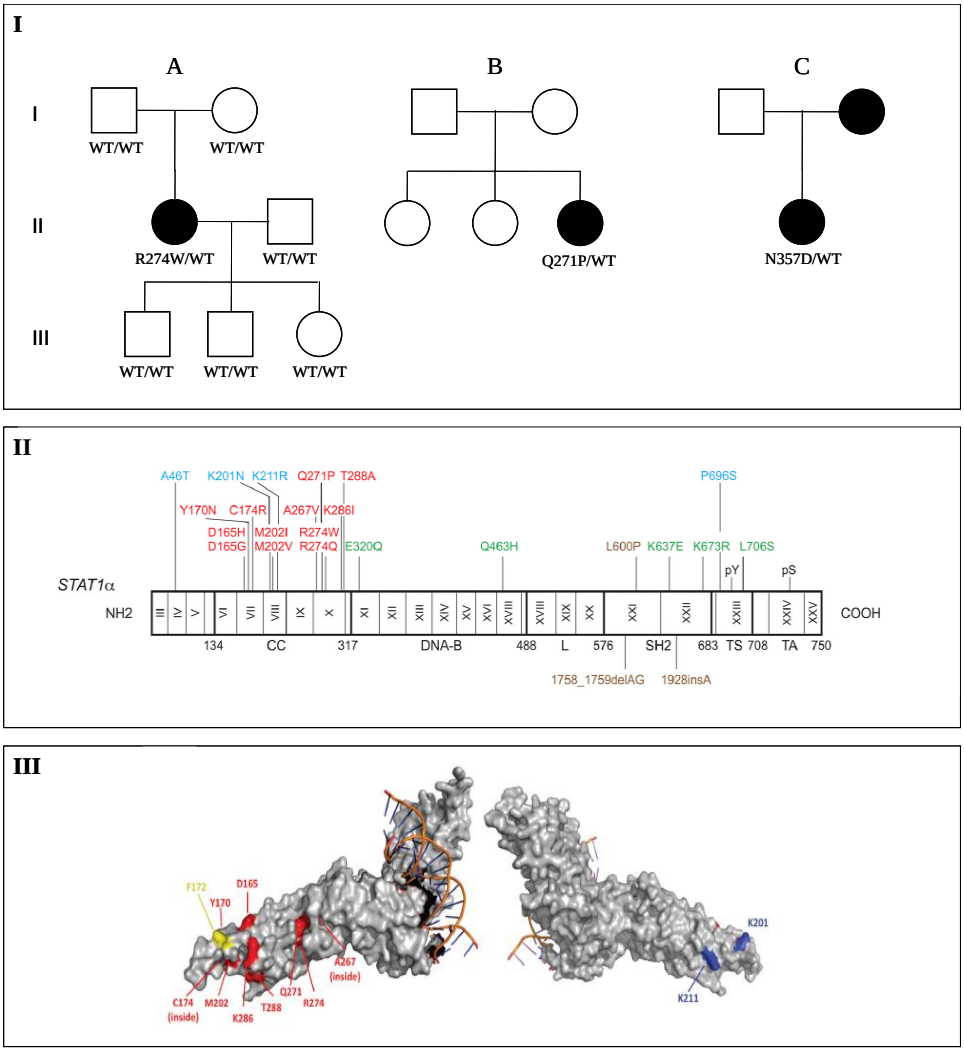


Figure 20: Heterozygous missense *STAT1* mutations in patients with isolated CMC

(I) Pedigree of three families with autosomal dominant "gain-of-function" *STAT1* mutations. Each patient is designated by a letter (A-C) and each generation is designated by a roman numeral (I-III). Black indicates patients with isolated CMC and when tested, the genotype for *STAT1* is presented below each individual **(II)** The human STAT1α isoform with its known pathogenic mutations. Coding exons are numbered with roman numerals and delimited by a vertical bar. Regions corresponding to the coiled-coiled domain (CC), DNA-binding domain (DNA-B), linker domain (L), SH2 domain (SH2), tail segment domain (TS) and transactivator domain (TA) are indicated, together with their amino-acids boundaries, and are delimited by bold lines. Tyr701 (pY) and Ser727 (pS) are indicated. Mutations in green are dominant and associated with partial STAT1 deficiency, mutations in brown are recessive and associated with complete STAT1 deficiency and mutations in blue are recessive and associated with partial STAT1 deficiency. Mutations in red are dominant and associated with a gain-of-function of STAT1 and isolated CMC **(III)** Three-dimensional structure of phosphorylated STAT1 in com-

plex with DNA. Connolly surface representation, amino acids highlighted in red indicate mutated amino acids in patients with isolated CMC. Adapted from Liu et al. (485)

4.2.6 PBMCs of CMC patients show a diminished STAT3 and enhanced STAT1 activation in response to IL-6 and IFN-γ

The ubiquitous cytokine signal transduction molecule STAT3 is involved in Th17 polarization and plays a central role in CMC-associated Hyper IgE syndrome (283, 284) where a Th17 defect has been described. Cytokines such as IFN-α/β, IFN-γ or IL-27 are known to inhibit Th17 rsponses via STAT1 (485-490) and in three tested iCMC patients in the present study a *STAT1* gain-of-function mutation could be identified. These observations lead to the question if DNA binding activity of STAT3 or STAT1 is disturbed in PBMCs of CMC patients.

To evaluate possible defects of STAT3 in CMC, PBMCs of two iCMC patients and four APS1 patients were stimulated with the STAT3-inducing cytokine IL-6 and STAT3 activation was examined by a TransAM system (figure 21). PBMCs of both iCMC patients and APS1 patients exhibited a decreased STAT3 DNA binding capacity compared to healthy controls (Fig.21, A,B), significant for APS1 patients (Fig.21, B). To investigate STAT1 activation PBMCs of iCMC patients were treated with the STAT1-inducing cytokine IFN-γ and the DNA binding activity of STAT1 was determined by a TransAM system (figure 21). PBMCs of iCMC patients showed an enhanced STAT1 activation capacity in comparison to healthy controls (Fig.21, C). Significances for STAT1 could not be determined due to the low number of tested patients with isolated CMC.

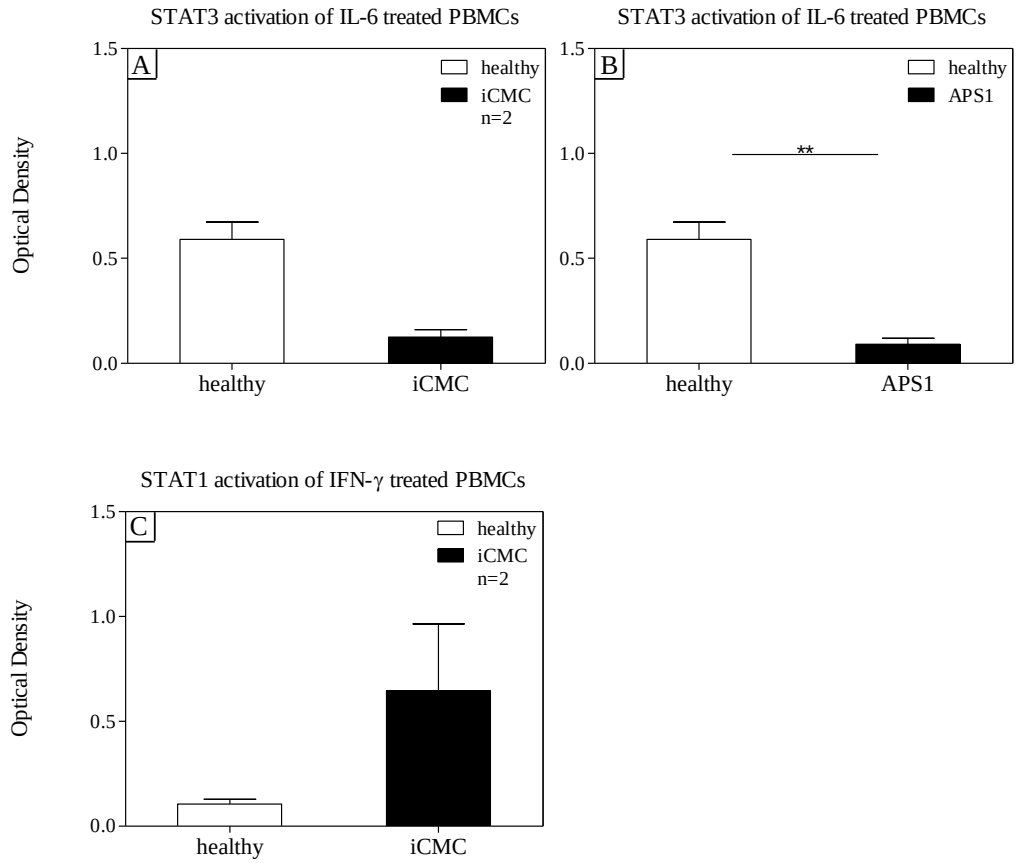


Figure 21: IL-6 induced STAT3 DNA binding is reduced and IFN-γ induced STAT1 activation enhanced in PBMCs of CMC patients

PBMCs of iCMC/APS1 patients (black bars) and healthy controls (white bars) were stimulated with rhIL-6 (20 ng/ml) or IFN-γ (100 U/ml) for one hour and lysed. Subsequently, 2-20 µg of nuclear cell lysate was loaded on a 96well plate precoated with ologonucleotide containing the STAT3 or STAT1 consensus site in a TransAM system. STAT3 activation was analyzed in 2 iCMC patients (A), 4 APS1 patients (B) and 6 healthy controls. STAT1 activation was determined in 2 iCMC patients and 2 healthy controls (C). Results are depicted as mean ± SEM. **, $p<0.01$, Mann Whitney test

4.3 Analysis of the dendritic cell function in CMC patients

A coordinated immune response involving both innate and adaptive mechanisms is required to defend *Candida*-infections (297). It is still not fully understood how the different components of innate and adaptive immunity result in the complex Th17-related pathogenesis of CMC. The previous data of this study demonstrate an impaired T cell cytokine secretion in CMC patients. Now it remains to be elucidated if this dysreglated cytokine production results from a direct T cell defect or aberrant T cell differentiation capacity of innate immune cells.

4.3.1 MoDC of CMC patients show a distinct cytokine profile in response to *Candida* and its cell wall compounds

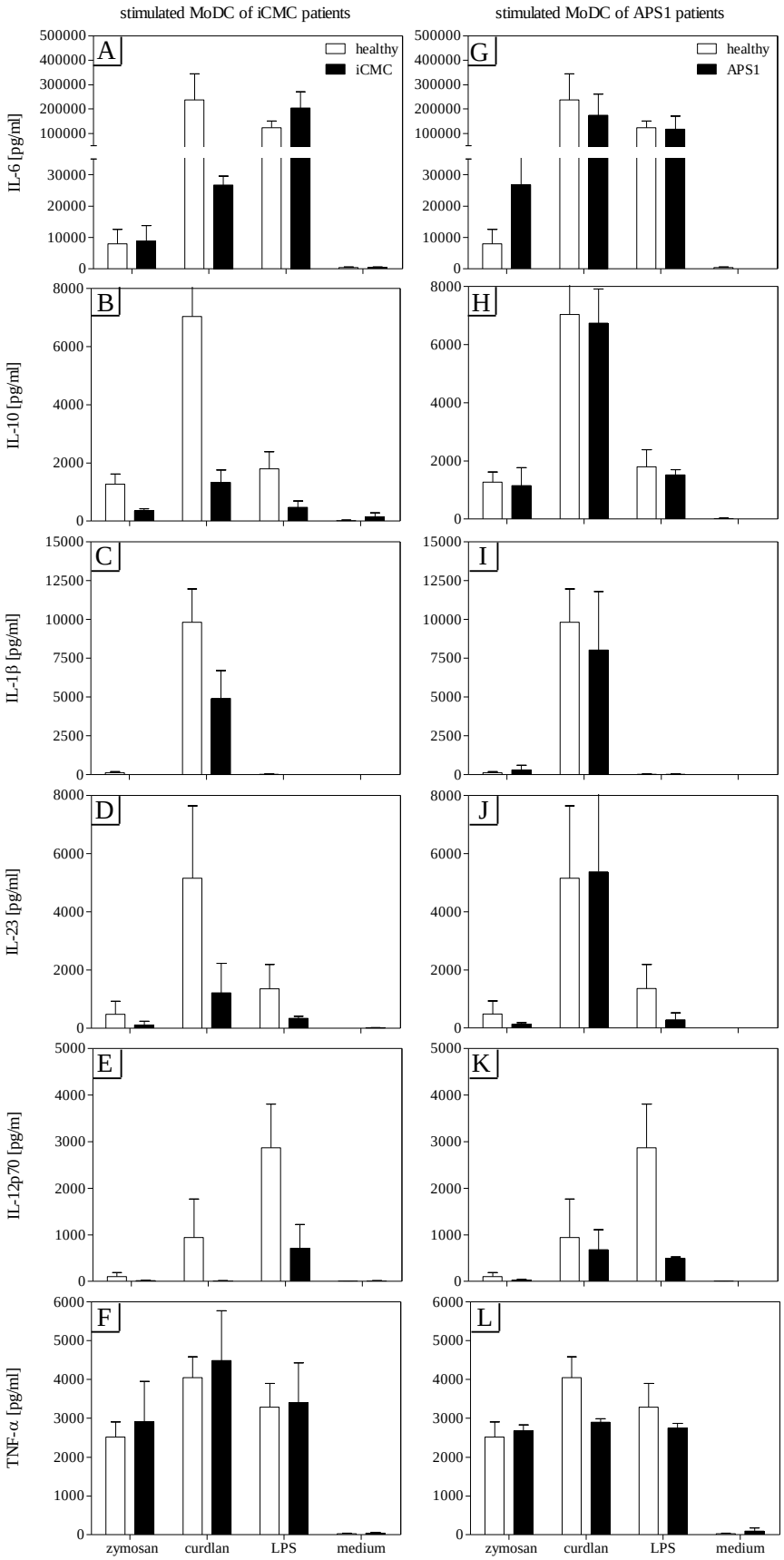
To analyze the cytokine profile of DCs of CMC patients in response to *Candida*, monocyte derived dendritic cells (MoDC) of CMC patients and healthy controls were stimulated with curdlan as dectin-1, zymosan as dectin-1/TLR2 or LPS as TLR4 agonist. Syk-CARD9 dependent dectin-1 triggering by glucanes induces the secretion of proinflammtory cytokines such as IL-2, IL-10, IL-6, TNF-α, IL-23, IL-1β but little IL-12 (205, 206). Cytokine secretion of stimulated MoDC was measured in the supernatant after 48 hours of incubation (figure 22).

IL-6 production in MoDC of iCMC patients induced by zymosan was comparable to healthy controls, IL-10 production was slightly decreased (Fig.22, A-B). In contrast, IL-6 secretion in zymosan-stimulated MoDC of APS1 patients was enhanced whereas no differences in IL-10 induction could be observed (Fig.22, G-H). Zymosan stimulation of MoDC only slightly induced the production of IL-1β, IL-23 and IL-12p70 in both iCMC and APS1 patients (Fig.22, C-E, I-K). MoDC of APS1 patients secreted TNF-α in a comparable manner to healthy MoDC in response to zymosan (Fig.22, L) whereas TNF-α production of iCMC MoDC was slightly increased (Fig.22, F).

Curdlan-treated MoDC of iCMC patients produced reduced amounts of IL-6, IL-10, IL-1β, IL-23 and IL-12p70 whereas TNF-α secretion was slightly enhanced compared to healthy MoDC (Fig.22, A-F). MoDC of APS1 patients showed a similar decreased cytokine profile in response to curdlan, amounts of IL-6, IL-10, IL-1β, IL-12p70 and TNF-α were diminished except for IL-23 production which was comparable to healthy

MoDC (Fig.22, G-L). The reduced cytokine production of APS1 MoDC after exposure to curdlan was less prominent as observed in iCMC MoDC.

Cytokine production of IL-10 and IL-23 was reduced under the TLR4 stimulus (LPS) (Fig.22, N,P), equally observed for both iCMC and APS1 patients (Fig.22, B,D,H,J). IL-1β was not upregulated by LPS in all tested MoDC. TNF-α production of LPS-exposed MoDC was comparable between iCMC patients and healthy controls (Fig.12, F) whereas MoDC of APS1 patients showed reduced TNF-α amounts in response to LPS (Fig.22, L). IL-6 was increased in MoDC of CMC patients compared to healthy MoDC after stimulation with LPS (Fig.22, A). APS1 MoDC secreted comparable amounts of IL-6 to healthy MoDC after LPS-stimulation (Fig.22, G-H). Interestingly, the amounts of IL-12p70 of LPS-matured MoDC were remarkable reduced in both iCMC and APS1 patients (Fig.22, E,K).


stimulated MoDC of iCMC patients
stimulated MoDC of APS1 patients
healthy
iCMC
APS1
A
B
C
D
E
F
G
H
I
J
K
L
IL-6 [pg/ml]
IL-10 [pg/ml]
IL-1β [pg/ml]
IL-23 [pg/ml]
IL-12p70 [pg/m]
TNF-α [pg/ml]
zymosan
curdlan
LPS
medium

compared condition	IL-6	IL-10	IL-1β	IL-23	IL-12	TNF-α
zymosan						
iCMC vs. healthy	0.4970	0.1939	0.5545	0.4709	1.0000	1.0000
APS1 vs. healthy	0.6303	0.9212	0.6970	0.2667	0.8175	1.0000
curdlan						
iCMC vs. healthy	0.1333	0.0848	0.2788	0.1939	0.656	1.0000
APS1 vs. healthy	0.7416	1.0000	0.7758	0.8889	0.8333	1.0000
LPS						
iCMC vs. healthy	0.2758	0.0848	0.8967	0.3758	0.1939	1.0000
APS1 vs. healthy	1.0000	0.7758	0.8967	0.8889	0.7758	1.0000

Figure 22: Zymosan-, curdlan- and LPS-induced cytokine response is impaired in MoDC of CMC patients compared to healthy controls

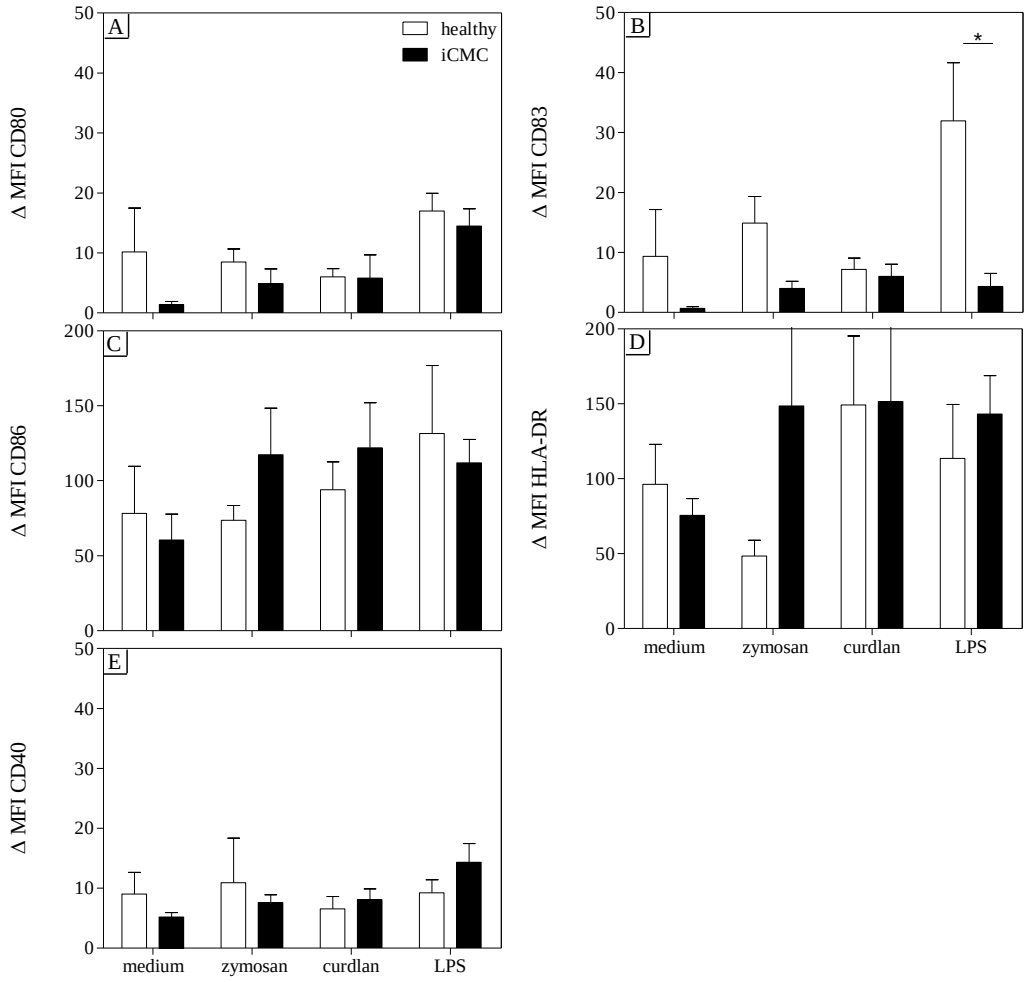
MoDC of CMC patients (black bars) and healthy controls (white bars) were incubated with zymosan (100 µg/ml), curdlan (300 µg/ml) or LPS (100 ng/ml) for 48 hours. Supernatants were recovered and amounts of IL-6, IL-10, IL-23, IL-1β, IL-12p70 and TNF-α were measured by ELISA. Results of 3 iCMC patients, 3 APS1 patients and 8 healthy controls are expressed as mean ± SEM. *, $p<0.05$, Mann Whitney test. Analyzed p values are depicted in the table

4.3.2 Maturation of *Candida*-stimulated MoDC of CMC patients is impaired compared to healthy controls

In order to investigate the maturation of MoDC in response to *Candida* cell wall compounds the surface markers CD40, CD80, CD83, CD86 and HLA-DR of zymosan-, curdlan- and LPS-stimulated MoDC of iCMC patients, APS1 patients and healthy controls were stained with the respective antibodies and analyzed by flow cytometry (figure 23 and 24).

The mean fluorescence intensity (MFI) of all activation markers in unstimulated cultures was lower in iCMC patients compared with healthy controls (Fig.23, A-E). All stimulation conditions led to an upregulation of all tested maturation markers compared to the unstimulated cultures in MoDC of iCMC patients (Fig.23, A-E). In contrast, Zymosan-treatment of healthy MoDC did not induce upregulation of CD80, CD86 and HLA-DR compared to unstimulated MoDC (Fig.23, A, C-D), curdlan-treatment failed to upregulate CD80 and CD83 (Fig.23, A-B). Both zymosan- and curdlan-stimulation led to a higher MFI of CD86 in MoDC of iCMC patients in comparison to healthy controls (Fig.23, C). Upregulation of CD80, CD83 and CD40 after exposure to zymosan was reduced on MoDC of iCMC patients whereas upregulation of these activation markers was comparable to healthy MoDC after curdlan-stimulation (Fig.23, A-B, E).

Notably, the MFI of HLA-DR was remarkable enhanced in zymosan-matured MoDC of iCMC patients compared to healthy controls, whereas curdlan-stimulation led to a comparable upregulation of HLA-DR (Fig.23, D). Concerning the maturation of MoDC in reponse to LPS the MFI of CD80, CD83 and CD86 was lower in MoDC of iCMC patients compared to healthy controls (Fig.23, A-C), significant for CD83. In contrast, LPS-stimulation led to a higher MFI of HLA-DR and CD40 in MoDC of iCMC patients compared to healthy MoDC (Fig.23, D-E).



ΔMFI	CD80	CD83	CD86	CD40	HLA-DR
zymosan	0.6475	0.0667	0.2667	0.1833	0.0667
curdlan	0.8192	0.7309	0.2667	0.8192	1.000
LPS	0.7758	0.0121	0.1939	0.3758	0.2788
medium	0.1833	0.2667	0.8333	1.000	0.5167

Figure 23: Zymosan-, curdlan- and LPS-induced maturation of MoDC is impaired in iCMC patients compared to healthy controls

MoDC of 3 iCMC patients (black bars) and 8 healthy controls (white bars) were incubated with zymosan (100 µg/ml), curdlan (300 µg/ml) or LPS (100 ng/ml) for 48 hours. Surface markers were stained and MFI was analyzed by flow cytometry. Results are expressed as mean MFI ± SEM. *, $p<0.05$, Mann Whitney test. Determined p values are presented in the table

The mean fluorescence intensity (MFI) of all activation markers in unstimulated cultures was lower in APS1 patients compared with healthy controls, except for the MFI of CD86 which was comparable to healthy controls (Fig.24, C). All analyzed stimulation conditions in MoDC of APS1 patients resulted in the upregulation of all maturation markers compared to the untreated condition (Fig.24, A-E). MoDC of APS1 patients showed a higher MFI of CD86 in response to zymosan and curdlan compared to healthy controls whereas CD86 upregulation after LPS-stimulation was comparable between MoDC of APS1 patients and healthy controls (Fig.24, C). MFI levels of CD80 were not significant different in MoDC of APS1 patients and healthy controls under all stimulation conditions (Fig.24, A). Upregulation of CD83 after exposure to curdlan or zymosan was comparable between MoDC of APS1 patients and healthy controls (Fig.24, B) whereas LPS-treated MoDC of APS1 patients showed a lower CD83 upregulation compared to healthy subjects (Fig.24, B). The curdlan- and LPS-induced HLA-DR upregulation in MoDC of APS1 patients was diminished compared to healthy MoDC, whereas zymosan-treatment led to a higher upregulation of HLA-DR in APS1 MoDC (Fig.24, D). The MFI of CD40 was remarkable high in curdlan- and LPS-matured MoDC of APS1 patients compared to healthy controls whereas CD40 upregulation was decreased in response to zymosan (Fig.24, E).

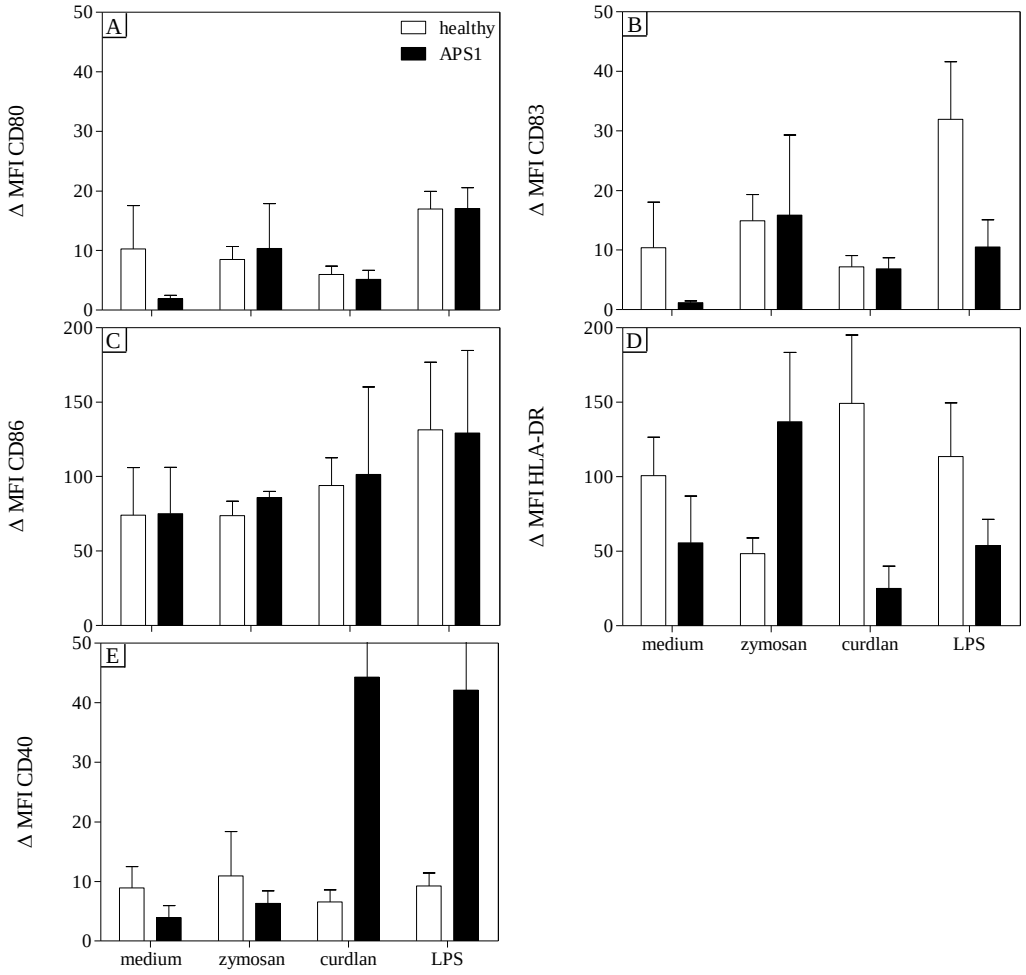


Figure 24: Zymosan-, curdlan- and LPS-induced maturation of MoDC is impaired in APS1 patients compared to healthy controls

MoDC of 8 healthy controls (white bars) and 2 APS1 patients (black bars) were incubated with zymosan (100 µg/ml), curdlan (300 µg/ml) or LPS (100 ng/ml) for 48 hours. Surface markers were stained and MFI was analyzed by flow cytometry. Results are expressed as mean MFI ± SEM. Statistical analysis could not be performed due to the low number of APS1 patients (n=2)

4.4 DC - T cell crosstalk in CMC patients is altered

DCs stimulated with *Candida* cell wall compounds such as curdlan or zymosan are known to induce Th1/Th17 cells in mice and humans (206). Because of the impaired cytokine profile analyzed in MoDC of iCMC patients the T cell response induced by these *Candida* cell wall-stimulated MoDC was examined.

4.4.1 Cytokine profile of naive T cells of CMC patients induced by *Candida*-stimulated MoDC differs from healthy controls

The polarization of T lymphocytes induced by zymosan-, curdlan- or LPS-matured MoDC of patients with isolated CMC was investigated performing an allogenic stimulation assay (ASA). After 48 hours stimulation with zymosan, curdlan or LPS MoDC were coincubated with naïve $CD4^+CD45RA^+$ T cells of iCMC patients and healthy individuals in different combinations: Healthy MoDC were incubated with healthy naïve T cells (n=5), MoDC of iCMC patients with healthy T cells (n=4) and healthy MoDC were cultured with iCMC T cells (n=3) (figure 25). Healthy naïve T cells instructed by healthy MoDC present the healthy control model. Because of the limited availability of patient blood samples and the complexicity of this experimental setup APS1 patients could not be investigated in these experiments. Cytokine release was determined by ELISA (figure 26).

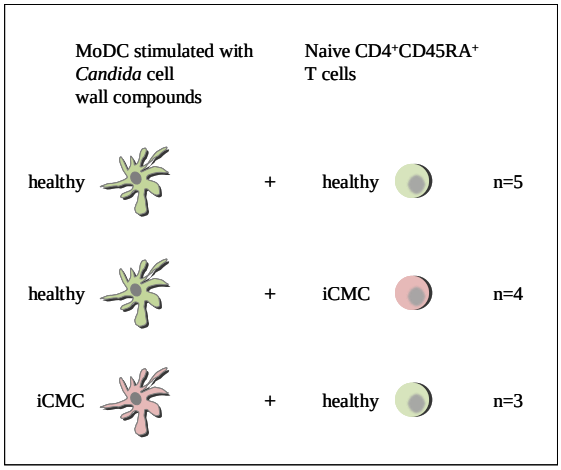


Figure 25: Different culture conditions of the allogenic stimulation assay with naïve T cells

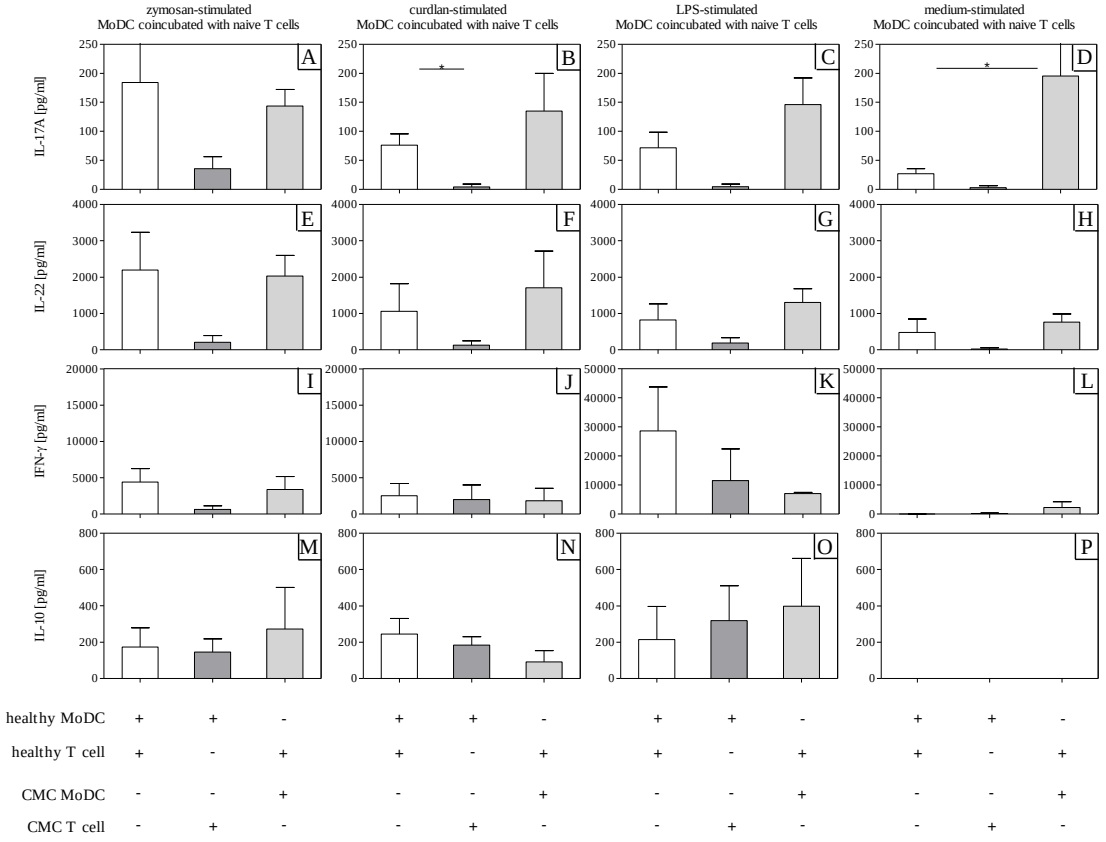
Both healthy and iCMC MoDC induced IL-17A and IL-22 secretion in healthy naïve T cells after stimulation with the *Candida* cell wall compounds zymosan and curdlan; and the TLR4 stimulus LPS (Fig.26, A-C). In contrast, iCMC T cells showed a reduced production of IL-17 and IL-22 after coincubation with both zymosan- and curdlan-matured healthy MoDC, significant for IL-17 under curdlan conditions (Fig.26, A-B, E-F). Healthy T cells compensated the observed DC defects of iCMC patients after stimulation with zymosan- or curdlan-matured MoDC showing an IL-17/IL-22 response comparable to healthy controls or even higher (Fig.26, A-C, E-G). Interestingly, these healthy T cells released significant increased amounts of IL-17A and IL-22 induced by MoDC treated with medium alone (Fig.26, D,H).

Both healthy T cells polarized by iCMC MoDC and iCMC T cells treated with healthy MoDC showed a tendency to produce decreased amounts of the Th1 cytokine IFN-γ under zymosan and curdlan conditions (Fig.26, I-J), and this effect was most obvious for iCMC T cells that were stimulated with zymosan-matured healthy MoDC (Fig.26, I).

Among all tested stimulation conditions LPS-polarized T cells produced the highest amounts of IFN-γ, iCMC T cells secreted lower amounts of IFN-γ compared to the healthy control model (Fig.26, K).

The production of regulatory IL-10 was slightly decreased in iCMC T cells differentiated by zymosan- or curdlan-stimulated MoDC compared to healthy T cells (Fig.24, M,N). Healthy T cells differentiated in the presence of zymosan- or LPS-treated iCMC MoDC secreted more IL-10 compared to the other donor conditions (Fig.26, M,O), whereas IL-10 production was decreased under curdlan conditions (Fig.26, N).

Notably, iCMC T cells polarized by LPS-matured MoDC showed higher IL-10 levels compared to healthy T cells (Fig.26, N-O). Analyzing the IL-10/IL-17 ratio of cytokine data as demonstrated in table 9, iCMC T cells were characterized by the highest IL-10/IL-17 ratios after coculture with zymosan-, curdlan- or LPS-stimulated MoDC in comparison to healthy T cells.



compared condition	IL-17A	IL-22	IFN-γ	IL-10
zymosan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.3929	0.1771	0.1429	0.8738
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	1.0000	0.7302	1.0000	0.8933
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.0571	0.0571	0.1143	0.8544
curdlan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.0357	0.3594	0.5412	0.7857
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.5556	0.4606	0.6228	0.2622
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.1991	0.1991	0.5821	0.2118
LPS				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.1991	0.4000	0.4000	0.8544
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.4857	0.5614	0.3429	0.3095
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.0571	0.1143	0.6286	1.0000
medium				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.1266	0.5252	1.0000	not detectable
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.0159	0.2683	0.1248	not detectable
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.0571	0.0571	0.5821	not detectable

Figure 26: iCMC T cells show a different cytokine profile after coincubation with *Candida* cell wall-matured MoDC

Zymosan-, curdlan- or LPS-stimulated MoDC were cocultured with purified naïve $CD4^+CD45RA^+$ T cells for five-six days in different combinations. IL-17 (A-D), IL-22 (E-H), IFN-γ (I-L) and IL-10 (M-P) were analyzed in the supernatants by ELISA. Results are given as mean ± SEM. *, $p<0.05$, Mann Whitney test. The table shows the determined significances for each condition

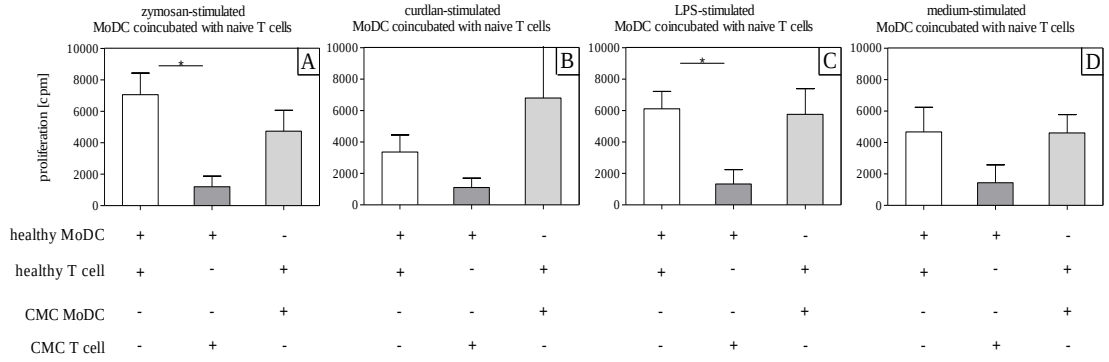
IL-10/IL-17 ratio of compared condition	zymosan	curdlan	LPS
healthy MoDC vs. healthy T cell	1.33	3.21	3.00
healthy MoDC vs. CMC T cell	5.14	40.96	68.42
CMC MoDC vs. healthy T cell	0.64	0.68	2.73

Table 9: iCMC T cells show an enhanced IL-10/IL-17 ratio after coincubation with C*andida* cell wall-matured MoDC in the allogenic stimulation assay

Zymosan-, curdlan- or LPS-stimulated MoDC were cocultured with purified naïve $CD4^+CD45RA^+$ T cells for five-six days in different combinations. IL-17A and IL-10 were analyzed in the supernatants by ELISA and the IL-10/IL-17 ratio was determined

4.4.2 Proliferation capacity of naive T cells of CMC patients induced by *Candida*-stimulated MoDC differs from healthy controls

After five to six days of incubation of the ASA proliferation capacity was measured by ^{3}H-thymidine assay (figure 27). Healthy MoDC matured by zymosan, curdlan or LPS induced proliferation in healthy naïve T cells (Fig.27, A-C). CMC MoDC also instructed healthy naïve T cells to proliferate in a similar manner (Fig.27, A-D). iCMC T cells polarized by healthy MoDC showed the lowest proliferation capacity among all tested donor conditions (Fig.27, A-D). Coculture of iCMC T cells with zymosan- or LPS-stimulated healthy MoDC led to a significant reduced proliferation in comparison to healthy T cells (Fig.27, A-C).



compared condition	p value
zymosan	
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.0357
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.4127

healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.1143
curdlan	
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.1429
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.5556
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.2286
LPS	
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.0357
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.9048
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.1143
medium	
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.1429
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	1.0000
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.2286

Figure 27: iCMC T cells show an impaired proliferation capacity after coincubation with *Candida* cell wall-matured MoDC compared to healthy controls

Purified naïve $CD4^+CD45RA^+$ T cells were cocultured with zymosan- (A), curdlan- (B) or LPS-stimulated (C) MoDC for five-six days in different combinations. Medium-stimulated MoDC incubated with naïve T cells served as control (D). Proliferation was examined performing the ^{3}H-thymidine incorporation assay. Results are given as mean ± SEM. *, $p<0.05$, Mann Whitney test. Determined p values are shown in the table

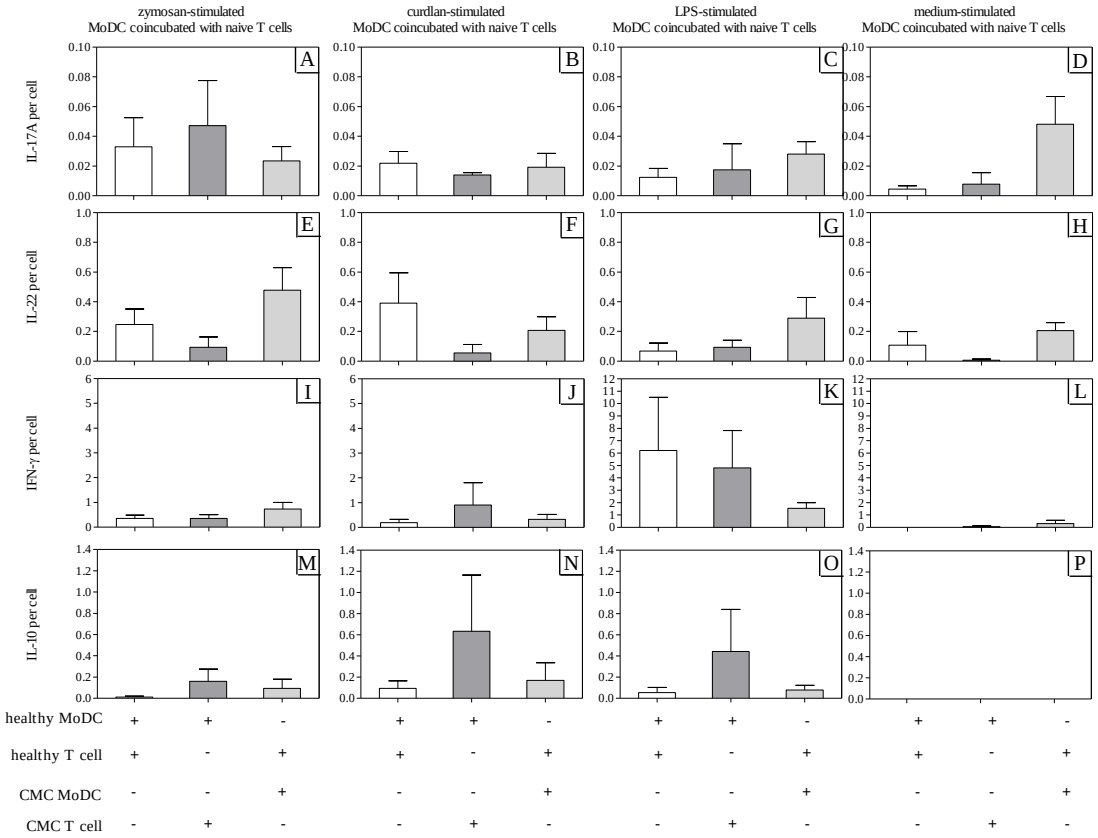
4.4.3 Different cytokine profile of naive T cells of CMC patients induced by *Candida*-stimulated MoDC in relation to proliferation capacity

Because of the observed reduced proliferation capacity of iCMC T cells the data of cytokine release were related to proliferation to determine the cytokine production per single cell (figure 28). Of note, the production of the Th17-cytokine IL-17A per iCMC T cell induced by zymosan-triggered MoDC was slightly increased compared to healthy T cells whereas the secretion of IL-22 per cell was diminished (Fig.28, A,E). iCMC T cells polarized by curdlan-matured healthy MoDC secreted decreased amounts of IL-17A and IL-22 per cell compared to healthy T cells (Fig.28, B,F). Under LPS-stimulation conditions poduced amounts of IL-17A and IL-22 per cell were comparable between iCMC T cells and healthy T cells (Fig.28, C,G).

T cells of iCMC patients stimulated with medium alone led to an increased IL-17A and IL-22 secretion per cell in naïve healthy T cells (Fig.28, D).

Concerning the production of effector cytokines per single cell the amounts of IFN-γ were only barely detectable in healthy and iCMC naïve T cells instructed by zymosan-stimulated MoDC (Fig.28, I, M). The release of inflammatory IFN-γ per single cell was enhanced in iCMC T cells after coculture with curdlan-matured MoDC (Fig.28, J,N).

iCMC T cells polarized by LPS-treated MoDC secreted lower amounts of IFN-γ per cell compared to healthy controls (Fig.28, K) whereas levels of regulatory IL-10 per cell were increased (Fig.28, O). The single-cell production of IL-10 was increased in iCMC T cells under all culture conditions compared to healthy controls (Fig.28, M-O).



compared condition	IL-17A	IL-22	IFN-γ	IL-10
zymosan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	1.0000	1.0000	0.4857	0.1241
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	1.0000	0.3429	0.3429	0.6198
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	1.0000	0.2000	0.6857	0.4596
curdlan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.8824	0.8824	1.0000	0.1143
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.8824	0.6857	0.8824	0.6573
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.8824	0.6857	0.8824	0.1102
LPS				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.8824	0.5614	1.0000	0.4857
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.2000	0.2000	0.8857	0.3094
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.8857	0.6857	0.3429	0.3005
medium				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.1143	0.3590	0.8255	not detectable
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.0571	0.4000	0.1241	not detectable
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.8544	0.1000	0.5821	not detectable

Figure 28: iCMC T cells show a different single-cell profile of cytokine production related to proliferation capacity compared to healthy controls

Purified naïve $CD4^+CD45RA^+$ T cells were cocultured with zymosan-, curdlan- or LPS-stimulated MoDC for five-six days in different combinations. The production of IL-17 (A-D), IL-22 (E-H), IFN-γ (I-L) and IL-10 (M-P) was analyzed in the supernatants by ELISA. Results of cytokine release are presented in relation to proliferation capacity and given as mean ± SEM. P values determined by Mann Whitney test are shown in the table

4.4.4 Cytokine profile of memory T cells of CMC patients induced by *Candida*-stimulated MoDC differs from healthy controls

The positively separated T cell fraction of naïve T cell isolation was used as memory T cells in the ASA to investigate the cytokine release of already polarized T cells coincubated with *Candida* cell wall-matured MoDC. Memory T cells were cocultured with MoDC in different combinations: Healthy MoDC were incubated with healthy naïve T cells (n=3), MoDC of iCMC patients with healthy T cells (n=3) and healthy MoDC were cultured with iCMC T cells (n=3) (figure 29). Healthy naïve T cells instructed by healthy MoDC present the healthy control model. Cytokine release was determined by ELISA (figure 30). Due to the low availability of patient material the ASA could not be performed with cells of APS1 patients.

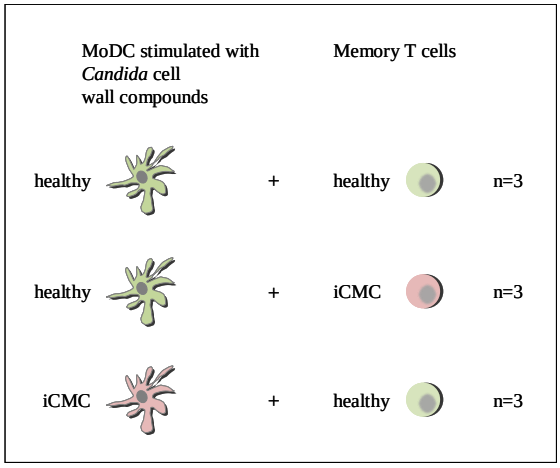
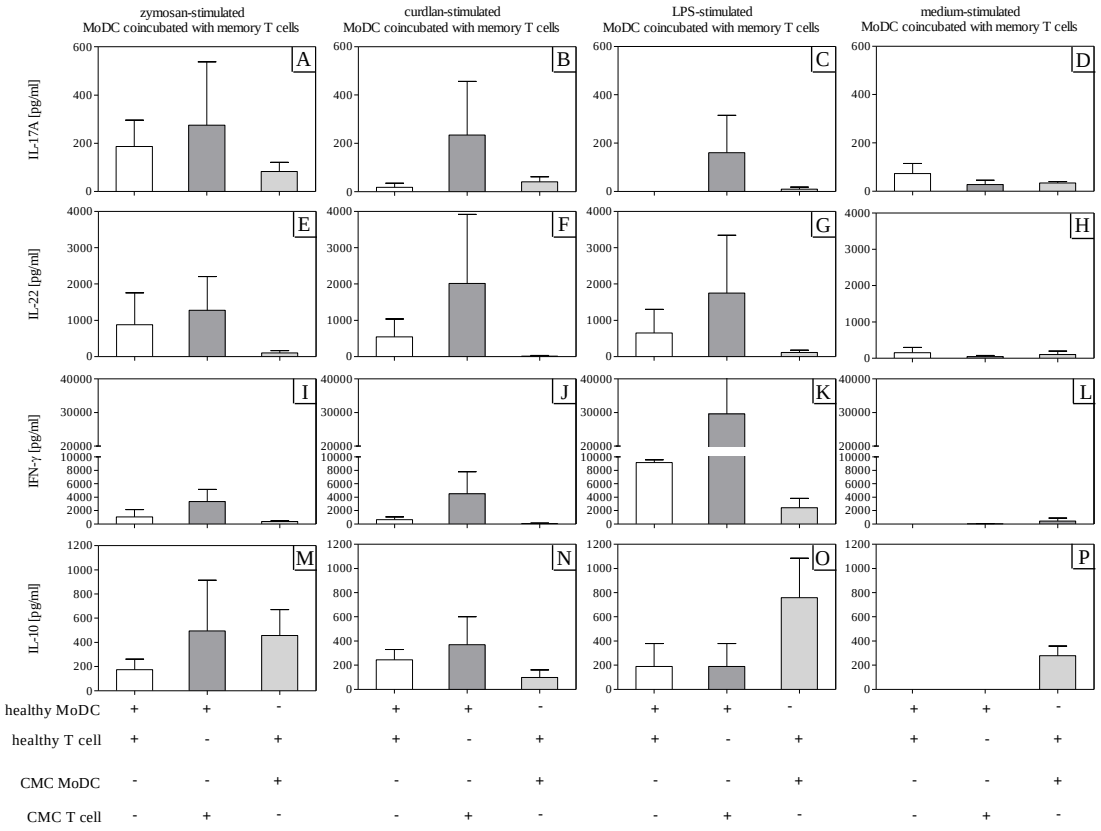


Figure 29: Different culture conditions of the allogenic stimulation assay with memory T cells

Healthy MoDC matured with zymosan, curdlan or LPS induced the highest amounts of IL-17A (Fig.30, A-C) and IL-22 (Fig.30, E-G) in memory T cells of iCMC patients

compared to the healthy control model and healthy memory T cells polarized by iCMC MoDC.

Concerning the production of effector cytokines IFN-γ and IL-10 (figure 30), Memory T cells of iCMC patients cultured with zymosan- or curdlan-treated healthy MoDC exhibited the highest amounts of effector cytokines IFN-γ (Fig.30, I-J) and IL-10 (Fig.30, M-N). The analysis of the IL-10/IL-17 ratio of cytokine data revealed the highest IL-10/IL-17 ratio in iCMC memory T cells in response to zymosan-, curdlan- or LPS-stimulated MoDC compared to healthy memory T cells (table 10).



compared condition	IL-17A	IL-22	IFN-γ	IL-10
zymosan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.8248	0.6428	0.3537	0.8248
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.7000	1.0000	0.6579	0.4000
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	1.0000	0.5066	0.7000	0.7000
curdlan				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.6428	0.8248	0.5066	1.0000
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.3537	0.3537	0.5066	0.4000
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.8248	0.3537	0.5066	0.5066

LPS				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.3537	1.0000	0.7000	0.2000
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	1.0000	1.0000	0.2000	not detectable
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.6428	0.5066	0.8000	not detectable
medium				
healthy MoDC+healthy T cell vs. healthy MoDC+CMC Tcell	0.5066	1.0000	1.0000	0.6531
healthy MoDC+healthy T cell vs. CMC MoDC+healthy Tcell	0.7500	1.0000	1.0000	not detectable
healthy MoDC+CMC T cell vs. CMC MoDC+healthy Tcell	0.7000	1.0000	1.0000	not detectable

Figure 30: iCMC memory T cells show a different profile of cytokine production after coincubation with *Candida* cell wall-matured MoDC in the ASA

Zymosan-, curdlan- or LPS-stimulated MoDC were cocultured with memory T cells for five-six days in different combinations. IL-17A (A-D), IL-22 (E-H), IFN-γ (I-L) and IL-10 (M-P) were analyzed in the supernatants by ELISA. Results are given as mean ± SEM. P values determined by Mann Whitney test are shown in the table

IL-10/IL-17 ratio of compared condition	zymosan	curdlan	LPS
healthy MoDC vs. healthy T cell	0.29	4.31	4.31
healthy MoDC vs. CMC T cell	4.85	39.56	24.37
CMC MoDC vs. healthy T cell	3.95	8.82	2.79

Table 10: Memory T cells of iCMC patients show an enhanced IL-10/IL-17 ratio after coincubation with C*andida* cell wall-matured MoDC in the ASA

Zymosan-, curdlan- or LPS-stimulated MoDC were cocultured with purified naïve $CD4^+CD45RA^+$ T cells for five-six days in different combinations. IL-17A and IL-10 were analyzed in the supernatants by ELISA and the IL-10/IL-17 ratio was determined

4.4.5 The proliferation capacity of memory T cells of CMC patients induced by *Candida*-stimulated MoDC is comparable to healthy controls

The proliferation capacity of memory T cells in the ASA was measured by ^{3}H-thymidine assay (figure 31). Healthy MoDC matured with zymosan, curdlan or LPS induced proliferation in iCMC memory T cells comparable to healthy memory T cells. Healthy memory T cells instructed by stimulated iCMC MoDC showed a slightly reduced proliferation capacity (Fig.31, A-C). Because almost no differences in proliferation capacity were detected, cytokine results were not related to the proliferation data.

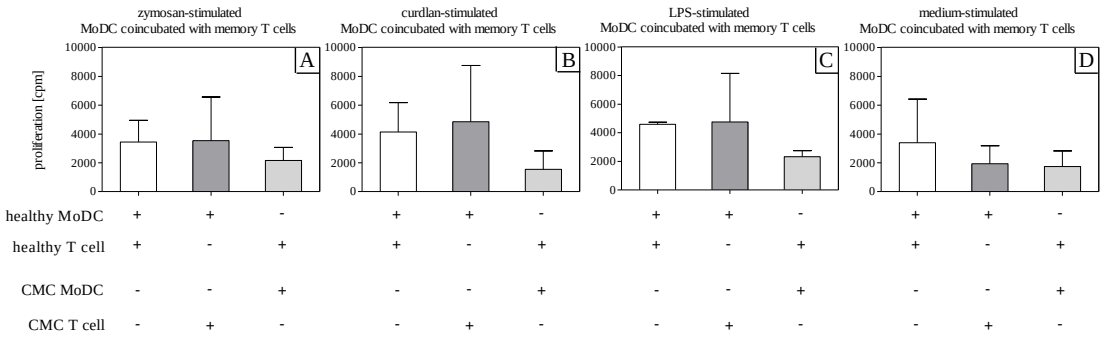


Figure 31: iCMC T cells show a normal proliferation capacity after coincubation with *Candida* cell wall-matured MoDC in the ASA

Memory T cells were cocultured with zymosan- (A), curdlan- (B) or LPS-stimulated-MoDC (C) for five-six days in different combinations. Medium-treated MoDC incubated with naïve T cells were concerned as control (D). Proliferation was assessed performing the ^{3}H-thymidine incorporation assay. Results are depicted as mean ± SEM

4.5 Analysis of T cell differentiation in CMC patients

The observation of an impaired production of IL-17 and IL-22 in CMC patients in PBMCs and in the allogenic stimulation assay raised the question if naïve T cells of CMC patients are able to differentiate to Th17 cells. The polarization capacity of naïve T cells of CMC patients to different T cell lines (Th1, Th2, Th17) in general was of interest.

4.5.1 The establishment of Th17 lines in CMC patients

Naïve $CD4^+CD45RA^+$ T cells of iCMC, APS1 patients and healthy controls were polarized into Th17 cells under serum-free conditions and cytokine release was determined by ELISA (figure 32). Priming of naïve T cells of iCMC patients under Th17-conditions led to a diminished secretion of IL-17A (Fig.32, A) and IL-22 (Fig.32, B) compared to healthy controls. Statistically significances could not be determined because of n=2 in CMC patients. Naïve T cells of APS1 patients polarized to Th17 cells also significantly produced less IL-17A (Fig.32, C) and IL-22 (Fig.32, D) compared to healthy controls whereas the reduced IL-22 secretion was more prominent. Differentiated Th17 cells of APS1 patients released more IL-17A but less IL-22 than generated Th17 cells of iCMC patients (Fig.32, A-D).

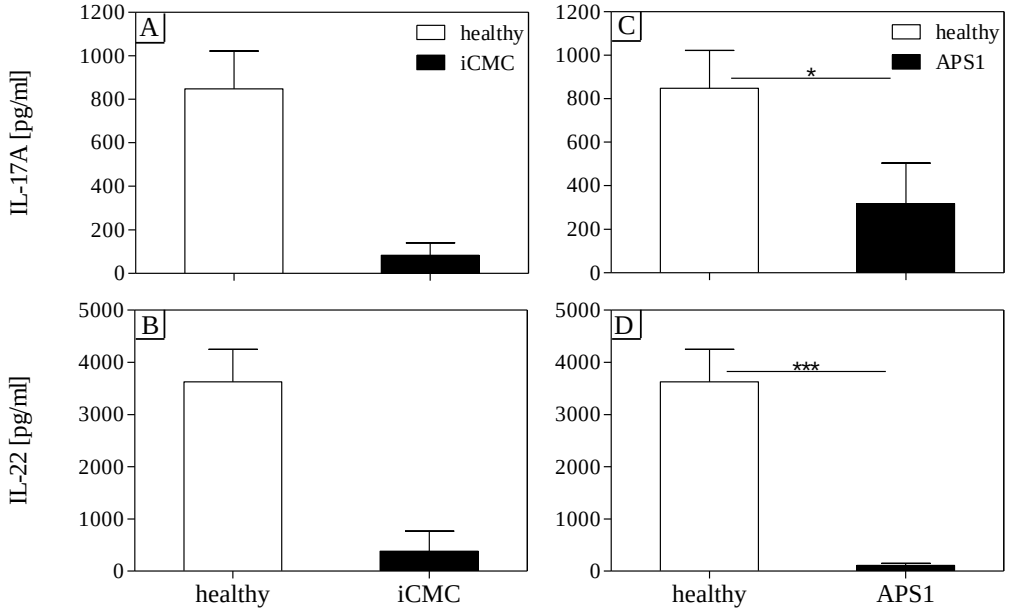


Figure 32: Generated Th17 cells of iCMC and APS1 patients are characterized by a diminished IL-17 and IL-22 production

Purified $CD4^+CD45RA^+$ T cells from CMC patients (black bars) and healthy controls (white bars) were incubated under anti-CD3/anti-CD28-stimulating conditions in serum-free medium with IL-6 (30 ng/ml), IL-1β (20 ng/ml), IL-23 (30 ng/ml), anti-IL-4 (5 µg/ml) and anti-IFN-γ (1 µg/ml) to induce Th17 cells. After seven days the release of IL-17A and IL-22 was determined in supernatant by ELISA. Results of 2 iCMC patients, 8 APS1 patients and 8 healthy controls are presented as mean±SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ Mann Whitney test

4.5.2 The establishment of T cell lines in CMC patients

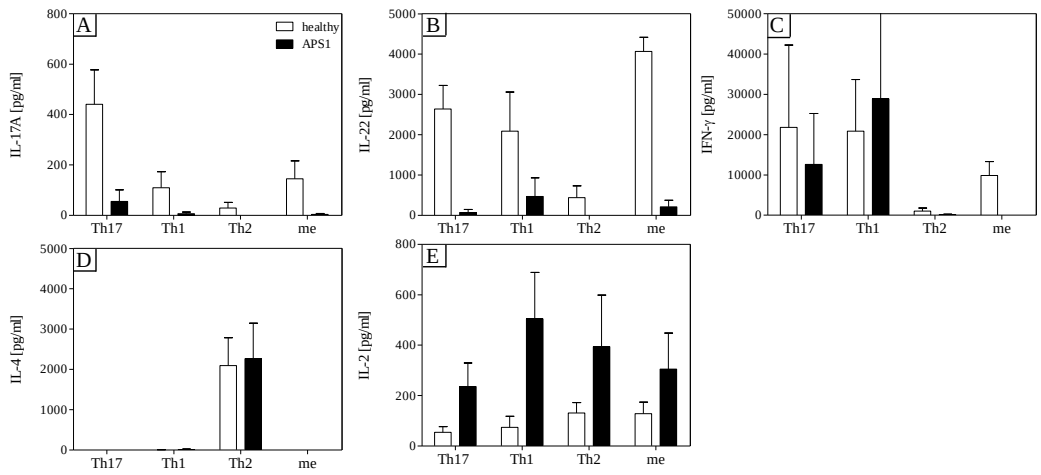
To examine the capacity of naïve T cells of CMC patients to polarize into different T cell lines, naïve $CD4^+CD45RA^+$ T cells of iCMC/APS1 patients and healthy controls were differentiated to Th17, Th1 and Th2 cells. Cytokine production was analyzed by ELISA (figure 33).

Differentiation of naïve T cells to Th1 cells led to an increased IFN-γ secretion in APS1 T cells compared to healthy T cells (Fig.33, IC) whereas generated Th1 cells of the iCMC patient produced only barely detectable amounts of IFN-γ (Fig.33, IIC). Priming of naïve T cells to Th2 cells induced a comparable production of IL-4 in APS1 and healthy T cells (Fig.33, ID). Differentiated Th2 cells of the iCMC patient secreted remarkable enhanced amounts of IL-4 compared to Th2 cells of healthy controls (Fig.33, IID).

However, naive T cells of both APS1 and iCMC patients could not be polarized into Th17 cells under adequate cytokine conditions, Th17 cells of APS1 and iCMC patients produced decreased amounts of IL-17A (Fig.33, IA, IIA) and IL-22 (Fig.33, IB, IIB) compared to generated Th17 cells of healthy controls.

Interestingly, polarized Th17, Th1 and Th2 cells of APS1 patients secreted enhanced levels of IL-2 in comparison to healthy controls (Fig.33, IE). Generated Th17 and Th1 cells of the iCMC patient produced increased amounts of IL-2 in comparison to healthy T cells whereas IL-2 secretion of polarized Th2 cells of the iCMC patient was decreased (Fig.33, IIE).

I



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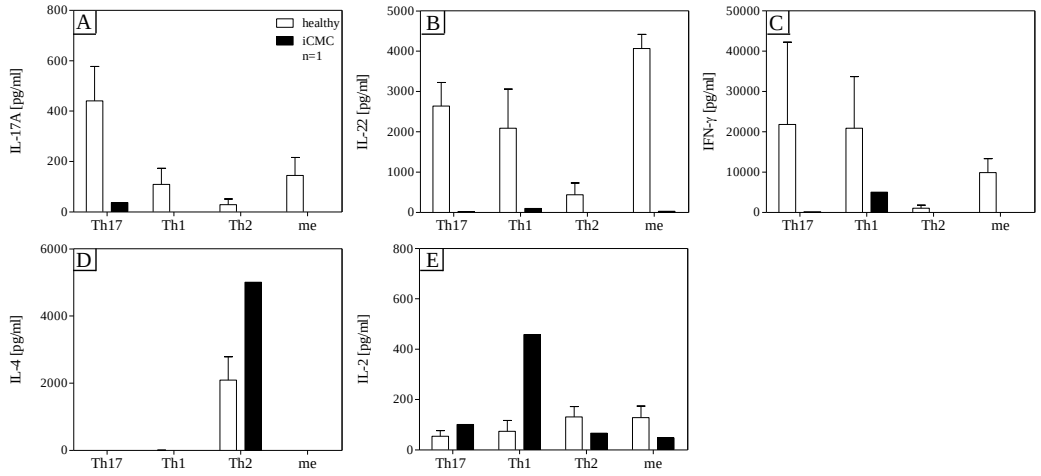


Significances for APS1 T cell lines	IL-17	IL-22	IFN-γ	IL-4	IL-2
Th17	0.0571	0.0571	0.5821	0.8255	0.2286
Th1	0.1991	0.6286	1.0000	0.8255	0.0571
Th2	0.5585	05585	0.5585	0.8571	0.4000

Figure 33: Naïve T cells of APS1 and iCMC patients can not be polarized into Th17 cells under adequate polarization conditions

Purified $CD4^+CD45RA^+$ T cells from CMC patients (black bars) and healthy controls (white bars) were incubated under anti-CD3/anti-CD28-stimulating conditions in serum-free medium with IL-12 (250 ng/ml) and anti-IL-4 (5 µg/ml) for Th1-polarization, with IL-6 (30 ng/ml), IL-1β (20 ng/ml), IL-23 (30 ng/ml), TGF-β (3 ng/ml), anti-IL-4 (5 µg/ml) and anti-IFN-γ (1 µg/ml) to induce Th17 cells. Medium-stimulated cells (me) served as control. After five-six days the release of IL-17, IL-22, IFN-γ, IL-4 and IL-2 was measured in supernatant by ELISA. **(I)** Cytokine results of generated T cell lines of 3 APS1 patients and 4 healthy controls are shown. Results are presented as mean ± SEM. *, $p<0.05$, Mann Whitney test. Analyzed p values are listed in the table **(II)** Cytokine results of generated T cell lines of 1 iCMC patient and 4 healthy controls are shown. Results are presented as mean ± SEM. Statistical significances could not be determined due to only one iCMC patient is tested

4.6 Keratinocytes of CMC patients are characterized by an impaired defensin production

One important mechanism to defence C*andida*-infections in the skin is the induction of antimicrobial peptides in human epithelial cells by IL-17, IL-22 and TNF-α (138, 139, 273). To explore the responsiveness of epithelial cells of iCMC patients to these cytokines, keratinocytes of CMC patients and healthy controls were challenged with IL-17A, IL-22 and TNF-α in different combinations and the secretion of human β-defensin 2 was assessed by ELISA (figure 34). Treatment of keratinocytes of iCMC patients with IL-17, IL-22 or TNF- α resulted in decreased β-defensin 2 production compared to healthy keratinocytes. The additive effect of IL-17 and IL-22 in defensin-induction was enhanced in iCMC patients, while the additive effect of TNF-α and IL-22 was reduced (Fig.34). No statistic significances could be detected because keratinocytes of only two iCMC patients could be tested.

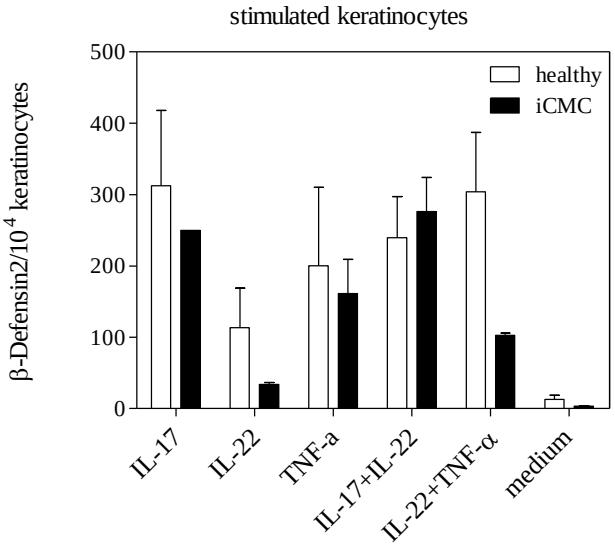


Figure 34: The production of human β-Defensin 2 is impaired in keratinocytes of iCMC patients

Keratinocytes of iCMC patients (n=2) and healthy controls (n=3) were stimulated with rhIL-17, rhIL-22 and rhTNF-α (all 50 ng/ml) in different combinations. Release of hβ-Defensin 2 was determined after 48 hours in supernatant by ELISA. Results are presented as mean ± SEM, two independent experiments are shown

4.7 Whole genome analysis of CMC patients and healthy controls

Untouched T cells of two iCMC patients and two healthy controls were stimulated with anti-CD3 and anti-CD28 for six hours and cell pellets were sent to Miltenyi for gene expression analysis using Agilent Whole Human Genome Oligo Microarrays (one color). Unstimulated T cells at timepoint 0 served as control for basal gene expression.

The differential gene expression Feature Extraction Software–derived output data files obtained from Miltenyi were further analyzed using the Gene Spring data analysis system (GeneSpring GX Software). Herein, upregulated genes in response to anti-CD3/anti-CD28 stimulation were compared between T cells of iCMC patients and healthy controls. Table 11 shows selected upregulated genes in stimulated T cells of iCMC patients compared to stimulated T cells of healthy controls, table 12 demonstrates selected downregulated genes in iCMC T cells.

Gene symbol	Gene name	Fold Change	P value
CXCL10	Homo sapiens chemokine (C-X-C motif) ligand 10	78.599	0.002
CXCL11	Homo sapiens chemokine (C-X-C motif) ligand 11	66.706	0.000
CXCL9	Homo sapiens chemokine (C-X-C motif) ligand 9	62.671	0.011
UBD	Homo sapiens ubiquitin D	40.078	0.010
IL1B	Homo sapiens interleukin 1	17.750	0.035
GPR56	Homo sapiens G protein-coupled receptor 56	14.001	0.031
CD33	Homo sapiens CD33 molecule	13.134	0.003
CX3CR1	Homo sapiens chemokine (C-X3-C motif) receptor 1	9.744	0.012
GZMB	Homo sapiens granzyme B	9.730	0.031
CD38	Homo sapiens CD38 molecule	8.703	0.046
IFNG	Homo sapiens interferon	8.530	0.006
CD70	Homo sapiens CD70 molecule	7.380	0.003
ICAM1	Homo sapiens intercellular adhesion molecule 1	7.085	0.040
IL7	Homo sapiens interleukin 7	7.079	0.013
LAG3	Homo sapiens lymphocyte-activation gene 3	6.736	0.020
FGL2	Homo sapiens fibrinogen-like 2	6.483	0.008
XAF1	Homo sapiens XIAP associated factor 1	4.435	0.038
PDE4A	Homo sapiens phosphodiesterase 4A	4.279	0.038

IL2	Homo sapiens interleukin 2	4.241	0.033
IRF1	Homo sapiens interferon regulatory factor 1	3.671	0.006
LGALS9	Homo sapiens lectin	3.634	0.015
MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8	3.591	0.029
TNF	Homo sapiens tumor necrosis factor	3.359	0.048
CDKN2A	Homo sapiens cyclin-dependent kinase inhibitor 2A	3.010	0.024
PDCD1	Homo sapiens programmed cell death 1	3.010	0.026
IL15	Homo sapiens interleukin 15	2.745	0.043
RIPK2	Homo sapiens receptor-interacting serine-threonine kinase2	2.741	0.017
TRIM96	Homo sapiens tripartite motif-containing 69	2.697	0.002
BCL2L11	Homo sapiens mRNA for Bim-alpha2	2.682	0.015
CYCS	Homo sapiens cytochrome c	2.466	0.031
SOCS3	Homo sapiens suppressor of cytokine signaling 3	2.433	0.028
BAX	Homo sapiens BCL2-associated X protein	2.407	0.024
TAP1	Homo sapiens transporter 1	2.405	0.009
BCL2L1	Homo sapiens Bcl2-like 1	2.350	0.022
PML	Homo sapiens promyelocytic leukemia	2.275	0.044
CREM	Homo sapiens cAMP responsive element modulator	2.179	0.048
SLAMF6	Homo sapiens SLAM family member 6	2.067	0.019
BCL2	Human B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene mRNA encoding bcl-2-beta protein	2.027	0.003
FOXD2	Homo sapiens forkhead box	2.021	0.032

Table 11: Upregulated genes in anti-CD3/anti-CD28 stimulated T cells of iCMC patients compared to healthy controls

Untouched T cells of two iCMC patients and two healthy controls were stimulated with anti-CD3 and anti-CD28 for six hours and cell pellets were sent to Miltenyi for gene expression analysis using Agilent Whole Human Genome Oligo Microarrays (one color). Unstimulated T cells at timepoint 0 served as control for basal gene expression. The differential gene expression Feature Extraction Software-derived output data files obtained from Miltenyi were further analyzed using the Gene Spring data analysis system (GeneSpring GX Software)

Gene symbol	Gene name	Fold Change	P value
HRH4	Homo sapiens histamine receptor H4	3.471	0.043
IGF1R	Homo sapiens insulin-like growth factor 1 receptor	2.977	0.013
IL7R	Homo sapiens interleukin 7 receptor	2.474	0.031
IL6ST	Homo sapiens interleukin 6 signal transducer	2.161	0.022
PPARG	Homo sapiens peroxisome proliferator-activated receptor gamma	2.070	0.014

Table 12: Downpregulated genes in anti-CD3/anti-CD28 stimulated T cells of iCMC patients compared to healthy controls

Untouched T cells of two iCMC patients and two healthy controls were stimulated with anti-CD3 and anti-CD28 for six hours and cell pellets were sent to Miltenyi for gene expression analysis using Agilent Whole Human Genome Oligo Microarrays (one color). Unstimulated T cells at timepoint 0 served as control for basal gene expression. The differential gene expression Feature Extraction Software-derived output data files obtained from Miltenyi were further analyzed using the Gene Spring data analysis system (GeneSpring GX Software)

In summary T cell responses induced by yeast such as *Candida albicans* can be deregulated as shown in this study on the basis of the disease model of chronic mucocutaneous candidiasis (CMC). Table 13 gives an overview about the main results of cell mediated immune responses analyzed in the present study in patients with isolated CMC and APS1 which are both subgroups of chronic mucocutaneous candidiasis.

Analyzed cells	iCMC	APS1
PBMCs	**> *Candida*-stimulus** IL-17 ↓, IL-22 ↓ IL-1β ↓, IL-6 ↔, CCL20 ↔ IL-10 ↑, IFN-γ ↓ **> PHA-stimulus** IL-17 ↓, IL-22 ↓ IL-1β ↓, IL-6 ↔, CCL20 ↔ IL-10 ↓, IFN-γ ↓ **Proliferation** > *Candida* ↓ > PHA↔ **STAT3 DNA binding**: ↓ **STAT1 DNA binding**: ↑	**> *Candida*-stimulus** IL-17 ↓, IL-22 ↓ IL-1β ↓, IL-6 ↔, CCL20 ↔ IL-10 ↓, IFN-γ ↓ **> PHA-stimulus** IL-17 ↓, IL-22 ↓ IL-1β ↓, IL-6 ↑, CCL20 ↔ IL-10 ↑, IFN-γ ↓ **Proliferation** > *Candida* ↓ > PHA↔ **STAT3 DNA binding**: ↓ **STAT1 DNA binding**: not analyzed

DCs	**> zymosan-stimulus** IL-6 ↔, IL-10 ↓ IL-1β, IL-23, IL-12p70 barely induced TNF-α ↑ **> curdlan-stimulus** IL-6 ↓, IL-10 ↓ IL-1β ↓, IL-23 ↓, IL-12p70 ↓ TNF-α ↑ **> LPS-stimulus** IL-6 ↑, IL-10 ↓ IL-1β not detectable IL-23 ↓, IL-12p70 ↓ TNF-α ↔	**> zymosan-stimulus** IL-6 ↑, IL-10 ↔ IL-1β, IL-23, IL-12p70 barely induced TNF-α ↔ **> curdlan-stimulus** IL-6 ↓, IL-10 ↓ IL-1β ↓, IL-23 ↔, IL-12p70 ↓ TNF-α ↓ **> LPS-stimulus** IL-6 ↔, IL-10 ↓ IL-1β not detectable IL-23 ↓, IL-12p70 ↓ TNF-α ↓
DC maturation	**> zymosan-stimulus** CD80 ↓, CD83 ↓, CD40 ↓ CD86 ↑, HLA-DR ↑ **> curdlan-stimulus** CD80 ↔, CD83 ↔, CD40 ↔ CD86 ↑, HLA-DR ↔ **> LPS-stimulus** CD80 ↓, CD83 ↓, CD86 ↓ CD40 ↑, HLA-DR ↑	**> zymosan-stimulus** CD80 ↔, CD83 ↔, CD86 ↑ CD40 ↓, HLA-DR ↑ **> curdlan-stimulus** CD80 ↔, CD83↔, CD86 ↑ CD40 ↑↑, HLA-DR ↓ **> LPS-stimulus** CD80 ↔, CD83 ↓, CD86 ↔ CD40 ↑↑, HLA-DR ↓
Generated Th17 cells	IL-17 ↓↓ IL-22 ↓↓	IL-17 ↓ IL-22 ↓↓
Generated T cell lines	**Th17**: IL-17+IL-22 ↓↓, IL-2 ↑ **Th1**: IFN-γ ↓, IL-2 ↑ **Th2**: IL-4 ↑, IL-2↓	**Th17**: IL-17+IL-22 ↓↓, IL-2 ↑ **Th1**: IFN-γ ↑, IL-2 ↑ **Th2**: IL-4 ↔, IL-2↑
Naïve T cells (coincubated with healthy *Candida* compound-stimulated DCs) (ASA) in relation to proliferation	**> zymosan-DCs** IL-17 ↓↓, IL-22 ↓↓ IFN-γ ↓, IL-10 ↓ **> curdlan-DCs** IL-17↓↓, IL-22 ↓↓ IFN-γ ↓, IL-10 ↓ **> LPS-DCs** IL-17 ↓↓, IL-22 ↓↓ IFN-γ ↓↓, IL-10 ↓ **Proliferation**: ↓ under all stimulation conditions **> zymosan-DCs** IL-17 ↑, IL-22 ↓ IFN-γ ↔, IL-10 ↑ **> curdlan-DCs** IL-17 ↓, IL-22 ↓ IFN-γ ↑, IL-10 ↑↑ **> LPS-DCs** IL-17 ↔, IL-22 ↔ IFN-γ ↓, IL-10 ↑	**not analyzed**

Memory T cells (coincubated with healthy *Candida* compound-stimulated DCs) (ASA)	> **zymosan-DCs** IL-17 ↑, IL-22 ↑ IFN-γ ↑, IL-10 ↑ > **curdlan DCs** IL-17 ↑, IL-22 ↑ IFN-γ ↑, IL-10 ↑ > **LPS-DCs** IL-17 ↑, IL-22 ↑ IFN-γ ↑, IL-10 ↔ **Proliferation**: normal under all stimulation conditions	**not analyzed**

Table 13: Summary of main results of cell mediated immune responses in iCMC patients and APS1 patients

PBMCs of iCMC and APS1 patients produced significant reduced amounts of IL-17 and IL-22 and showed an impaired cytokine profile of IL-10 and IFN-γ secretion. Moreover PBMCs of iCMC/APS1 patients were characterized by a decreased STAT3 and increased STAT1 activation that was associated with *STAT1* mutations in three analyzed iCMC patients. Stimulation of MoDC of iCMC/APS1 patients with *Candida* cell wall compounds and LPS resulted in an impaired maturation and cytokine profile. Co-incubation of naïve T cells of iCMC patients with these *Candida* compound-stimulated MoDC revealed an impaired proliferation capacity and decreased amounts of the Th17-associated cytokines IL-17 and IL-22 compared to healthy T cells. Noticeable, cytokine secretion of memory T cells of iCMC patients was not impaired. Naïve T cells isolated from iCMC/APS1 patients showed an impaired ability to differentiate into Th17 cells as generated Th17 cells of these patients produced decreased amounts of IL-17 and IL-22 in comparison to healthy Th17 cells. Th1 polarization was detectable in APS1 patients whereas Th1 cells from the iCMC patient secreted diminished amounts of IFN-γ. Th2 differentiation could be observed in both iCMC and APS1 patients with normal or even enhanced amounts of IL-4 in comparison to healthy Th2 cells.

These observed defects of innate and adaptive immunity in response to *Candida* give further insights in the pathology of CMC and may open new strategies for the treatment of the disease. Lactic acid bacteria (LAB) have been discussed to prevent or treat *Candida*-infections. For that purpose basic investigations on the influence of LAB on the DC-T cell-axis are necessary and the results of these investigations are shown in the next part of this study.

4.8 Effects of lactic acid bacteria (LAB) on the human immune system

Growing evidence suggests that dysregulated host-microbial interactions lead to chronic inflammation and commensal bacteria such as LAB may prevent infection. LAB are known to modulate the immune system of the host - mainly on the level of dendritic cells that induce specific T cell responses. Therefore the effect of the two lactic acid bacteria strains IMS1 and IMS2 were investigate for their ability to modulate the function of human healthy MoDC and the ensuing T cell responses.

4.8.1 Analysis of human DCs stimulated with LAB

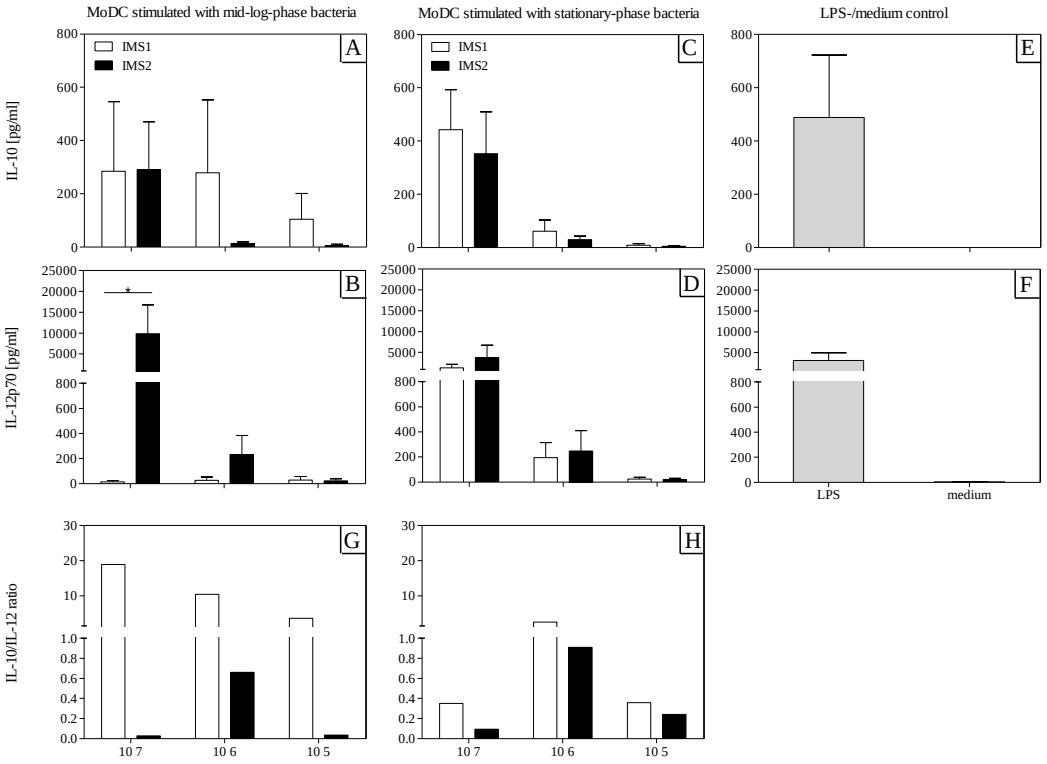
4.8.1.1 LAB strains differently induce IL-10 and IL-12 in human MoDC

To investigate the impact of LAB on the cytokine production of human dendritic cells, immature monocyte-derived DCs (MoDC) were exposed to the bacterial strains IMS1 and IMS2 in different concentrations (colony forming units, cfu) from different growth phases for 48 hours. Cell-free supernatants were assayed for IL-12p70 and IL-10 by ELISA (figure 35).

Exposure of immature MoDC to IMS1 in the mid-log phase induced a higher in total release of IL-10 than stimulation of MoDC with IMS2 (Fig.35, A) and this effect was most obvious at concentrations of 10^6 cfu and 10^5 cfu. Concerning MoDC stimulation with bacteria strains from the stationary phase, IL-10 levels were dose-dependently enhanced in both strains (Fig.35, C). IMS1 led to a higher IL-10 release compared to IMS2 at concentrations of 10^7 cfu and 10^6 cfu, only low amounts of IL-10 were detectable at a bacterial concentration of 10^5 cfu (Fig.35, C). Under no bacterial stimulation (medium control), no IL-10 secretion was detected in supernatants of MoDC. Comparison of IL-12p70 production by MoDC matured with LAB revealed that IMS2 from both mid-log and stationary growth phase induced more IL-12p70 than IMS1 (Fig.35, B,D). This IL-12p70-inducing effect of IMS2 was more prominent in the mid-log phase strains, MoDC treated with IMS2 at a concentration of 10^7 cfu secreted significantly increased amounts of IL-12p70 compared to IMS1-triggered MoDC (Fig.35, B). Medium-stimulated MoDC did not secrete detectable levels of 12p70 (Fig.35, F). LPS-treated MoDC served as positive control for stimulation, but both strains did not induce more IL-10 in MoDC compared to the LPS control (Fig.35, A-E). IL-12p70 de-

tection in supernatant of IMS1-/IMS2-matured MoDC was not enhanced compared to LPS conditions, except for the concentration of 10^7 cfu (Fig.35, B-F).

The IL-10/IL-12 ratio for mid-log phase and stationary phase cultures was determined showing IMS1 was more potent in inducing a high IL-10/IL-12 ratio in human MoDC (Fig.35, G-H).



IMS1 vs. IMS2 (mid-log phase)	IL-10	IL-12p70
10^7 cfu	0.3429	0.0294
10^6 cfu	0.8824	0.2186
10^5 cfu	0.8778	0.8687

IMS1 vs. IMS2 (stationary phase)	IL-10	IL-12p70
10^7 cfu	0.6857	0.6857
10^6 cfu	0.8846	0.6631
10^5 cfu	0.8778	0.6446

Figure 35: MoDC stimulated with IMS1 are characterized by an enhanced IL-10 and decreased IL-12p70 production

Immature MoDC from healthy volunteers were stimulated with IMS1 (white bars) and IMS1 (black bars) from the mid-log (A-B) and stationary (C-D) growth phase in different concentra-

tions (10^7-10^5cfu) for 24 hours. Medium-treated MoDC served as negative control, MoDC matured by LPS (100 ng/ml) as positive control for stimulation (E-F). Supernatants were recovered and amounts of IL-10 and IL-12p70 were measured in supernatant by ELISA. Results are presented as mean ± SEM, four independent experiments are shown. *, $p<0.05$, Mann Whitney test. Determined p values are indicated in the tables. The IL-10/IL-12 ratio was determined of mean values of cytokine production (G-H)

4.8.1.2 LAB strains differently upregulate maturation markers in human MoDC

To test whether the bacterial strains IMS1 and IMS2 induce a different maturation pattern in MoDC, immature MoDC were stimulated with different concentrations of the bacterial strains IMS1 and IMS2 for 48 hours. The maturation marker and costimulatory molecules CD80, CD83, CD86, CD40 and HLA-DR were stained and analyzed by flow cytometry (figure 36).

CD80, CD83, CD86 and CD40 were dose-depently upregulated in MoDC stimulated with IMS1 and IMS2 in the mid-log phase (Fig.36, A-D). The bacterial strains at the highest concentration induced the highest percentage of MoDC positive for upregulation of maturation marker. IMS1 induced a slightly higher expression of CD80, CD83, CD86, CD40 and HLA-DR in MoDC compared to IMS2 (Fig.36, A-D).

Concerning DC maturation by IMS1 and IMS2 in the stationary phase also a dose-dependent induction of CD80- CD83-, CD86- and CD40- expression in MoDC could be detected (Fig.36, F-I). Respectively, rather a higher MoDC activation was observed after stimulation with IMS2 in the stationary growth phase (Fig.36, F-I). Statistic significances of the effects could not be anlayzed because only one pilot experiment was performed.

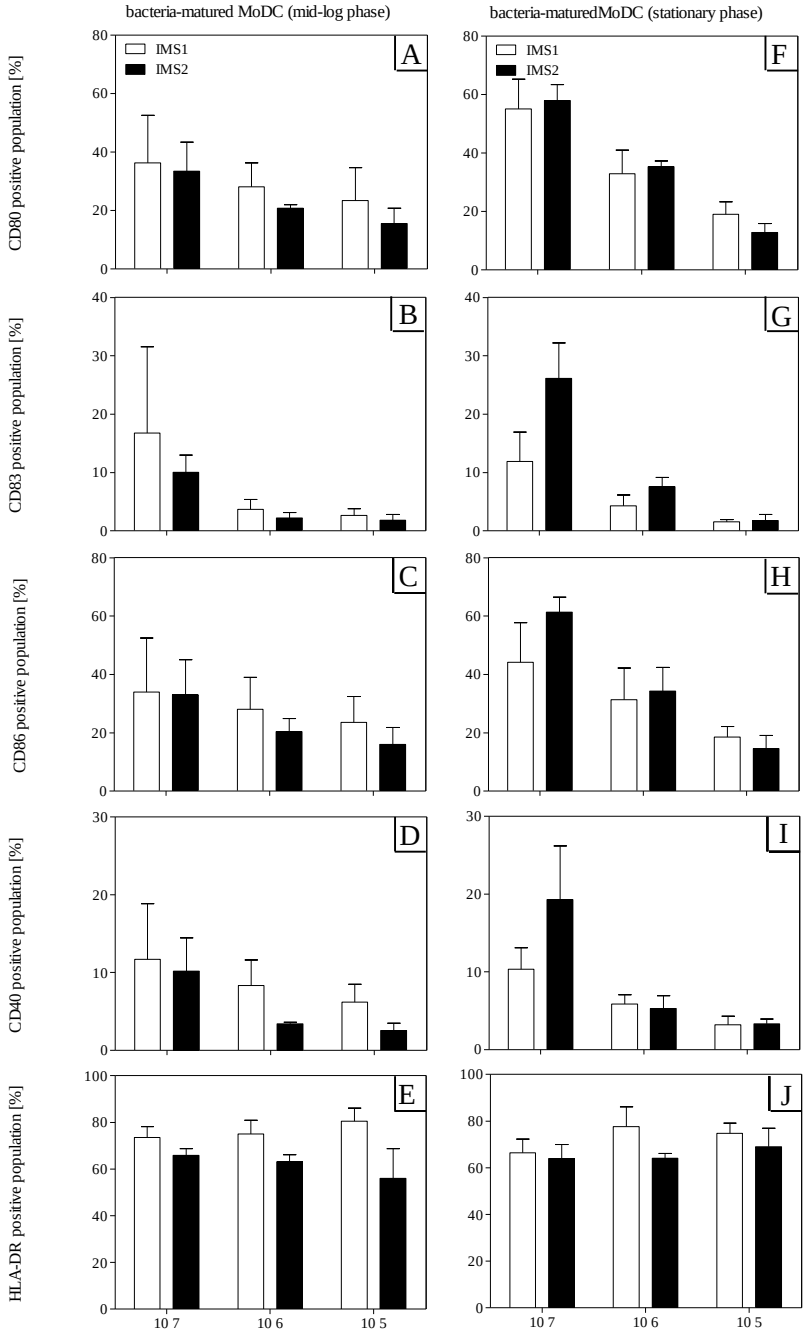


Figure 36: Maturation markers are differently expressed in MoDC stimulated with IMS1 and IMS2

Immature MoDC from healthy volunteers were stimulated with IMS1 (white bars) and IMS2 (black bars) from the mid-log (A-E) and stationary (F-J) growth phase in different concentrations (10^7-10^5cfu) for 24 hours. The expression of maturation markers was assessed by flow cytometry. Results are presented as mean percentage of positive stained cells ± SEM, one pilot experiment is shown

4.8.2 Analysis of T cells induced by LAB-stimulated MoDC

LAB have been described to promote Th1 and Treg cells due to their ability to induce IL-10 and IL-12 production in dendritic cells. Because of the different IL-10/IL-12 ratios of MoDC matured in the presence of IMS1 and IMS2 the phenotype of primary T cell responses induced by these MoDC was examined.

4.8.2.1 Impact of LAB on T cell differentiation

To examine the influence of LAB on T cell differentiation, MoDC of non-atopic volunteers were primed with the bacterial strains IMS1 and IMS2 from different growth phases at different concentrations (10^7-10^5cfu) and cocultured with purified naïve $CD4^+CD45RA^+$ T cells in an allogenic stimulation assay at a DC:T cell ratio of 1:10 for seven days. Naïve $CD4^+CD45RA^+$ T cells were stimulated with LPS- or medium-matured MoDC in the presence of rhIL-2, rhTGF-β, anti-IL-12 and anti-IFN-γ to obtain a positive control for regulatory T cells (Tregs). In order to get a positive control for Th1 polarization naive T cells were primed with MoDC stimulated with LPS plus anti-IFN-γ and anti-IL-4 mAb was added to the coculture. T cells induced by medium- or LPS-treated MoDC alone served as controls.

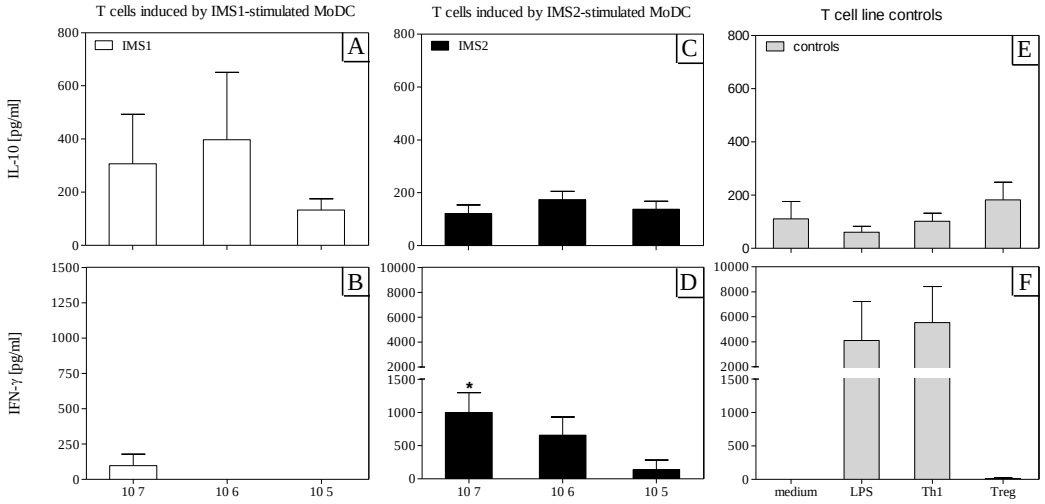
To determine the possible directions of T cell polarization the key cytokines of Th1 cells (IFN-γ) and Tregs (IL-10) were determined by ELISA and intracellular staining, expression of Foxp3 was assessed by flow cytometry. Cytokine production of IL-10 and IFN-γ of T cells induced by mid-log phase bacteria (figure 37) and by stationary phase bacteria (figure 38) was analyzed by ELISA.

4.8.2.1.1 Induction of T cells by LAB in the mid-log phase

Immature MoDC exposed to IMS1 from the mid-log growth phase induced a cytokine profile in naïve T cells dominated by enhanced amounts of IL-10 (Fig.37 A) and almost no detectable level of IFN-γ (Fig.37, B). At bacterial concentrations of 10^7 cfu and 10^6 cfu IL-10 amounts produced by IMS1-instructed T cells were higher compared to IL-10 released by IMS2-instructed T cells. At a bacterial concentration of 10^5 cfu IL-10 induction in naïve T cells was comparable between IMS1 and IMS2 (Fig.37, A,C). Coculture of IMS1-primed MoDC with naïve T cells resulted in higher amounts of IL-10 compared to the Treg control (Fig.37, A-E).

In contrast, IMS2-stimulated MoDC polarized T cells were characterized by low levels

of IL-10 (Fig.37, C) and highly increased amounts of IFN-γ (Fig.37, D) compared to IMS1. MoDC matured by IMS2 at a concentration of 10^7 cfu promoted significant increased IFN-γ secretion in naïve T cells in comparison to IMS1-triggered MoDC (Fig.37, C-D). The potency of IMS1 and IMS2 to induce IFN-γ was lower in comparison to the generated Th1 control and LPS-induced T cells (Fig.37, B-F).



mid-log phase bacteria IMS1 vs. IMS2	IL-10	IFN-γ
10^7 cfu	1.0000	0.0294
10^6 cfu	1.0000	0.1241
10^5 cfu	0.4857	0.8676

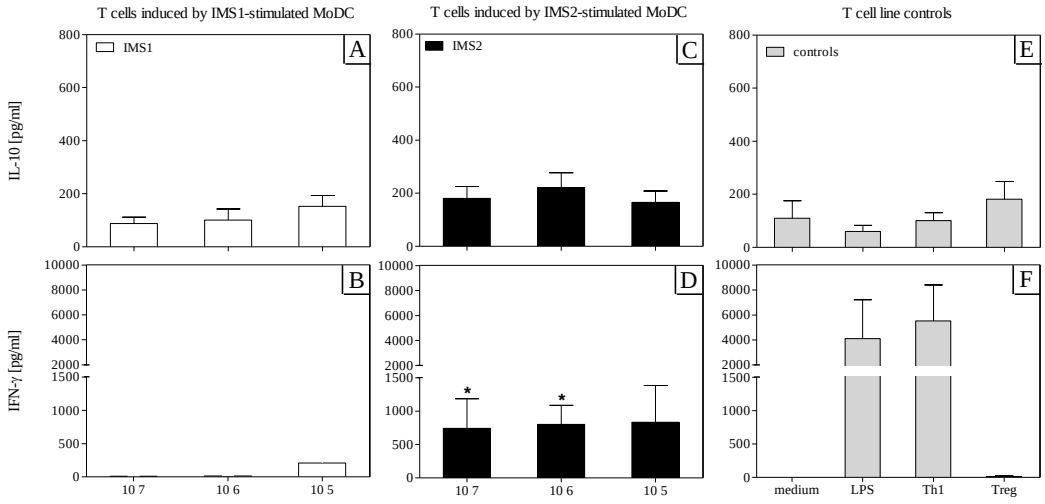
Figure 37: T cells induced by IMS1 (mid-log phase)-stimulated MoDC are characterized by a high IL-10/IFN-γ ratio

Immature MoDC stimulated with IMS1 (white bars, A-B) or IMS2 (black bars, C-D) at different concentrations (10^7-10^6 cfu) from mid-log phase cultures were cocultured with purified naïve $CD4^+CD45RA^+$ T cells in an allogenic stimulation assay at a T cell:DC ratio of 1:10 for seven days. MoDC pretreated with LPS (100 ng/ml) and IFN-γ (100 U/ml) (Th1 control) and LPS/medium (100ng/ml) (Treg control) were co-incubated with purified naïve $CD4^+CD45RA^+$ T cells to generate T cell line controls (E-F). Neutralizing anti-IL-4 (5 µg/ml) and rhIL-12 (250 ng/ml) to the Th1 control and neutralizing anti-IL-12 (5 µg/ml) and anti-IFN-γ (1µg/ml), rhIL-2 (200 ng/ml) and rhTGF-β (5 ng/ml) was added to the Treg control (E-F). IL-10 and IFN-γ were analyzed in the supernatants by ELISA. Results are given as mean ± SEM and four independent experiments are shown. *, $p<0.05$, Mann Whitney test. Analyzed p values are listed in the table

4.8.2.1.2 Induction of T cells by LAB in the stationary phase

In order to compare T cell polarization induced by LAB from different growth phases T cell differentiation of naïve T cells induced by IMS1 and IMS2 from the stationary phase was investigated (figure 38). The coincubation of IMS1-matured MoDC with naive T cells led to IL-10 production and almost no detectable amounts of IFN-γ (Fig.38, A-B). The IL-10 inducing effect of IMS1-stimulated DCs on T cells was slightly diminished in the stationary phase bacteria cultures (Fig.38) compared to the mid-log phase cultures (Fig.37).

Priming of naïve T cells with IMS2-stimulated MoDC revealed IL-10 amounts comparable to IMS1-instructed T cells (Fig.38, A,C), but these IMS2-polarized T cells were characterized by highly enhanced levels of IFN-γ compared to IMS1-instructed T cells (Fig.38, B,D). IMS2 at a concentration of 10^7 cfu and 10^6 cfu promoted signficant more IFN-γ production in naïve T cells compared to IMS1 (Fig.38, B,D).



stationary phase bacteria IMS1 vs. IMS2	IL-10	IFN-γ
10^7 cfu	0.2000	0.0265
10^6 cfu	0.1143	0.0265
10^5 cfu	0.6857	0.3005

Figure 38: T cells induced by IMS1 (stationary phase)-stimulated MoDC are characterized by a high IL-10/IFN-γ ratio

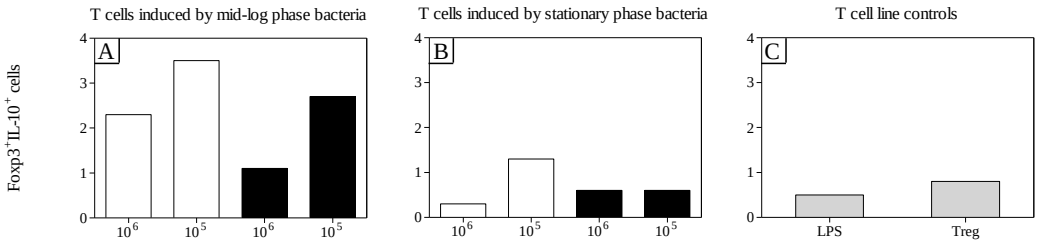
Immature MoDC stimulated with IMS1 (white bars, A-B) or IMS2 (black bars, C-D) at different concentrations (10^7-10^6 cfu) from stationary phase cultures were cocultured with purified

naïve $CD4^+CD45RA^+$ T cells in an allogenic stimulation assay at a T cell:DC ratio of 1:10 for seven days MoDC pretreated LPS (100 ng/ml) and IFNγ (100 U/ml) (Th1 control) and LPS/medium (100ng/ml) (Treg control) were co-incubated with purified naïve $CD4^+CD45RA^+$ T cells to generate T cell line controls (E-F). Neutralizing anti-IL-4 (5 µg/ml) and rhIL-12 (250 ng/ml) to the Th1 control and neutralizing anti-IL-12 (5 µg/ml) and anti-IFN-γ (1 µg/ml), rhIL-2 (200 ng/ml) and rhTGF-β (5 ng/ml) was added to the Treg control. IL-10 and IFN-γ were analyzed in the supernatants by ELISA. Results are given as mean ± SEM and four independent experiments are shown. *, $p<0.05$, Mann Whitney test. Analyzed p values are listed in the table

4.8.2.1.3 IMS1-matured MoDC induce a higher percentage of $Foxp3^+IL\text{-}10^+$ cells

Naïve T cells polarized by IMS1-stimulated MoDC are characterized by enhanced amounts of IL-10 and decreased levels of IFN-γ. Next step was to examine if these cells are also positive for Foxp3 and IL-10 expression - phenotypical characteristics for Tregs. On day seven of the ASA cells were restimulated with anti-CD3/anti-CD28, stained intracellularly and analyzed for expression of Foxp3 and IL-10 by flow cytometry (figure 39). MoDC stimulated with IMS1 in the mid-log phase induced a higher percentage of $Foxp3^+IL\text{-}10^+$ cells in naïve T cells compared to IMS2 in both tested concentrations 10^6 cfu and 10^5 cfu (Fig.39, IA and IIA,B). And this percentage of $Foxp3^+IL\text{-}10^+$ T cells polarized by LAB from the mid-log growth phase is higher than the $Foxp3^+IL\text{-}10^+$ percentage of generated Tregs and T cells induced by LPS-triggered MoDC (Fig.39, IC and IIC). Concerning the stationary phase cultures IMS1-primed MoDC led only to an enhanced percentage of $Foxp3^+IL\text{-}10^+$ cells at a concentration of 10^5 cfu in comparison to IMS1-primed MoDC (Fig.39, IB). The percentage of $Foxp3^+IL\text{-}10^+$ cells in general is higher in T cells instructed by LAB from the mid-log growth phase.

I)



II)

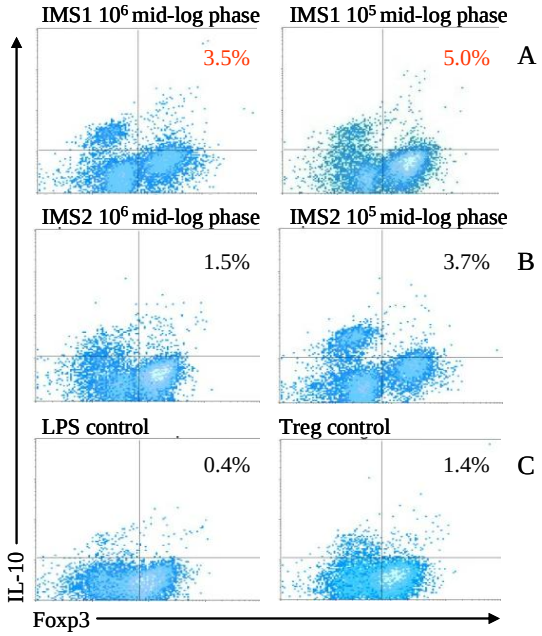


Figure 39: T cells induced by IMS1-stimulated MoDC are characterized by a higher percentage of Foxp3$^+$IL-10$^+$ cells

Immature MoDC stimulated with IMS1 (white bars) or IMS2 (black bars) from mid-log or stationary phase cultures at different concentrations (10^6-10^5 cfu) were cocultured with purified naïve CD4$^+$CD45RA$^+$ T cells in an allogenic stimulation assay at a T cell:DC ratio of 1:10. MoDC pretreated with LPS/medium were co-incubated with purified naïve CD4$^+$CD45RA$^+$ T cells (LPS control) and anti-IL-12 (5 µg/ml) and anti-IFN-γ (1 µg/ml), rhIL-2 (200 ng/ml) and rhTGF-β (5 ng/ml) was added to generate the Treg control. On day seven of coculture cells of the ASA were restimulated with anti-CD3/anti-CD28 (1 µg/ml) for 48 hours followed by intracellular staining of Foxp3 and IL-10. **(I)** The percentage of Foxp3$^+$IL-10$^+$ cells induced by mid-log and stationary phase bacteria is demonstrated graphically. One representative experiment of two is shown. **(II)** The percentage of Foxp3$^+$IL-10$^+$ cells is shown by FACS analysis. One representative staining of two experiments is depicted showing T cells induced by mid-log phase bacteria at the concentrations of 10^6 and 10^5 cfu

4.8.2.1.4 IMS1-matured MoDC induce a higher percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells

Tregs are characterized by the expression of the surface markers CD4 and CD25 and the transcription factor Foxp3. On day seven of the ASA cells were restimulated with anti-CD3/anti-CD28, stained intracellularly and analyzed for expression of CD4, CD25 and Foxp3 by flow cytometry (figure 40). Generated Tregs showed the highest percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells (92 %) compared to the bacteria-induced T cells (42 %-76 %) and the medium control (26 %). IMS1 (log-phase and stationary phase)-stimulated MoDC induced a higher percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells in compari-

son to IMS2-triggered MoDC at the tested bacterial concentrations of 10^6 cfu and 10^5 cfu.

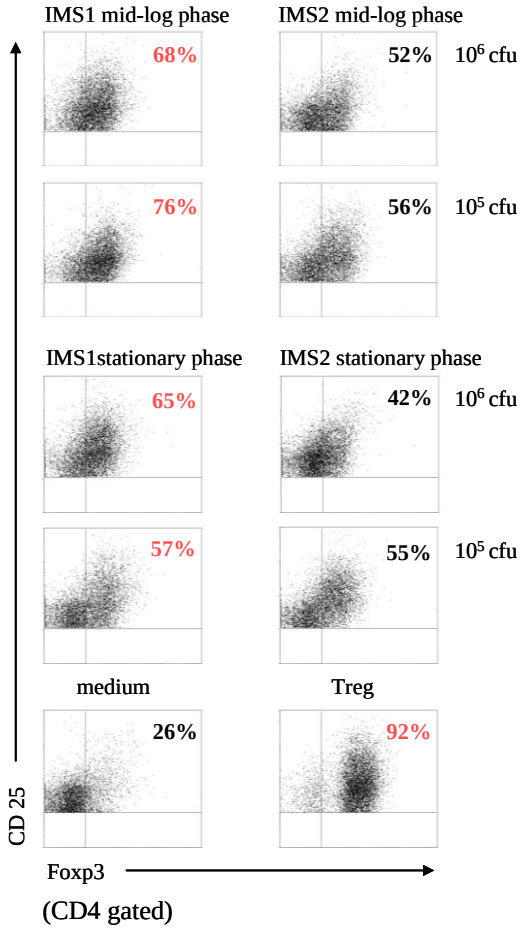


Figure 40: T cells induced by IMS1-stimulated MoDC are characterized by a higher percentage of $CD4^+CD25^+Foxp3^+$ cells

Immature MoDC stimulated with IMS1 or IMS2 from mid-log or stationary phase cultures at different concentrations (10^6-10^5 cfu) were cocultured with purified naïve $CD4^+CD45RA^+$ T cells in an allogenic stimulation assay at a T cell:DC ratio of 1:10. MoDC pretreated with LPS/medium (Treg control) were co-incubated with purified naïve $CD4^+CD45RA^+$ T cells to generate the Treg control. On day seven of coculture cells of the ASA were restimulated with anti-CD3/anti-CD28 (1 µg/ml) for 48 hours followed by intracellular staining of CD4, CD5 and Foxp3. $CD4^+$ gated $CD25^+Foxp3^+$ cells are demonstrated. One representative of two experiments is shown

4.8.2.1.5 T cells induced by IMS-1 stimulated MoDC are functional suppressive

To verify whether the higher percentage of IL-10 producing $CD4^+CD25^+Foxp3^+$ cells differentiated by IMS1-matured MoDC is also functional, cultured cells were coincu-

bated with CFSE-labelled $CD4^+$ responder T cells in a suppression assay (figure 41). IMS1-stimulated MoDC at both concentration of 10^6 cfu and 10^5 cfu promoted T cells that were able to suppress proliferation of $CD4^+$ responder T cells more than T cells induced by IMS2-primed MoDC (Fig. 41). But these IMS1-primed T cells did not reach the suppressive capacity of natural Tregs isolated from peripheral blood (Fig.41).

IMS1-induced suppression of responder T cells was more efficient than the suppressive ability of T cells generated with LPS- or medium-treated MoDC.

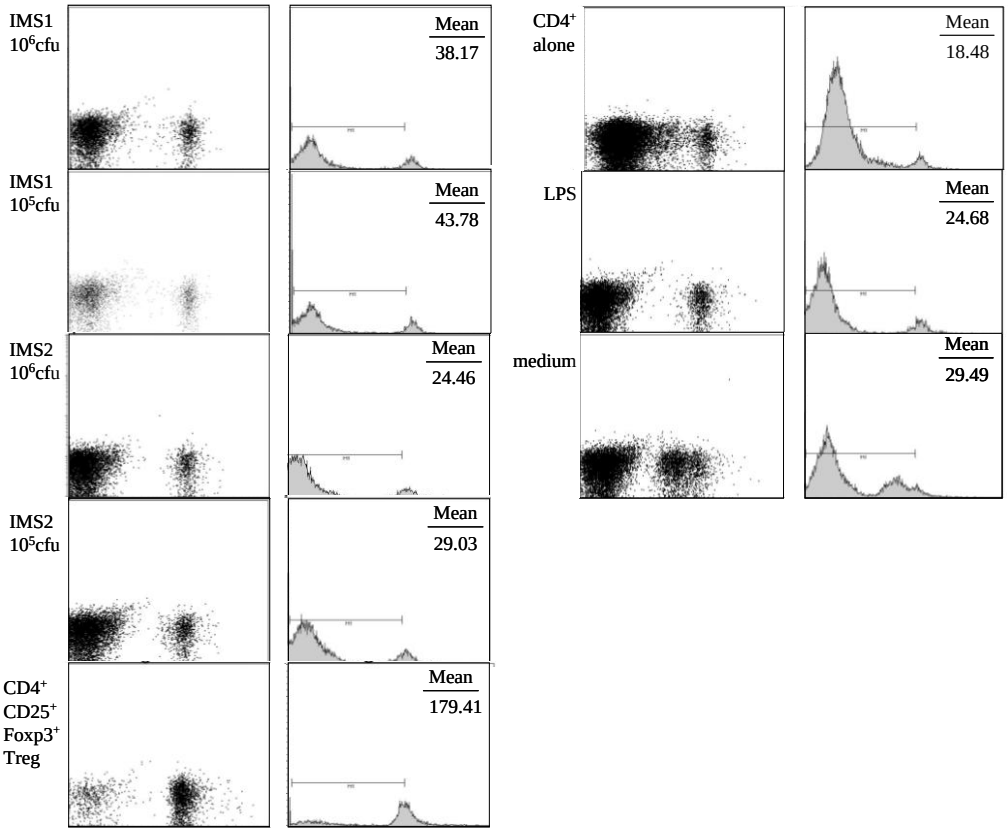


Figure 41: T cells induced by IMS1-stimulated MoDC display suppressive function

$CD4^+$ responder T cells were labelled with CFSE (0.5 µM) and cocultured with effector T cells from the ASA at a Teff:Tresp ratio of 1:1 in the presence of 1 µg/ml anti-CD3/anti-CD28 and 20 U/ml rhIL-2. Generated Tregs from the ASA were cultured with CFSE-labelled responder T cells in the presence of 100 U/ml rhIL-2. $CD4^+CD25^+Foxp^+$ cells were isolated from peripheral blood and coincubated with CFSE-labelled $CD4^+$ responder T cells, 100 U/ml rhIL-2 was added. Proliferation capacity of $CD4^+$ responder T cells was anlayzed after five days by flowcytometry. One representative experiment of two is shown

To summarize, these results show how LAB are able to instruct human MoDC strain-dependent and therefore prime different T cell responses. The bacterial strain IMS1

shows immune-regulatory characteristics by the induction of increased amounts of IL-10 and decreased level of IL-12p70 in healthy human MoDC. In contrast, IMS2 is a more pro-inflammatory strain by inducing high amounts of IL-12p70 in MoDC. IMS1-stimulated MoDC instructed naïve T cells to produce high levels of IL-10 and decreased amounts of IFN-γ whereas IMS2-matured MoDC promoted enhanced IFN-γ amounts in T cells. IL-10 producing T cells induced by IMS1 showed phenotypically characteristics of Tregs by the highest expression of the Treg marker CD4, CD25 and Foxp3 compared to IMS2-polarized T cells. These IMS1-induced regulatory T cells were also functional suppressive showing the highest suppression activity of responder T cells compared to IMS2-instructed T cells. These human *in vitro* data give first important indications for immune-regulatory capacities of the two tested bacterial strains in the human. Further investigations are needed to proof the use of these bacteria in food or to treat different diseases such as allergy, atopic dermatitis or *Candida*-infections which can be chronic as in the case of chronic mucocutaneous candidiasis (CMC). T cell responses involved in CMC will be discussed in the next part of the study.

5 Discussion

Patients with CMC suffer from recurrent or chronic infections of skin, nails and mucosal tissues with *Candida albicans*. The involvement of Th17 cells in clearing *Candida albicans* infections has been reported in mice and human (260-263). Th17 have been described as a lineage of $CD4^+$ lymphocytes producing IL-17A, IL-17F and IL-22 (16, 491) that are important for neutrophil recruitment and mucosal host defense against organisms as *Candida albicans* (192, 274).

5.1 PBMCs of CMC patients show a distinct cytokine profile in response to *Candida albicans*

Peripheral blood mononuclear cells (PBMCs) of CMC patients and healthy controls were screened for cytokine secretion in response to *Candida albicans*. This study affirms the importance of IL-17 producing cells showing that PBMCs of patients with a current *Candida*-infection (Candidiasis) secreted enhanced amounts of IL-17 and IL-22 compared to healthy controls with no *Candida*-infection. In contrast, PBMCs of both isolated CMC (iCMC) and APS1 patients were characterized by a significant reduced IL-17/IL-22 production after *Candida* stimulation compared to Candidiasis patients and healthy controls pointing to a defect Th17 differentiation. The remarkable diminished amounts of IL-17 and IL-22 might be due to decreased numbers of IL-17 secreting cells as several studies described a reduced number of IL-17 producing $CD4^+CCR4^+CCR6^+$ cells in iCMC patients (257, 264). The observed IL-17A deficiency in iCMC patients is consisting with previous data (257, 264) whereas the reduced amounts of IL-17A in APS1 patients are contrary to studies describing normal (265) or enhanced IL-17A amounts in PBMCs of APS1 patients (264, 492). Therefore IL-22 has been suspected to be more important in *Candida* defense especially due to its role at epithelial surfaces (137, 274, 325, 493). Reduced T cell derived IL-17/IL-22 was not exclusively to *Candida* antigen stimulus. Stimulation of PBMCs with mitogen also led to almost no detectable amounts of IL-17A and IL-22 suggesting that the observed IL-17/IL-22 deficiency concerns the whole T cell compartment.

The secretion of tissue-instructing cytokines such as IL-17, IL-22 and IFN-γ by Th17 cells induces the production of antimicrobial peptides by epithelial cells following re-

cruitment of phagocytic neutrophils (139, 274, 494) and the observed Th17 defect in CMC patients supposes an disturbed immune response against *Candida* at epithelial surfaces.

In humans Th17 cells differentiate in the presence of IL-6 and IL-1β (16, 494) and the detected IL-17 deficiency could be due to a dysregulated or defective production of these cytokines. This study demonstrates that *Candida*-stimulated PBMCs of iCMC patients, APS1 patients and matched control groups are able to produce comparable amounts of IL-6 and slightly decreased levels of IL-1β making a defect secretion of Th17-differentiating cytokines rather unlikely. These data are in line with Ng et al showing also no significant differences in IL-6 production in PBMCs of iCMC and APS1 patients after exposure to *Candida* species (264). On the contrary, previous studies found enhanced levels of IL-6 in CMC patients but these discrepancies could be explained by the use of different *Candida* compounds (246, 357). Th17 cells are characterized by the expression of the chemokine receptor CCR6 and binding of its ligand CCL20 provides migration of Th17 cell subsets to sites of infection (16, 495). Concerning CCL20 secretion no differences could be detected between *Candida*-/PHA-stimulated PBMCs of iCMC patients, APS1 patients and control subjects excluding IL-17 deficiency as a consequence of a reduced Th17-recruitment to inflamed sites.

A Th1 response is important to defense *Candida*-infections and IFN-γ-deficient mice have an enhanced susceptibility to *Candida*-infections (286, 354, 496). The present study shows that PBMCs of patients with iCMC are characterized by diminished amounts of inflammatory IFN-γ and enhanced amounts of regulatory IL-10 in response to *Candida* (but not to PHA) suggesting impaired *Candida*-specific Th1 responses. These data of an impaired IFN-γ/IL-10 secretion are in concordance with various studies reporting a dysregulated T cell response in CMC patients including our own (246, 247, 264, 288, 357, 358). In contrast, in PBMCs of APS1 patients both cytokines IFN-γ and IL-10 were reduced after stimulation with *Candida*, findings that are consistent with Ng et al. demonstrating reduced IFN-γ amounts in PBMCs of APS1 patients (264). Of note, PBMCs of APS1 patients produced diminished levels of IFN-γ under both stimulation conditions suggesting that the IFN-γ defect is not specific for the *Candida* antigen. However, these findings of an altered Th1 response differ from recently recorded data of an unchanged Th1 reactivity in response to *Candida* in both iCMC and APS1 patients (492). CMC is a very heterogeneous disease consisting of many subgroups and observed discrepancies in cytokine release could be due to this complexicity of the dis-

ease and depends on the subgroups of CMC patients that are enrolled in the study. A better phenotyping of CMC patients represent a prerequisite to better understand the immunological phenotype and hopefully genotype in the future.

Different types of Tregs control Th1, Th2 and Th17 cells and are thus essential for immunity against *Candida* (231, 287, 289). The enhanced level of IL-10 and decreased IFN-γ/IL-10 ratio in iCMC patients in response to *Candida* antigen might reflect a negative feedback mechanism via Tregs leading to an enhanced IL-10 production. The decreased proliferation capacity observed in *Candida*-stimulated PBMCs of iCMC patients might also indicate the *Candida*-specific involvement of suppressive Tregs. However, IL-10 secretion after mitogen stimulation was comparable between iCMC patients and control groups pointing to a *Candida*-specific immune response in iCMC patients which is characterized by enhanced IL-10 and decreased IFN-γ production. In contrary, PBMCs of APS1 patients produced lower amounts of IL-10 in response to *Candida* antigen (not in response to PHA) compared to control subjects pointing to different *Candida*-specific T cell and cytokine responses in iCMC and APS1 patients. Defective Treg have been described to be involved in the pathogenesis of APS1 that could explain the decreased *Candida*-specific amounts of IL-10 detected in PBMCs of APS1 patients (497, 498).

5.2 PBMCs of CMC patients produce no IL-17 and IL-22 in the auto- and heterologues system

Recently autoantibodies against IL-17 and IL-22 have been described to be responsible for the observed IL-17/IL-22 deficiency in APS1 patients (265, 266). The present study shows that PBMCs of one iCMC and three APS1 patients did not produce IL-17A or IL-22 in response to *Candida* or PHA in medium supplemented with autoantibody-containing autologous serum. These data are in line with the studies by Kisand et al. and Puel et al. suggesting that autoantibodies contained in the autologous serum bind IL-17 and IL-22 and hence are not detectable anymore by ELISA (265, 266). But PBMCs of iCMC and APS1 patients in the present study also did not produce IL-17A and IL-22 under heterologous conditions when the medium did not contain neutralizing autoantibodies. Therefore these results exclude that only autoantibodies affect IL-17 secretion in the tested CMC patients in the present study. The presence of autoantibodies against IL-17 and IL-22 in the serum of the CMC patients included in the present study was not investigated so far and remains to be further analyzed.

5.3 PBMCs of CMC patients show an impaired proliferation capacity

Concerning the proliferation capacity PBMCs of iCMC and APS1 patients were characterized by a normal proliferation in response to PHA compared to healthy controls. To allow a more confident comparison of the tested individuals the proliferation of PBMCs in response to PHA was assumed as maximum proliferation capacity and proliferation to the specific antigen *Candida* was regarded relative to this proliferation. Therefore the relative stimulation index was determined as a ratio of proliferation to PHA and proliferation to *Candida*. PBMCs of both iCMC and APS1 patients showed a slightly decreased proliferative response to *Candida* compared to healthy controls confirming a diminished immune response of T cells. IL-10 is known to suppress proliferation. Thus the *Candida*-specific increased amounts of IL-10 analyzed in PBMCs of iCMC patients could be an explanation for the diminished proliferative capacity to *Candida* as the proliferative response to mitogen was comparable to healthy controls. The ability of T cells from CMC patients to proliferate in response to the antigen *Candida* is controversial discussed: some studies describe a reduced (258) or normal (247) proliferation to both stimuli PHA and *Candida*, some report a *Candida*-specific increased proliferation and normal proliferation of T cells in response to PHA (288). The different results of proliferation described in these studies may result from different analyzed phenotypes of CMC patients. Because of the complex heterogeneity of CMC including the different subgroups such as iCMC and APS1, a precise characterization of CMC patients is required to acquire a valuable knowledge. Another aspect concerning the varying proliferation data could be the use of different *Candida* antigens (hyphal or yeast form) in several studies.

5.4 PBMCs of CMC patients are characterized by an impaired expression of surface markers compared to healthy controls

Autoimmune endocrine diseases are often associated with characteristic changes of the peripheral blood lymphocytes, in many cases an impaired ratio of $CD4^+$ to $CD8^+$ cells has been reported (Ilonen et al. CEI 1991). Thus, the percentage of T ($CD3^+$), B ($CD19^+$) and NK ($CD56^+$) cells, $CD4^+$ and $CD8^+$ T lymphocytes, naïve ($CD45RA^+$) and memory ($CD45RO^+$) T cells, Th17 ($CD161^+$), monocytes ($CD14^+$) of PBMCs of one iCMC and two APS1 patients was determined by analyzing the mean fluorescence intensity (MFI) by flow cytrometry. Because results of staining were similar between iCMC and APS1 both types of CMC patients are considered together in one group.

All tested patients showed a reduced mean fluorescence intensity (MFI) of these surface markers with respect to healthy controls except for the surface molecules CD19 and CD14. Only rare and contradicting data exist concerning the alteration in lymphocyte populations in patients with APS1 or isolated CMC. A higher percentage of T lymphocytes due to a higher MFI of $CD3^+$ or $CD4^+$ cells as described in Perniola et al. (484) could not be confirmed, but in concordance was the elevated $CD4^+/CD8^+$ ratio in iCMC/APS1 patients compared to healthy controls. The ratio of $CD4^+/CD8^+$ in CMC patients has been reported to be both enhanced and decreased in different studies (247, 484, 499-501). The increased $CD4^+/CD8^+$ ratio in iCMC/APS1 patients might suggest a diminished activity of cytotoxic cells or the accumulation of these cells at inflamed sites, therefore reduced in peripheral blood.

The findings of a decreased MFI of CD45RO and CD45RA in APS1 PBMCs are in contrast to previous data of higher numbers of $CD4^+CD45RO^+$ cells in APS1 patients (484). iCMC/APS1 patients in this study revealed a remarkable enhanced $CD45RA^+/CD45RO^+$ ratio indicating an increased activity of naïve T cells in CMC patients as $CD4^+CD45RA^+CD29^-$ T cells have been described to represent unprimed $CD4^+$ T cells which respond poorly to recall antigen and show suppressor activity (502, 503). The detection of enhanced $CD45RA^+$ cells in iCMC/APS1 patients could also amount for a defect maturation or activation capacity of naïve T cells as activated T cells down-regulate CD45RA expression and exert T helper cell function (503). This might explain the reduced proliferative response of PBMCs and naïve T cells of CMC patients to *Candida* antigen analyzed in the present study which could result in decreased effector

T cell responses against the *Candida* antigen. An unresponsivness of naïve T cells in CMC patients might explain the lower MFI of CD45RO representing decreased circulating $CD45RO^+$ memory T cells responding strongly to antigens (504, 505). These results are in common with other studies showing reduced numbers of circulating $CD4^+$ memory cells in CMC patients (247) and in patients with X-linked gammaglobulinaemia (XLA) suffering from cutaneous anergy (506). Another explanation for the decreased level of $CD45RO^+$ cells in peripheral blood of iCMC/APS1 patients could be that these cells accumulated at sites of infection and are hence limited present in peripheral blood.

Various reports exist concerning the percentage of $CD19^+$ B cells in CMC patients (331, 500, 501). In the present study iCMC/APS1 patients were characterized by a comparable MFI of CD19 to healthy controls making a role of B cells in the pathogenesis of the tested iCMC/APS1 patients rather unlikely.

The decreased MFI of the Th17 marker $CD161^+$ (24) is concordant with the diminished production of IL-17 and IL-22 in PBMCs of iCMC/APS1 patients and might point to a reduced number of Th17 cells in these patients.

The IL-7 receptor CD127 and its interaction with IL-7 modulates the homeostasis of peripheral $CD4^+$ and $CD8^+$ T cells (507, 508). The IL-7R has been shown to be downregulated in human Tregs with suppressive activity on effector T cells in autoimmune diseases such as multiple sclerosis. Moreover the IL-7R is a marker for identification and purification of Treg populations (68, 509). The reduced MFI of CD127 detected in PBMCs of iCMC/APS1 patients compared to healthy controls could therefore indicate an impaired homeostasis of peripheral $CD4^+$ and $CD8^+$ T cells or an impaired Treg activity in comparison to healthy subjects which would be in line with several studies reporting a reduced function of Tregs in APS1 (497, 510).

The observed reduced expression of the natural killer (NK) cell marker CD56 in iCMC/APS1 patients of the present study is contradicting to reports describing normal NK cell counts in patients with APS1 (511) suggesting a diminished activity of NK cells or reduction in peripheral blood due to enhanced migration to infected sites of inflammation.

Data of $CD14^+$ monocytes in APS1 patients varies as enhanced $CD14^+$ frequencies in APS1 patients have been described by several studies (310, 481) whereas Wolff et al. reported variations in $CD14^+$ monocytes in APS1 (512). CD14 was rarely detectable on

PBMCs of both iCMC/APS1 patients and healthy controls making any conclusions difficult.

The described abnormalities of surface molecule expression in the present study might indicate a general decreased cell activity in iCMC/APS1 patients compared to healthy subjects. Discrepancies to already existing data might be due to the low number of tested individuals or different analyzed phenotypes of CMC compared to other studies. Differences also might depend on different phases of the pathogenesis of CMC or on certain manifestations. As already mentioned, only a restricted number of patients was examined in this experiment and a more extensive research is necessary to validate these results.

5.5 PBMCs of CMC patients show a gain-of-function *STAT1* mutation and are characterized by a diminished STAT3 and enhanced STAT1 activation in response to IL-6 and IFN-γ

In three tested iCMC patients of the present study "gain-of-function" mutations in *STAT1* were identified in cooperation with Jean-Laurent Casanova, Rockefeller University New York using whole exome sequencing (485). At the same time *STAT1* mutations have been identified as a new genetic etiology of CMC affecting Th1/Th17 immunity against *Candida* in a study by van de Veerdonk et al. (489). In this cooperative study with Jean-Laurent Casanova heterozygous germline mutations of *STAT1* were found in 47 patients from 20 kindreds with iCMC. The three-dimensional structure of phosphorylated STAT1 molecules revealed that the iCMC-linked missense mutations affected residues located in a speficic pocket of the coiled-coil domain which plays a role in unphosphorylated STAT1 dimerization and STAT1 nuclear dephosporylation (485, 513-518). iCMC-causing *STAT1* alleles were functional characterized using STAT1-deficient U3C fibrosarcoma cells transfected with the WT allele, a Mendelian susceptibiliy to mycobacterial disease (MSDM)-causing-loss-of-function *STAT1* allele or the iCMC-causing *STAT1* alleles. This analysis revealed that cells transfected with the iCMC-causing *STAT1* allele had an increased gamma interferon activation site (GAS)-binding activity in response to IFN-α, IFN-γ and IL-27 stimulation compared to WT allele transfected cells. Moreover these cells showed a higher level of STAT1 phosphorylation and increased transcription of the CXCL9 and CXCL10 target genes indicating that the iCMC-linked *STAT1* mutations are intrinsically gain-of-function (485) which means that the mutated STAT1 gene has a new or abnormal function. Whole genome analysis of activated T cells of iCMC patients carrying the *STAT1* mutant alleles performed in the present study also revealed that gene expression of CXCL9 (62-fold) and CXCL10 (78-fold) was higher than in healthy activated T cells underlining the involvement of an impaired IFN-γ-STAT1-axis in the pathogenesis of the herein tested iCMC patients. IFN-γ-stimulated PBMCs of these two iCMC patients with known *STAT1* mutations exhibited an enhanced STAT1 DNA binding capacity in the TransAM system supporting the functionality of gain-of-function *STAT1* mutations in iCMC patients. Moreover, this gain-of-function, which manifests itself on DNA binding

activity, induction of reporter and target genes, may not necessarily enhance the transcription of target genes, possibly even leading to the repression of some genes.

STAT1 plays a central role in cellular responses induced by variuos cytokines as IFN-α/β, IFN-γ or IL-27 which have been described to inhibit the generation of Th17 cells via STAT1 (486-488, 490). Gain-of-function iCMC causing *STAT1* alleles may therefore increase cellular responses to cytokines activating STAT1 that might be involved in defective Th17 responses in iCMC. PBMCs of the three iCMC patients with identified gain-of-function *STAT1* alelles displayed decreased amounts of IL-17 and IL-22 in response to *Candida*-/PHA-stimulation and generated Th17 cell of these patients were characterized by a reduced ability to produce IL-17 and IL-22 suggesting that these *STAT1* mutations provide a new molecular mechanism involved in Th17 responses. Therefore it has to be examined how these STAT1-associated cytokines mediate their Th17 inhibiting effects. IFN-γ or IL-27 with anti-Th17 activity have been described and also Th17 inhibitory molecules such as the growth factor independent (Gfi-1) gene, Ets1 and sphingosine 1-phosphate (S1P) proposing these mechanisms could play a role in Th17 immunity in iCMC patients with *STAT1* mutations (490, 519-522). In line with the identified *STAT1* mutation is the increased expression of the IFN-γ gene in activated T cells of iCMC patients analyzed by Whole Genome Analysis in the present study. IFN-γ as a hallmark cytokine of Th1 cells signals via STAT1 (523) suggesting a prominent role of inflammatory and cytotoxic T cell responses in iCMC that might be involved in the observed Th17 defect. Contradicting to the identification of a gain-of-function *STAT1* mutation in iCMC are the reduced amounts of IFN-γ measured in activated PBMCs and generated Th1 cells of iCMC patients. How these findings are linked to each other remains to be further investigated. One speculation would be that proposed *Candida*-specific Tregs or cytotoxic cells are involved in STAT1-mediated immune responses during *Candida*-infection.

Mutations of *STAT3*, which is an ubiquitous cytokine signal transduction molecule involved in Th17 polarization (524, 525), have been described to cause the CMC-associated hyper-IgE syndrome (HIES) and these HIES patients are characterized by the inability to differentiate Th17 cells (283, 284, 317, 318). Generated Th17 cells of both iCMC and APS1 patients have been shown in the present study to produce reduced amounts of IL-17 and IL-22 suggesting an association between STAT3 and Th17 development in iCMC/APS1 patients. Analysis of STAT3 activation in IL-6 stimulated PBMCs of iCMC and APS1 patients in a TransAM system revealed that all patients

exhibited a striking reduced STAT3 DNA binding activity compared to healthy controls supporting a possible link between STAT3 and the deregulated Th17 differentiation in iCMC and APS1 patients. The underlying mechanisms that result in an impaired STAT3 activity remain to be further investigated. Suppressor of cytokine signalling (SOCS) 3 for example was reported to constrain Th17 differentiation by attenuating the activity of STAT3 that normally binds to the IL-17A/F-promotor and is involved in the generation of Th17 cells (526). Whole genome analysis of activated T cells of iCMC patients revealed a 2-fold upregulation of the SOCS3 gene compared to healthy T cells suggesting that SOCS3 overexpression might be involved in the impaired function of STAT3 in iCMC patients. *STAT3* mutations in iCMC patients haven't been sequenced so far which needs further investigation.

STAT3 might also be associated with the analyzed *STAT1* mutations. The decreased STAT3 and enhanced STAT1 activity in combination with *STAT1* mutations analyzed in the present study might affect cellular responses induced by cytokines as IFN-α/β, IFN-γ or IL-27 that predominantly activate STAT1 over STAT3, as well as IL-6 and IL-21 which predominantly induce STAT3 activation over STAT1. *STAT1* mutant alleles have been described to increase also cellular response to IL-6 and IL-21 that normally induce Th17 cells via STAT3 rather than STAT1 (485, 527). These data propose that enhanced STAT1-dependent cellular responses to these two groups of STAT1/STAT3 activating cytokines are involved in impaired Th17 responses. The decreased STAT3 and increased STAT1 activity observed in iCMC patients tested in the present study suggests an antagonism of STAT3-dependent Th17 inducing cytokines IL-6, IL-21 and IL-23 by STAT1 (485, 527). It remains to elucidate how STAT1- and STAT3-dependent cytokines either individually or in combination affect Th17 development in iCMC patients included in this study.

Moreover, the effect of the aryl hydrocarbon receptor (Ahr) on Th17 development might also be affected by gain-of-function *STAT1* alleles as it has been shown that the Ahr, that is involved in the regulation of Th17, Treg and Th22 cells, interacts with STAT1 under Th17 polarizing conditions (22, 39, 41, 42, 528). The development of Th17 cells is STAT1-dependently suppressed in AhR deficient mice (42). These findings propose a possible role for AhR in the pathogenesis of iCMC due to the observed *STAT1* mutations in combination with an impaired Th17 response. Mechanisms by which Ahr signalling might affect *Candida*-infections is largely unknown. Therefore the influence of Ahr agonists on Th17 development in iCMC patients with *STAT1* muta-

tions in the presence or absence of STAT1 antibodies represents an interesting aspect that remains to be further analyzed.

These data indicate that *STAT1* mutations cause isolated CMC by impairing Th17 responses and further investigations on this mutation might open new strategies to treat patients with isolated CMC. Therefore one the one hand the effect of STAT1 antibodies on Th17 development and *Candida* defense is one interesting aspect that needs further investigation. One the other hand, the genome-wide influence of these *STAT1* mutations on various cell types stimulated with a range of STAT1/STAT3-associated cytokines remains to be elucidated.

5.6 MoDC of CMC patients show a distinct cytokine profile in response to *Candida* and its cell wall compounds

A coordinated immune response involving both innate and adaptive mechanisms is required for defending *Candida*-infections. It is of interest to verify if the dysregulated T cell cytokine secretion observed in CMC patients results from a direct T cell defect or an affected interaction with APC.

The *Candida* cell wall is mainly composed of carbohydrates such as glucan, chitin and mannan that are recognized by a variety of pattern recognition receptors (PPRs) on DCs including dectin-1/2 and TLRs (209, 529, 530). The present study focused on the C-type-lectin receptor dectin-1 on DCs. Dectin-1 plays a mayor role in immunity against *Candida* since DCs stimulated with purified β-glucans induce IL-17/IFN-γ producing T cells (206, 211). Dectin-1 signals through two distinct pathways, the spleen tyrosine kinase (syk)-caspase recruitmet domaine 9 (CARD9) pathway and the Raf1 pathway leading to the activation of NFκB (213-216). Syk-CARD9 dependent dectin-1 triggering by β-glucans induces the secretion of IL-2, IL-6, IL-10, IL-23, IL-1β but little IL-12 (206, 218). In order to get a better insight in the intracellular pathways the specific *Candida* cell wall compounds zymosan and curdlan were used as pure receptor ligands in this study. Curdlan is a β-1,3-glucan compound of the *Candida albicans* cell wall derived from the bacterium *Alcaligense faecalis* that acts as a selective dectin-1 agonist and contains no TLR2 or TLR4 ligands (206, 240, 531). Investigation of the syk-dependent dectin-1 pathway showed that MoDC of iCMC patients produced less amounts of IL-10 and IL-6 in response to curdlan. This points to a defect syk-CARD9 signalling given that curdlan-induced IL-10 and IL-6 secretion is syk-dependent (214, 215) (figure 42).

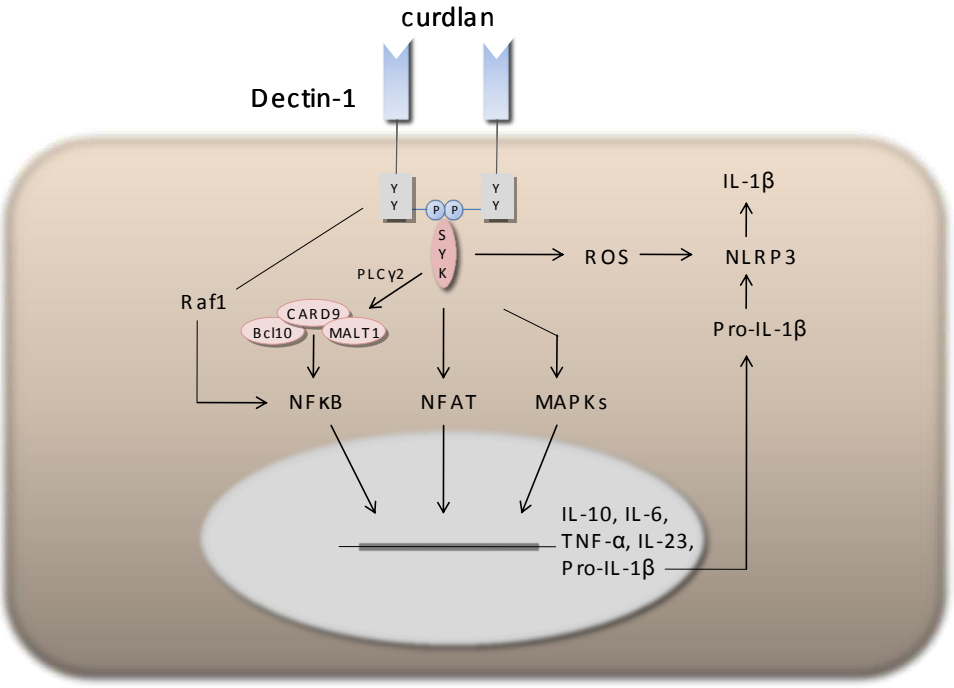


Figure 42: Dectin-1 signalling in antigen presenting cells in response to curdlan
Adapted from Osorio et al. (207)

On the contrary, MoDC of APS1 patients showed only slightly decreased levels of IL-10 and IL-6 upon curdlan-stimulation compared to healthy controls that may reflect a different impaired dectin-1 signalling in comparison to MoDC of iCMC patients. Curdlan-stimulation of MoDC of iCMC patients resulted also in decreased amounts of the Th17-differentiating cytokines IL-1β and IL-23 further supporting a deregulated dectin-1 signalling followed by an inadequate Th17 differentiation. The proposed dectin-1 defect in iCMC patients is further underlined by the fact that dectin-1 and CARD9 deficiency in mice and human has been reported to be associated with an enhanced susceptibility to *Candida*-infections (210-212, 215). A contradicting report showed that DC of iCMC patients secrete increased level of IL-6 and diminished amounts of IL-23 after *Candida*-stimulation (309) but these discrepancies could be explained by different stimuli (TLR2/6 ligand, heat-killed *Candida* hyphae) used in the study. In the present study in MoDC of APS1 patients slightly diminished amounts of IL-1β and normal level of IL-23 could be found in comparison to healthy subjects. Priming of MoDC with curdlan led to a decreased production of IL-12p70 in both iCMC and APS1 patients indicating a disturbed Th1 induction of these MoDC. The production of TNF-α in response to curdlan was slightly enhanced in MoDC of iCMC patients compare to healthy controls whereas MoDC of APS1 patients secreted reduced amounts of TNF-α. This is concor-

dant with a recent study demonstrating that dectin-1 induced TNF-α production is decreased in curdlan-stimulated PBMCs of APS1 patients suggesting an impared function of the dectin-1/syk pathway while Raf-1 signalling was not affected. Moreover AIRE has been described to be involved in these processes as reduced AIRE expression resulted in a significant diminished TNF-α secretion in a human monocytic cell line indicating an extrathymic role for AIRE in fungal infections in APS1 patients (336).

The second dectin-signalling pathway, independent of syk, activates a Raf1-dependent pathway that integrates with syk-CARD9-pathway at the level NFκB activation (figure 43).

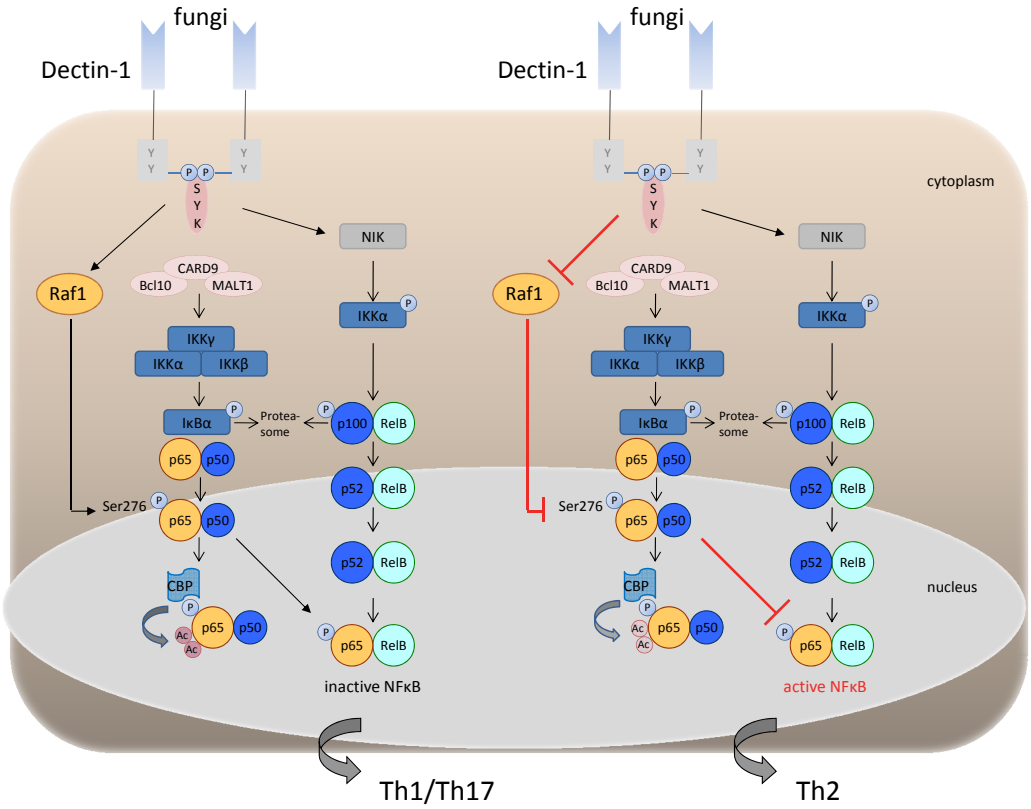


Figure 43: Dectin-1 signalling through Raf1 in antigen presenting cells in response to fungi
Adapted from Geijtenbeek et al. (209)

Dectin-1 induced Raf1 signalling involves the phosphorylation of p65 followed by enhanced p65 transactivation and repression of syk-induced RelB-activity. P65-phosphorylation regulates cytokine production at two different levels: RelB-inactivation and p65-acetylation. RelB inactivation leads to the secretion of IL-1β and IL-12p40 whereas p65-acetylation induces the production of IL-10 and IL-6. The syk/Raf1 signal-

ling pathways after dectin-1 stimulation with *Candida* or curdlan are important to induce the production of Th1-/Th17-differentiating cytokines whereas inhibition of Raf1 and increased RelB-activity promote Th2-instructing cytokines (209, 213) (figure 43). The decreased amounts of IL-1β, IL-12p70 and IL-23 in curdlan-stimulated MoDC of iCMC patients suggest an impaired Raf1 signalling pathway at the level of an enhanced RelB activity and the observed reduced production of IL-10 and IL-6 proposes an impaired p65-acetylation pointing to a defect Th1/Th17 polarization. The cytokine results of curdlan-stimulated MoDC of APS1 patients showed distinct results concerning the dectin-1 induced Raf1 signalling pathway: The only slight differences in IL-1β, IL-12p70, IL-23, IL-10 and IL-6 production between APS1 patients and healthy controls are less prominent as observed in iCMC MoDC that might reflect a Raf1 signalling pathway not much affected as seen in iCMC patients.

Dectin-1 activation alone is sufficient to induce antifungal responses but can also collaborate with TLRs resulting in synergistically cytokine induction. Published reports have shown that the syk- and Raf1-pathways induced by dectin-1 are involved in TLR-crosstalks by p65-acetylation and syk-induced RelB inhibition (213). However, further studies are necessary to analyze the different dectin-1-TLR crosstalks in iCMC/APS1 patients. The syk-CARD9 pathway can also activate the pyrin domain containing 3 (NLRP3) inflammasome that induces the conversion of pro-IL-1β to active IL-1β by caspase 1 (figure 42). NLRP3 deficient mice have been described to have a higher susceptibility for *Candida*-infections (218). Herein IL-1β secretion of curdlan-triggered MoDC is decreased in iCMC and to a lesser degree in APS1 patients suggesting an impaired NLRP3 activity. Furthermore Tassi et al. showed that PLCγ2 is involved in syk-dependent dectin- signalling (figure 42). PLCγ2 deficient mice are characterized by decreased level of IL-12, IL-23 and IL-6 and are not able to generate Th1/Th17 cells in response to glucan (219). The diminished amounts of these cytokines in the studied iCMC patients and to a lesser degree in APS1 patients may indicate also a defect of the syk-CARD9 signalling pathway at the level of PLCγ2. Therefore, the impaired cytokine production of curdlan-matured MoDC of iCMC and APS1 patients may result from a defect Dectin-1/syk-CARD9 signalling that can occure on different levels of the signalling pathway and needs more specific investigation.

The *Candida* cell wall compound zymosan analyzed in this study is mainly composed of β-1,3-glucans and β-1,6-glucans but also of mannans and chitins and zymosan specifically binds to dectin-1 and TLR2/TLR6 (235, 236, 241, 532) (figure 44).

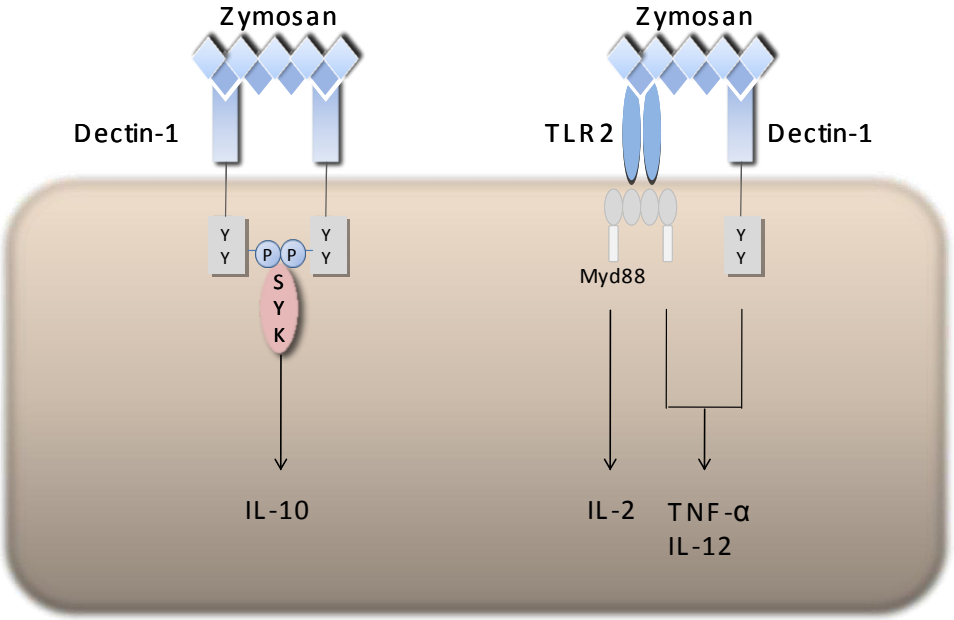


Figure 44: Cytokine induction in DCs mediated by dectin-1 and TLR2 activation in response to zymosan

Adapted from Brown et al. (205)

Zymosan induces IL-10 in a TLR-independent and syk-dependent manner (214). The finding of a diminished IL-10 production in MoDC of iCMC patients supports a deregulated syk-signalling downstream of dectin-1 whereas IL-10 induction in MoDC of APS1 patients was normal. IL-1β, IL-23 and IL-12p70 production in MoDC of both iCMC and APS1 patients were low in response to zymosan. Notably, zymosan does not reflect the true complexity of the *Candida* cell wall (205). TNF-α is induced by zymosan in a syk- and TLR2-dependent manner (205, 215), thus slightly enhanced TNF-α amounts in MoDC of iCMC and normal levels of TNF-α in MoDC of APS1 patients might reflect a functional TLR2 pathway in both CMC subgroups. A deregulated dectin-1 pathway combined with a functional TLR2 signalling could lead to a compensation of the defect dectin-1 pathway through TLR2 which is described to mediate an increased susceptibility to *Candida*-infection by the induction of suppressive Tregs (231, 232). As mentioned above, MoDC of APS1 patients secreted normal amount of TNF-α compared to healthy control MoDC making a defective dectin-1/TLR2 crosstalk in APS1 unlikely. The cytokine results of APS1 MoDC in this study except for IL-1β and IL-12p70 after curdlan- or zymosan-stimulation differ from previous data describing reduced amounts of IL-6, IL-10, IL-1β and IL-12p70 of MoDC in APS1 patients in response to *Candida*

albicans (359). One the one hand these differences could be attributed to the analysis of different phenotypes of CMC patients. On the other hand the use of different *Candida* compounds in the present study such as zymosan and curdlan instead of e.g. *Candida albicans* hyphae might explain the variety of results among different studies.

Concerning the cytokine response to the TLR4 stimulus LPS, a decreased IL-12p70 production of both iCMC and APS1 MoDC after stimulation with LPS was detected suggesting a defect TLR4 signalling. This further supports the theory of an impaired Th1 response in the iCMC/APS1 patients analyzed in the present study and might reflect the reduced amounts of IFN-γ detected in activated PBMCs of these patients. TLR4 plays also a role in antifungal immunity due to its recognition of *O*-linked mannosyl polymers of the *Candida albicans* cell wall (figure 45).

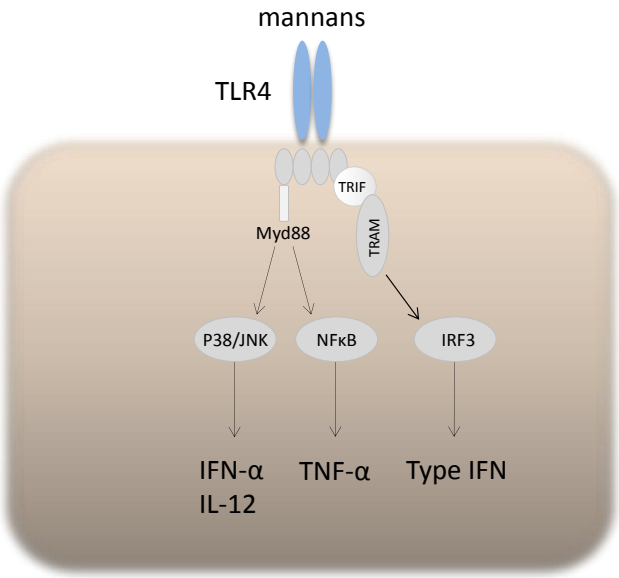


Figure 45: Cytokine production mediated by TLR4 signalling in response to mannan
Adapted from Van de Veerdonk et al. (195)

Dectin-1 is known to collaborate with many TLRs including TLR2 and TLR4 (235, 236, 240, 241). A previous report of TLR4 deficient mice with an enhanced susceptibility to *Candida*-infections (228) further supports this observed defect of TLR4 signalling that may contribute to disturbed interactions with dectin-1. In contrast to these findings Ryan et al. demonstrated that DCs of CMC (non-APECED) patients produced high IL-12 levels after LPS-challenging whereas APS1 patients showed no abnormalities of IL-12 production (309). These discrepancies could be due to different used stimuli and

the interindividual differences that could play a role concerning PRR expression and signalling pathways.

Another interesting aspect concerning the cytokine secretion of *Candida*-stimulated DCs is the production of TGF-β and IL-27 which haven't been investigated so far in this study. IL-27 has been described to promote the differentiation of Th1 cells but inhibits Th17 development and both effects are STAT1 dependent (487, 490, 533, 534). IL-27 has also been reported to induce together with TGF-β regulatory Tr1 cells and the Il10 promoter is known to have a binding site for STAT1 (38, 109, 535, 536). With respect to the analyzed *STAT1* mutations in iCMC patients in the present study IL-27 secretion by *Candida*-stimulated DCs would be of great interest because it might be involved in the STAT1 mediated development of Th1 and Tr1 cells suppressing Th17 responses.

Interestingly, it has been described that *Candida*-stimulation of mouse bone marrow derived conventional DCs induces syk-dependent the activation of TLR7, thereby stimulating a MyD88-IRF1-dependent signalling pathway which results in INF-β secretion. INF-β subsequently drives the IFNα/β receptor (IFNAR1)-mediated activation of intracellular STAT1 and IRF7 expression (227) suggesting that the observed *STAT1* mutations in iCMC patients could also play a role on the level of *Candida*-stimulated DCs. Moreover IFN-β has been reported to be involved in the inhibition of Th17 cells (488) which makes this recently described signalling pathway of *Candida*-treated DCs interesting for its investigation in human *Candida*-mediated Th17 responses.

The innate immune response to fungi is very complex due to the variety of PRR expression and crosstalks between the PRRs leading to *Candida* defense. Hong et al. reported a normal expression of PRRs involved in *Candida* recognition in DCs of CMC patients (310). The variety of PRRs on DCs involved in the recognition of *Candida*, signalling pathways such as the dectin-1 pathway and possible mutations have to be further analyzed in CMC patients enrolled in this study. One aspect would be the dectin-2 receptor which binds to α-mannans and has been described to be also involved in *Candida* recognition and following induction of Th1/Th17 cells (220, 221).

Therefore further studies on dectin-2 function in MoDC of CMC patients would be useful to get more insight in *Candida*-mediated innate immune responses in these patients.

Taken together, the impaired cytokine production observed in MoDC of iCMC and APS1 patients indicates a deregulated *Candida*-induced receptor signalling compared to healthy MoDC that might lead to impaired T cell responses including Th17, Th1 and Treg cells followd by an insufficient *Candida* defence. The altered cytokine profile of MoDC of APS1 patients in response to *Candida* compounds is different to the impaired cytokine pattern observed in MoDC of iCMC patients. These data support the theory that MoDC of iCMC patients are characterized by different impaired receptor signalling including dectin-1 in response to *Candida*. Therefore iCMC patients and APS1 patients have to be considered separately concerning immune responses against *Candida*.

5.7 Maturation of *Candida*-stimulated MoDC of CMC patients is impaired compared to healthy controls

Scarce data exist on DC maturation in iCMC and APS1 patients. The finding of a lower mean fluorescence of all tested maturation markers in unstimulated cultures in MoDC of iCMC and APS1 patients compared to healthy controls observed in this study is in common with a previous report of Ryan et al. which also considered CMC patients separately as APECED and non-APECED patients (309). Herein activation markers have been described to be decreased under unstimulated conditions except for a comparable expression of CD83 and an increased upregulation of CD86 in all patients which is simlar to the findings of the present study.

CD83 is a characteristic marker for DC maturation (537, 538). The poorly detectable, but lower MFI of CD83 in iCMC MoDC compared to healthy MoDC in response to zymosan and curdlan suggests an impaired maturation capacity of iCMC MoDC in response to *Candida* cell wall compounds. The MFI of CD83 was comparable between MoDC of APS1 patients and healthy controls under both conditions zymosan and curdlan indicating functional maturation of MoDC in APS1 patients in response to *Candida* compounds. LPS-stimulated MoDC of both iCMC and APS1 patients in this study show a reduced MFI of CD83 that is in concordance with Ryan et al. and proposes an affected maturation capacity in response to the TLR4 stimulus LPS which is reflected by a decreased IL-12p70 production in response to LPS (309). The enhanced expression of CD86 in zymosan-/curdlan-treated MoDC of iCMC patients might reflect a higher *Candida*-specific costimulatory activity for T cell interactions compared to healthy MoDC. Also curdlan-/zymosan stimulated MoDC of APS1 patients tended to show an enhanced MFI of CD86 in response to the *Candida* cell wall compounds that is consistent with the study by Ryan et al. describing an increased expression of CD86 in APECED patients in response to *Candida* hyphae-stimulation. Whereas curdlan-treated MoDC of iCMC patients show a comparable expression of the costimulatory CD40 to healthy controls, the remarkable increased MFI of CD40 in MoDC of APS1 patients points to an enhanced capacity to induce proliferative responses in T cells (539). The analysis of a differently expression of HLA-DR in MoDC of iCMC and APS1 patients in response to zymosan-, curdlan- and LPS-stimulation differs from Ryan et al. reporting no differences in HLA-DR expression in non-APECED and APECED patients

compared to healthy subjects (309). All these described discrepancies could be due to the use of specific *Candida* cell wall compounds as pure receptor ligands in the present study in contrast to the use of the hyphal *Candida* morphotype as in the case of Ryan et al. where β-1,3-glucans are masked.

The observed impaired activation of iCMC/APS1 MoDC in combination with an impaired cytokine response might result in an altered T cell responsiveness contributing to an insufficient *Candida* defence. But it is important to mention that only a small number of iCMC/APS1 patients was analyzed so far and a larger patient collective is necessary to draw general conclusions. Again, MoDC of iCMC and APS1 patients are characterized by a differential induced maturation program in response to *Candida* compounds reflecting the different obtained cytokine results. These data support that iCMC and APS1 patients have to be immunologically considered as two different subgroups.

5.8 Alteration of DC - T cell crosstalk in CMC patients

In response to different PRR agonists DCs produce cytokines that favor T cell development such as Th1, Th2, Th17 and Treg (234, 540). Dectin-1 stimulation of DCs results in the generation of Th1 and Th17 cells (92, 206, 234, 541). Therefore the effect of zymosan-, curdlan- and LPS-matured MoDC on T cell differentiation was investigated in an allogenic stimulation assay (ASA) with iCMC patients using different donor conditions: Healthy MoDC were incubated with healthy naïve T cells resembling the healthy model, MoDC of iCMC patients were cultured with healthy T cells and healthy MoDC with iCMC T cells. ASA experiments could only be performed with iCMC patients and not with APS1 patients due to the low availability of patients' material.

In concordance with several studies describing the Th1/Th17 inducing effect of dectin-1 stimulated DCs is that zymosan- and curdlan-matured MoDC promoted IL-17A, IL-22 and IFN-γ in naïve T cells in the healthy model. In contrast, iCMC T cells that were cocultured with functionally curdlan-/zymosan-primed MoDC of healthy subjects showed a reduced Th17 profile supporting a defect on the T cell level in iCMC. Healthy T cells seem to compensate the observed DC defect of iCMC patients regarding the comparable or even enhanced amounts of IL-17/IL-22 compared to the healthy model. IFN-γ induction in response to *Candida* compounds was decreased in iCMC T cells suggesting that also induction of Th1 cells by *Candida* compounds is impaired in iCMC. LPS-stimulated DCs induced high amounts of IFN-γ and a more Th1 response in the healthy model. The reduced amounts of IL-12p70 in iCMC DCs after LPS challenge led to a decreased IFN-γ secretion in healthy T cells compared to the healthy model suggesting that healthy T cells were not able to compensate the observed DC defect in iCMC DCs after LPS stimulation. These data of reduced IFN-γ in the iCMC culture conditions in response to LPS propose an impaired Th1 response in iCMC patients and are consistent with the observed decreased amounts of IFN-γ in PBMCs of iCMC patients after *Candida*-stimulation (246, 247, 288, 357).

Zymosan signals via dectin-1/TLR2 and according to the DC results TLR2 signalling seems to be normal in iCMC patients. IL-10 induction in naïve T cells was nearly comparable or only slightly decreased in iCMC T cells under zymosan or curdlan conditions. Moreover T cells of iCMC patients polarized by LPS-triggered DCs were characterized by a diminished IFN-γ and enhanced IL-10 production compared to healthy

T cells indicating that T cells of iCMC patients tend to develop a more regulatory T cell type in response to LPS-matured DCs. No data on Treg function exist on T cells from iCMC patients and the hypothesis of an involvement of *Candida*-specific Tregs in iCMC patients could be underlined by determing an enhanced IL-10/IL-17 ratio in iCMC T cells that were instructed by healthy zymosan-, curdlan- or LPS-matured DCs. This increased IL-10/IL-17 ratio in iCMC patients suggests that impaired amounts of the inhibitory IL-10 might be responsible for the proposed dysregulation of Th1 and Th17 responses in chronic *Candida*-infections. Netea et al. reported that TLR2 deficient mice have a higher susceptibility to disseminated candidiasis due to a decreased Treg population (231). The proposed hypercompensation of TLR2 in iCMC due to a defect dectin-1 signalling may indicate that Treg responses and suppression are imbalanced in iCMC patients. Therefore the impact of neutralizing IL-10 antibodies on T cell responses and proliferation in the ASA needs further investigation which was not possible so far due to the limitation of patients' material and the complexity of the experimental setup.

Analysis of proliferation in the ASA revealed that healthy DCs induced a robust proliferation in healthy naïve T cells. On the contrary, iCMC T cells polarized by zymosan-, curdlan- or LPS-matured healthy DCs showed a reduced proliferation capacity suggesting that the reduced cytokine production including IL-17/IL-22 in iCMC T cells is possibly due to a reduced proliferation capacity as already observed in *Candida*-stimulated PBMCs of iCMC patients. These data propose that naïve T cells of iCMC patients might have a decreased cell activity compared to healthy T cells. Moreover this observed hypoproliferative capacity further underlines the supposed imbalanced regulatory T cell responses in iCMC that might be involved in the inhibition of Th17 responses.

The hypothesis of a proliferation dependent IL-17 defect of iCMC T cells might also be assumed concerning the cytokine results of the ASA in response to *Candida* compounds in relation to the proliferation capacity. Now the secretion of IL-17A and IFN-γ per cell of iCMC T cells was comparable or even enhanced to healthy T cells. The reduced production of IL-22 even in relation to proliferation points to a more prominent defect of Th17 cells regarding the secretion of IL-22. The markedly enhanced IL-10 secretion per cell after zymosan-, curdlan- or LPS-stimulation in iCMC patients further highlights the tendency of Treg induction rather than Th17 differentiation in response to *Candida*.

In contrast to experiments with naive T cells, memory T cells of iCMC patients incubated with healthy DCs produced considerable amounts of Th17-associated cytokines IL-17, IL-22 and effector cytokines IFN-γ and IL-10 that is not consistent with the analyzed reduced level of IL-17, IL-22 and IFN-γ and increased amounts of IL-10 in *Candida*-pulsed PBMCs. One reason for the different obtained results could be that specific *Candida* cell wall compounds were used in the ASA whereas PBMCs were treated with *Candida albicans* and the manufacturer could not provide any information about the morphotype of the used *Candida* antigen. On the other hand discrepancies could be attributed to different cell types contained in the used memory cell fraction. The naïve T cells isolation kit is an indirect magnetic labelling system for isolation of naive untouched T cells. The labelled, non-naïve fraction was used as memory T cells in the ASA and contains $CD45RO^+$ and non-$CD4^+$ cells which are positive for CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD56, CD123, TCRγ/δ, HLA-DR and CD235a expression. For example NK T cells express CD56 and NK cells are known to express CD56 and CD16 on their surface (542-544). Though it can be assumed that the used memory fraction contains amongst others $CD8^+$ T cells, γ/δT cells, NK T cells and NK cells that are capable of IL-17 production (16) and proposes that these cell types are functional in iCMC memory T cells due the detectable amounts of IL-17 and IL-22 in the ASA. Martin et al. described that dectin-1 is also able to directly trigger IL-17 production by a subset of γ/δT cells (545). Moreover it has been shown that dectin-1-activated DCs can also prime $CD8^+$ cytotoxic T cells in mice suggesting that these cells among the memory fraction in the ASA could be activated by dectin-1 triggered MoDC (546).

Taken together, the content of different cell types in the memory fraction might explain the different cytokine results compared to experiments with naïve T cells. The proliferative capacity of these iCMC memory cells was not affected. This might indicate that the proliferative defect in iCMC is more specific for naive T cells. Of note, also memory T cells of iCMC patients were characterized by increased amounts of IL-10 and IFN-γ in response to *Candida* compounds and LPS in comparison to healthy T cells suggesting that the effect of enhanced IL-10 amounts in iCMC patients is not restricted to naïve T cells.

5.9 Generation of T17 cells and T cell lines in CMC patients

IL-6, IL-1β and IL-23 are required for the generation and maintenance of Th17 cells and lead to activation of STAT3. The function of TGF-β as Th17-differentiating cytokine and its need in Th17 differentiation in human was initially controversially discussed (16), though the first experiments of Th17 differentiation in this study were done without TGF-β. Both iCMC and APS1 patients were characterized by an impaired capacity to generate Th17 cells from naïve T cells compared to healthy controls suggesting that the observed IL-17/IL-22 deficiency could result from an altered ability of Th17 differentiation.

The analysis of Th17 generation adding TGF-β, IL-6, IL-1β and IL-23 revealed a better outcome of IL-17 producing T cells confirming Th17 polarization requires TGF-β (28, 29, 31). Therefore following experiments of Th17 differentiation were performed in the presence of TGF-β. Also under ameliorated conditions naïve T cells of both iCMC and APS1 patients failed to differentiate to Th17 cells compared to healthy subjects. The data of an impaired Th17 differentiation further underline the requirement of Th17 cells in fungal infections and suggest that signalling pathways required for RORC and Th17 induction are disturbed in naïve CMC T cells. Patients with the CMC-associated hyper IgE syndrome (HIES) show defective Th17 responses due to mutations of STAT3 which plays a central role in Th17 generation (283, 284). The STAT3 activity is also strikingly reduced in the herein analyzed iCMC/APS1 patients confirming that STAT3 mutations are associated with a higher susceptibility to *Candida*-infections and the inability to generate Th17 cells in the analyzed patients of the present study (284, 547).

The *in vitro* generated Th17 cells of iCMC patients carrying gain-of-function *STAT1* alleles were characterized by a reduced production of IL-17 and IL-22 suggesting that enhanced cellular responses mediated by STAT1 are associated with the detected impaired capacity of naïve iCMC T cells to develope Th17 cells. Cytokines such as IFN-α/β, IFN-γ or IL-27 which inhibit the generation of Th17 cells via STAT1 might be involved *in vivo* (486, 488, 490).

Also Th1 differentiation was impaired in the analyzed iCMC patient with known *STAT1* mutation as generated Th1 cells secreted diminished amounts of IFN-γ, while a Th2 polarization was possible. These data are in line with the decreased amounts of IFN-γ and enhanced amounts of IL-10 observed in *Candida*-stimulated PBMCs of iCMC pa-

tients. How these observations are linked to the identified gain-of-function *STAT1* mutations remains to be further investigated as IFN-γ signals via STAT1 (523). The impaired capacity of Th1 polarization might be attributed to the reduced ability of iCMC DCs to produce IL-12 in response to LPS as shown in the present study.

IL-12 as a key signal for Th1 differentiation induces STAT4-dependent the expression of Tbet and IFN-γ, and enhances IL-12Rβ2 expression which are key events for Th1 polarization (548, 549). IFN-γ controls the expansion and death of $CD4^+$ (550, 551) and $CD8^+$ T cells (552) but IFN-γ has also been suggested to control Th1 cell death by itself (553). Naïve T cells of iCMC patients might have a reduced cell activity as already observed in the low proliferation capacity in the ASA of the present study or IL-12/IFN-γ responsiveness might be disturbed. Another speculation would be that Th1 differentiation at early stages starts but IFN-γ might act in an autocrine manner mediating the death of IFN-γ producing Th1 cells. However these data are contradicting to Ng et al. showing that $CD4^+$IFN-γ^+ cells and IFN-γ producing $CD4^+CCR6^+CXCR3^+$ cells are comparable to healthy subjects (264). It is important to mention that the generation of different T cell lines is only tested in one iCMC patient so far due to the rareness of the disease and low availability of patients' material. A larger panel of iCMC patients is necessary in order to better understand the association between the identified *STAT1* mutations and T cell differentiation.

In contrast to the iCMC patient, naïve T cells of APS1 patients were able to polarize to Th1 and Th2 cells in a comparable manner to healthy controls. These findings of a functional Th1 and Th2 polarization in APS1 patients are consistent with data of HIES patients demonstrating no changes in the presence of Th1 and Th2 cells (283, 284). Another study also described a normal number of IFN-γ producing $CD4^+CCR6^+CXCR3^+$ cells in APS1 patients (264). But this functional Th1 differentiation is not in line with the proposed Th1 defect in APS1 patients according to the decreased IFN-γ levels as seen in stimulated PBMCs of the present study. Naïve T cells of APS1 patients could be induced *in vitro* to differentiate into the Th1 lineage in the presence of IL-12 and anti-IL-4 indicating that Th1 inducing cytokines *in vivo* fail or are deregulated during the *Candida* immune response in APS1 patients. The present study revealed that LPS-stimulated MoDC of APS1 patients produce reduced amounts of IL-12p70 indicating that defects on APC level might lead to inappropriate Th1 responses although naïve T cells are able to differentiate to Th1 cells in the presence of IL-12 and anti-IL-4.

Amounts of produced IL-2 are enhanced in all generated T cell lines of APS1 patients that might indicate cells are highly proliferating and active or they have a decreased cell activity and decreased resistance to cell death that would explain the deregulated Th17 differentiation but not the functional Th1 polarization. Interestingly, IL-2 production of generated Th17 and Th1 cells of the iCMC patient is increased while IL-2 secretion of polarized Th2 cells is reduced. These data rather suggest that the impaired Th1/Th17 differentiation in iCMC patients is reflected in enhanced IL-2 amounts due to the low activity of these cells. Therefore extended research on cell death and IL-2 secretion in polarized T cell lines is necessary.

These data of an impaired Th17 differentiation support the observed IL-17/IL-22 defect in CMC patients which might be due to a deregulated activity or hyporesponsivness of naïve CMC T cells to Th17 differentiating factors or STAT1-mediated inhibitory effects might be involved in the case of iCMC. Both groups iCMC and APS1 patients share the inability to differentiate to Th17 cells whereas the capacity to polarize Th1 and Th2 cells seem to be different which needs further investigation and underlines that both groups suffering from chronic *Candida*-infection and IL-17/IL-22 deficiency have to be immunological considered separately.

5.10 Keratinocytes of CMC patients are characterized by an impaired defensin production

During colonization of mucosal surfaces, *Candida* interacts with epithelial cells, which in turn respond to the infection with the secretion of proinflammtory cytokines and antimicrobial peptides to defence the growth of *Candida* (554).

IL-17 and IL-22 produced by Th17 cells are important key cytokines concerning the regulation of immune responses in the tissue and induce the production of IL-6, CXCL8, MCP-1 and antimicrobial peptides such as S100 proteins and human β-defensin 2 (HBD2) by epithelial cells (139, 140, 267-274). HBD2 is an antimicrobial peptide mainly produced by mucosal epithelia and has antimicrobial activity against viruses, bacteria and fungi such as *Candida albicans*. Absence or deficiency of such antimicrobial peptides could be responsible for recurrent infections (555, 556). IL-17 and IL-22 act synergistically on the upregulation of HBD2 in the skin (273). IL-22 together with TNF-α has been shown in a *in vitro* infection model to effectively inhibit the growth of *Candida albicans* in keratinocytes (138).

The analysis of keratinocyte function revealed that the synergistically effect of IL-17 and IL-22 on HBD2 expression is enhanced in iCMC patients compared to healthy controls indicating that epithelial cells of iCMC patients are able to upregulate antimicrobial responses in case of receiving IL-17 and IL-22 signals but defence mechanisms in the skin by these tissue-cytokines might be disturbed due to the observed IL-17/IL-22 deficiency in iCMC and results in chronic *Candida* colonization.

It was also reported in mice and human that keratinocytes are able to express dectin-1 (201, 202) which has been shown to be upregulated in human keratinocytes by its ligand β-glucan followed by the production of IL-1α, IL-8 and defensin (201). Therefore dectin-1 signalling in keratinocytes is another interesting aspect to investigate due to proposed defect of dectin-1 signalling in DCs of iCMC patients.

STAT3 has been shown to play a central role in skin homeostasis. It is known that IL-22 induces STAT3 phosphorylation in keratinocytes, but not STAT1 or STAT5, while exposure to IFN-γ leads to induction of STAT1- and STAT5 phosphorylation (139). *STAT1* mutations and the dysfunctional activity of STAT3 and STAT1 analyzed in the present study propose a possible deregulation of these STAT pathways in keratinocytes of iCMC patients that has to be further investigated. Furthermore the effect of super-

natants from the ASA on keratinocytes is of great interest and therefore experimental setups need to be further established.

The immunological mechanisms underlying keratinocyte-*Candida* interactions are complex and disclose an open field with many new aspects that requires extended research. Moreover larger patient cohorts are needed to be analyzed in order to clarify the function of epithelial cells in iCMC patients.

5.11 Whole genome analysis of CMC patients and healthy controls

Gene expression analysis of anti-CD3/anti-CD28 stimulated T cells of two iCMC patients and two healthy controls was performed using the Agilent Whole Human Genome Oligo Microarray (one color) by Miltenyi. Data analysis of expressed genes using the Gene Spring data analysis system (GeneSpring GX Software) revealed that several genes were up- and downregulated in activated T cells of iCMC patients compared to healthy controls. Selected regulated genes in iCMC T cells will be discussed in the following part of the study.

CXCL9, CXCL10, CXCL11

The three IFN-γ inducible CXC chemokines CXCL9 (Mig), CXCL10 (IP-10) and CXCL-11 (I-TAC) are T cell chemoattractants that bind to the CXCR3 receptor on B cells, NK cells and T cells (mainly on Th1), as well as on several tissue cells (557-559). Normally IFN-γ-producing Th1 cells induce the secretion of CXCL9/10/11 by different cell types and these chemoattractants in turn induce the recruitment of Th1 cells to sites of inflammation suggesting a loop between Th1 cells and tissue cells secreting CXCR3-binding chemokines to amplify ongoing Th1 responses (560, 561). CXCL10 is known to upregulate the production of Th1 cytokines leading to enhanced inflammatory reactions characterized by the secretion of IFN-γ which is 8-fold higher expressed in stimulated iCMC T cells (table 12). IFN-γ and TNF-α which are both upregulated in activated iCMC T cells (table 12) have been described to induce CXCL10 via STAT1 (562) as the CXCL10 promotor has a binding site for IFN-γ-induced STAT1 (523, 563, 564). Moreover IFN-γ is able to enhance the transcriptional activity of CXCL9 via STAT1 (565). The gain-of-function *STAT1* mutations identified in the analyzed iCMC patients suggest that these mutations are involved in the upregulation of IFN-γ-induced genes such as CXCL9 and CXCL10. The analyzed increased expression of these chemokines (CXCL9: 62-fold, CXCL10: 78-fold and CXCL11: 66-fold, table 12) combined with the also increased expression of TNF-α (3-fold) and IFN-γ (8-fold) might reflect a strong upregulation of inflammatory reactions and dominance of Th1 mediated responses in iCMC T cells that can be linked to the the identified gain-of-funtion *STAT1* mutations. iCMC T cells might respond under this inflammatory circumstances with an enhanced transcription of these chemokines. These proposed reinforced inflammatory Th1 biased mechanisms in activated iCMC

T cells are possibly compensatory effects due to defect Th17 pathways or might result in impaired Th17 responses.

UBD

The ubiquitin-like molecule UBD is a small ubiquitin-like modifier of the UBL family which is constitutively expressed on B cells and DCs (566, 567), but can also be induced by TNF-α and IFN-γ (568, 569) and functions as a signalling protein for rapid degradation of substrate proteins through the proteasome in an ubiquitin-independent manner (570). In mice UBD has been reported to be involved in caspase-mediated apoptosis and inhibition of proteasome activity results in accumulated UBD (568-570). Moreover UBD has been described as a downstream element of Foxp3 in human activated Tregs contributing to their anergic phenotype and expression of the β-galactoside binding lectin LGALS3 as a possible new Treg marker (571). In the presence of ionomycin overexpression of UBD in T cells results in the induction of IL-1R ressembling Foxp3-transduced T cells and Tregs (571).

The 40-fold upregulation of UBD in stimulated T cells of iCMC patients (table 12) in combination with the increased expression of IFN-γ (8-fold) and TNF-α (3-fold) suggests that UBD might be upregulated due to anti-CD3/anti-CD28 stimulation or due the enhanced IFN-γ/TNF-α expression indicating an enhanced susceptibility for apoptosis in iCMC T cells. UBD expression could also be upregulated by an inhibited proteasome activity. One the other hand the higher UBD expression might indicate a higher prevalence of Treg-like cells in iCMC patients regarding UBD as a downstream element of Foxp3.

IL-1β

IL-1β and TNF-α are considered to be key mediators in chronic autoimmune diseases including rheumatoid arthritis, Crohn's disease, and multiple sclerosis (572). IL-1β is involved in the induction of Th17-producing cells (16, 573). Th17 cells have been described to express the IL-1β receptor components IL-1 receptor type I (IL-1RI) and IL-1 receptor accessory protein (IL-1RAcP) compared to Th1 or Th2 cells (574). Lamacchia et al. reported that excessive IL-1β activity results in the development of Th1 and Th17 responses (575). Moreover it was shown that IL-1β is critical for the phenotypical conversion of natural Treg cells to Th17 cells (576, 577). IL-1β expression is 17-fold upregulated in stimulated T cells of iCMC patients (table 12) pointing to a more promi-

nent inflammatory state in these T cells. The IL-17 inducing effects of IL-1β are contradicting to the observed Th17 defect in iCMC and one explanation for enhanced IL-1β expression could be a deregulated function of IL-1β receptors leading to accumulation of IL-1β.

GPR56

The superfamily of G-protein coupled receptors (GPCRs) is one of the most diverse families of proteins (578, 579). The ligands for GPCRs range from ions, organic odorants, amines, peptides, proteins, lipids, nucleotides, and photons which are all able to activate GPCRs (578, 579). GPR56 was shown to be specific expressed on cytotoxic NK and T cells including $CD8^+$, $CD4^+$ and γλT cells (580) pointing to an increased GPR56 mediated cell signalling in iCMC T cells due to its 14-fold upregulation in activated T cells of iCMC patients (table 12).

CD33

Members of the sialic acid-binding immunoglobulin-like lectin (Siglec) family family including CD33 bind to specific glucan structures containing sialic acid (581) and are usually considered myeloid-specific but has also been described on lymphoid cells (582, 583). CD33 contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits SHP-1 and SHP-2 and acts as a regulatory molecule to suppress proliferation by inhibition of tyrosine phosphorylation and $Ca2^+$ mobilization (583-588). A study by Orr et al. could show that the suppressor of cytokine signaling (SOCS)3 blocks CD33-mediated inhibition of proliferation in a cytokine-inducible cell line by binding to the phosphorylated ITIM of CD33 followed by an enhanced CD33 proteasomal degradation (583). CD33 normally inhibits cytokine-induced proliferation, but Orr et al. suggest that during an inflammatory response, SOCS3 - which is 2-fold upregulated in iCMC T cells (table 12) - can suppress the inhibitory effect of CD33 on cytokine signalling by enhancing receptor degradation (583).

Not many data exist concerning the biological function of CD33 in the context of T cells but its 13-fold upregulation in activated iCMC T cells (table 12) might indicate an enhanced inhibition of cytokine-induced proliferation whereas the 2-fold upregulation of SOCS3 might be also involved in the reduction of CD33-mediated effects. The specific functional role of CD33 in T cells of iCMC patients needs to be further analyzed.

CX3CR1

The chemokine receptor 3 (CXCR3) is a 7-transmembrane domain G protein-coupled receptor (GPCR) and binds to Fractalkine (CX3CL1) (589). CX3CR1 is classified as an inflammatory chemokine receptor because of its selectively expression on effector leukocytes including cytotoxic $CD8^+$ and $CD4^+$ T cells, major subset of human NK cells, as well as on mouse and human monocytes (590-594). CXCR3 has been described to be preferentially expressed on Th1 cells (559, 595). CX3CR functions as an adhesion molecule (591) and mediates integrin- and G protein-independent adhesion of receptor-bearing leukocytes to CX3CL1-expressing endothelial cells and is induced by IFN-γ and TNF-α which are both upregulated in iCMC T cells (table 12) (596, 597).

The 9-fold upregulation in activated iCMC T cells compared to healthy controls might be due to the enhanced expression of IFN-γ (8-fold) and TNF-α (3-fold) indicating a higher migration potential of iCMC T cells to sites of inflammation (table 12). The preferentially expression of CX3CR on Th1 cells is in line with possible Th1-associated upregulated genes in iCMC T cells pointing to an enhanced inflammatory response in iCMC patients compared to healthy controls.

GZMB

GZMB, a cytolytic effector protein, is mainly produced by cytotoxic T cells (CTLs), natural killer cells (NK cells) and γλT cells to eliminate pathogenic cells resulting in apoptosis of target cells. GZMB directly cleaves caspase-3 or Bid that along with Bax is translocated to the mitochondria. Both mechanisms end in lysis of the target cell (598). The increased expression of GZMB (9-fold) in activated T cells of iCMC patients (table 12) suggests that CTLs could play a more potent role in iCMC than in healthy subjects or iCMC T cells are characterized an enhanced susceptibility to cell death. On the other hand suppressive mechanisms of Tregs can be GZMB dependent (71, 73). Cao et al. reported that 5 %-30 % of Treg cells in a tumor microenvironment express GZMB and these Treg cells are lytic for NK cells and CTLs in a GZMB- and perforin- dependent manner (72). Therefore the enhanced GZMB expression in iCMC T cells might also point to an enhanced suppressive activity of Tregs.

CD38

The lymphoid surface antigen CD38 is a NAD^+ glycohydrolase involved in the metabolism of cyclic ADP-ribose and plays a signalling role in T- and B cells (599). CD38 has

been described to be induced by IFN-γ and TNF-α (600) and human CD38 signals through direct or indirect interactions with the T-cell receptor (599, 601, 602). Ligation of CD38 on PBMCs and T cell lines has been described to result in activation and proliferation signals and the transcription of cytokines such as IL-1β, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) was induced at similar levels compared to triggering T cell receptor CD3 (603-606).

The increased 8-fold expression of CD38 in stimulated T cells of iCMC patients (table 12) could be associated with anti-CD3/anti-CD28 stimulation and therefore might be associated with an increased cell activity but its specific functional role in iCMC patients needs to be further examined.

IFN-γ, IRF-1, ICAM-1, TAP1

The proinflammatory cytokine IFN-γ is a type II IFN and is produced by activated T cells, NK cells and macrophages. After binding of IFN-γ to its receptor STAT1 gets phosphorylated in response to JAK1/JAK2 signalling and binds to the Gamma Activation Sequence (GAS) followed by recruiting transcriptional coactivators to enhance transcriptional activity (523, 607-609). IFN-γ modulates T cell responses by direct effects on T cells or via the modulation of APCs. IFN-γ leads to the reduction of proliferation and survival of activated T cells (523, 610). Th1 cells are characterized by the production of high amounts of IFN-γ (611) and IFN-γ has been described to inhibit IL-17 producing $CD4^+$ effector cells (612, 613). The anti-proliferative action of IFN-γ is STAT1-mediated as STAT1 leads to the induction of the cell cycle inhibitor CDKN1A (614). Another mechanism by which IFN-γ mediates the suppression of cell growth is IRF1 signalling. IFN-γ also induces the transcriptional regulator and tumor repressor gene IRF1 which is 3-fold upregulated in activated T cells of iCMC patients (table 12) (615). IRF1 is a target gene of STAT1 and plays an important role in immune responses and oncogenesis (616, 617). STAT1 activation results in IRF1 synthesis and IRF1 binds then to IFN-stimulated regulatory elements followed by the expression of several IFN-γ-inducible genes such as COX2, TAP1, etc. (616, 618, 619).

The IFN-γ-inducible gene TAP1 which is 2-fold upregulated in activated iCMC T cells (table 12) is involved in the MHC antigen processing pathway. Peptides for MHC loading are degraded in the cytosol and transported to the endoplasmatic reticulum by the heterodimeric transporter consisting of transporter associated with antigen processing (TAP)1 and (TAP)2 subunits (523, 620). MHC-I expressed on the surface of most nu-

cleated cells, is upregulated by IFN-γ which is an important mechanism for cytotoxic lymphocytes to recognize antigenic peptides (563). STAT1 and IRF1 are required for the IFN-γ mediated induction of TAP-1 and the enhanced expression of INF-γ (8-fold), IRF-1 (3-fold) and TAP1 (2-fold) in combination with the observed gain-of-function *STAT1* mutations in the analyzed iCMC patients argue for a reinforced effect of IFN-γ in iCMC T cells. Enhanced IFN-γ signalling might result in an enhanced MHC-I regulation and activity of cytotoxic lymphocytes. But upregulated IFN-γ and following STAT1-IRF-1 signalling is also involved in cell growth suggesting an increased susceptibility of iCMC T cells for cell death.

The leukocyte adhesion molecule ICAM-1 is also an IFN-γ-inducible gene as it possesses a functional GAS in its promoter (523, 621). The intercellular adhesion molecule (ICAM)-1 (CD54) is a Ig-like cell adhesion molecule expressed on endothelial cells, epithelial cells, fibroblasts, T cells, B cells, DCs, macrophages and eosinophils (622-624). ICAM-1 plays a role in inflammatory responses and T cell mediated host defense system (624). The presence of ICAM-1 on T cells and T cell activation may play an important role as tyrosine phosphorylation was found to induce cross-linking of ICAM-1 or ICAM-3 on T-cells (625). ICAM-1 is weakly expressed on resting T cells (626), whereas mitogen and antigen-specific stimulation (627), cytokines such as IFN-γ, IL-1β, IL-2 and TNF-α (628) and viruses (629, 630) result in the up-regulation of ICAM-1 on the T cell surface. It has been described that anti-ICAM-1 mAb inhibits *in vitro* the secretion of TNF-α, IFN-γ and IL-1β by PHA-activated human T cells. The presence of ICAM-1 on $CD8^+$ T cells is associated with cytotoxic activity and production of IFN-γ (622, 631).

The 7-fold upregulated expression of ICAM-1 in activated iCMC T cells associated with several other upregulated genes such as TNF-α, IFN-γ and IL-1β (table 12) points to an enhanced inflammatory and cytotoxic cell activity in T cells of iCMC patients compared to healthy controls.

IL-7/IL-7R

IL-7 is known to modulate T cell lymphopoiesis and homeostasis of peripheral $CD4^+$ and $CD8^+$ T cells as persistence and survival of naive and memory T cell populations depends on the presence of IL-7 (507, 508). IL-7 signals via the IL7R which is expressed on all resting peripheral $CD4^+$ and $CD8^+$ T cells (507, 632) and it has been demonstrated that IL-7 can directly promote the survival and proliferation of activated

human $CD4^+$ T cells (633, 634). $CD4^+$ effector cells activated *in vitro* express little or no IL-7Rα but regain expression when they are rested *in vitro* or *in vivo* (635).

Lu et al. described that naive $CD4^+$ T cells expressed high levels of the IL-7Rα chain, which was further upregulated after stimulation with IL-7 in the presence of anti-CD3/anti-CD28. IL-7 promoted the proliferation of naïve $CD4^+$ T cells in STAT1,3- and -5-dependent manner and maintained the survival of activated $CD4^+$ T cells (636).

The IL-7-IL-7R pathway has been linked to multiple autoimmune disorders in humans (637, 638) and has been implicated as a risk factor for multiple sclerosis (639) and also as a predisposing factor in rheumatoid arthritis and colitis (640). Genes encoding LAG3 and IL-7R have been identified to confer susceptibility to multiple sclerosis implying that these gene variations are involved in the derangement of immune homeostasis (641). The expression of inhibitory LAG3 is also upregulated in activated T cells of iCMC patients (6-fold) indicating that both genes might play a role in deregulated immune responses in iCMC. Laakso et al. described an IL-7 dysregulation and loss of $CD8^+$ T cell homeostasis in patients with APS1. Herein, the investigated patients had increased plasma levels of IL-7 and a drastically diminished expression of the IL-7R on $CD8^+$ T cells suggesting that IL-7 leads to pathological changes in the $CD8^+$ T cell population by decreasing T cell activation threshold and promoting proliferation which is reflected as a decreased IL-7R expression (642).

The 7-fold increased upregulation of IL-7 (table 12) and the 2-fold downregulated expression of the IL-7R (table 13) in stimulated iCMC T cells suggests that iCMC T cells might be characterized by an impaired immune homeostasis. The supposed dysregulation of the IL-7-IL-7R axis might play an important role in the pathogenesis in iCMC which needs further investigation. Moreover the 4-fold upregulated expression of IL-2 which is described to downregulate the IL-7R (643) could play a role in iCMC T cells.

On the other hand studies have shown that IL-7Rα is downregulated in human Tregs with suppressive activity on effector T cells in multiple sclerosis and other autoimmune diseases. Moreover the IL-7R is a marker for identification and purification of Treg populations (68, 509). In the study of Walline et al. $IL\text{-}7R^{-/-}$ mice were found to have an increased proportion of Treg cells in an EAE model of multiple sclerosis (644).

Therefore downregulated IL-7R expression in iCMC T cells could also point to an enhanced proportion or activity of Tregs in iCMC T cells with suppressive capacity against *Candida*-defending immune responses.

CD70

CD70 is a member of the tumor necrosis factor superfamily and is expressed on activated B- and T cells and mature DCs (645-650). Binding to its receptor CD27 is important in priming, effector functions, differentiation and memory formation of T cells and plasma and memory B cell generation (651-654). CD27 is constitutively expressed on the majority of peripheral blood T cells, on 25 % of peripheral blood B cells and NK cells (648, 650). CD70 may activate $CD8^+$ T cells while Th cells are largely unaffected (655). A direct inhibitory effect on T cell activation has also been described (656). CD70-CD27 interactions are also involved in mediating apoptosis (657). CD70 has been reported to be induced at low levels on CD3 monoclonal antibody-stimulated T cells *in vitro* (648). Van Oosterwijk et al. showed that CD27-CD70 interactions with signals delivered through the TCR-CD3 complex enhanced the Th1-specific transcription factor Tbet and caused upregulation of the IL-12Rβ2 chain and IFN-γ producing T cells in response to IL-12 (658). Another study described that direct contact between $CD45RO^+$ T cells and $CD45RA^+$ T cells occurs via CD27/CD70 which results in the activation of $CD45RA^+$ T cells by CD25 upregulation suggesting that the engagement of CD27 plays an important regulatory role in the communication of subsets of $CD45RO^+$ and $CD45RA^+$ T cells (651).

CD70 is 7-fold upregulated in stimulated T cells of iCMC patients (table 12) which might be due to anti-CD3/anti-CD28 stimulation. But the upregulation of CD70 compared to healthy T cells could point to an increased cell activity and enhanced ability of iCMC T cells to enter CD70-CD27 interactions followed by increased T cell-T cell interactions and T cell development including a possibly enhanced Th1 or Treg response in iCMC T cells. One the other hand enhanced CD70 expression could also indicate a higher susceptibility to cell death.

LAG3

LAG3 also known as CD223 is described to play a role in Treg mediated suppression. LAG3 binds to MHC-II molecules on immature DCs and leads to ITAM-mediated inhibitory signals resulting in the suppression of maturation and immunestimulatory capacity (71, 78). Moreover it has been suggested that Tregs can also suppress effector cells due to the fact that activated human T cells are able to express MHC-II (71, 78). Therefore the 6-fold increased expression of LAG3 in activated T cells of iCMC patients (table 12) might indicate an increased suppressive activity of Tregs in iCMC pa-

tients. This hypothesis is supported by the 2-fold downregulated IL-7R which has also been described to be a Treg marker (68, 509).

FGL2

Fibrinogen-like protein 2 (FGL2) is a member of the fibrinogen superfamily and has been described to be secreted by Tregs affecting DC function. $CD25^+$ T cells have been reported to express 6-fold higher FGL2 mRNA compared to $CD25^-$ T cells and it has been shown that FGL2 inhibits T cell proliferation in the presence of anti-CD3/anti-CD28 and alloantigen stimulation. FGL2 defective mice show a reduced proliferative suppression capacity of Tregs and a polyclonal anti-FGL2 leads to a completely inhibition of suppressive activity of Treg cells in a dose-dependent manner. FGL2 has been supposed to represent an important Treg product downregulating DC function as treatment of DCs from wild-type mice with FGL2 leads to a diminished expression of CD80 and CD86, whereas FGL2 treatment had no inhibitory effect on DCs from FcgRIIB-deficient mice (71, 78, 659).

The 6-fold higher expression of FGL2 in activated T cells of iCMC patients compared to healthy subjects (table 12) might indicate a more prominent role of suppressive Tregs in iCMC resulting in a defect DC function as it has been observed in iCMC patients in the present study.

XAF1

The XIAP associated factor 1 (XAF1) is an antagonistic regulator of the anti-caspase activity of the x-linked inhibitor of apoptosis (XIAP/BIRC4) determing susceptibility to apoptosis (660, 661). Members of the inhibitors of apoptosis proteins (IAP) family including the x-linked inhibitor of apoptosis (XIAP/BIRC4) are key regulators of apoptosis, cytokine production and signal transduction as they suppress apoptosis through the inhibition of the caspase cascade (662). XIAP consists beside the BIR domain of the RING domain which enables the proteins to function as E3 ubiquitin ligases by promoting the ubiquitination and proteasomal degradation of caspases, TRAF2 and several other molecules (662, 663). Moreover XIAP has a unique function as it directly binds activated caspases such as caspase-3 or -7 and inhibits substrate binding and subsequent catalysis (663). Furthermore XIAP promotes NFκB activation by increasing the translocation of NFκB in the nucleus (664). The two main antagonists of IAP proteins are Smac/DIABLO and XAF1 which play a role in balancing and regulation of apoptotic

stimuli. XAF1 has been shown to restrain the anti-apoptotic function of XIAP by sequestering XIAP from the cytoplasma to the nucleus. XAF1 has been discussed to be important in mediating the apoptosis resistance of cancer cells and the relative expression ratio of XIAP/XAF1 determines susceptibility for apoptosis (661, 665).

XAF1 expression is 4-fold upregulated in activated iCMC T cells compared to healthy controls (table 12) suggesting the involvement of XAF1 in restraining the anti-apoptotic effect of IAPs in iCMC and points to an enhanced susceptibility for apoptosis in iCMC.

PDE4

Cyclic nucleotide hydrolyzing phosphodiesterase (PDEs) comprise a large superfamily of 11 families termed PDE1-PDE11 and are expressed in inflammatory cells including monocytes and T cells. PDEs play an important role in the regulation of T cell signalling by attenuating the negative straint of cAMP (666-668). Several cAMP-hydrolizing PDEs (PDE1, PDE3, PDE4, PDE7 and PDE8) have been identified in T cells. PDE4 represents the predominant family including the subtypes PDE4A, PDE4B, PDE4C, and PDE4D which have distinct roles in modulating inflammation and immune cells (669, 670). PDE4 inhibitors increase intracellular cAMP levels and target inflammatory cells affecting T cell function by inhibition of cytokine release and proliferation (671, 672). The role of PDE4 in T cells is still unclear and remains speculative, although it has been shown that stimulation of the TCR and CD28 in human peripheral T cells results in the recruitment of PDE4A4, PDE4B2, PDE4D1, and PDE4D2 suggesting a potential role of the individual PDE4 isoforms in opposing the TCR-induced production of cAMP (673). Untreated human $CD4^+$ express PDE4 and anti-CD3/anti-CD28 stimulation enhances the expression of the PDE4 subtypes PDE4A, PDE4B and PDE4D (674).

The 4-fold increased expression of PDE4 in activated T cells of iCMC patients (table 12) might be due the activation by anti-CD3/anti-CD28 and could indicate an impaired TCR signalling in iCMC T cells compared to healthy T cells.

IL-2

The pleiotropic cytokine IL-2 which is 4-fold upregulated in activated T cells of iCMC patients (table 12), is primarly produced by $CD4^+$ T cells following antigen-activation and acts on lymphoid populations including T cells, B cells, and NK cells, as well as on hematopoietic populations such as neutrophils (675-680). IL-2 modulates activation-induced cell death, development of Tregs and cytotoxic T lymphocytes, expansion of

memory $CD8^+$ T cells and T cell differentiation (678). IL-2 signals via the three pathways phosphoinositol 3-kinase (PI 3-K)/AKT, Ras-MAP kinase, and JAK-STAT pathway mediating cell growth, survival and differentiation, but also activation-induced cell death. JAK1, JAK3 and STAT5 are the principal molecules involved, although STAT3 and STAT1 can also be activated by IL-2 (677, 678, 681). IL-2 is able to promote immunostimulatory effects and increased cell activity by upregulation of anti-apoptotic molecules such as Bcl-2 (682, 683). IL-2 can modulate receptor expression by inducing IL-4Rα and IL-12Rβ2 while repressing IL-6Rα/gp130 and IL-7Rα (643, 678). The observed 2-fold downregulation of IL-7R and 2-fold decreased expression of gp130 in activated T cells of iCMC patients might be due to the enhanced IL-2 expression. Repression of IL-7R might lead to reduced survival signals followed by activation-induced cell death which requires pre-exposure to IL-2 (678). Therefore the increased IL-2 expression in iCMC T cells is hardly to interpret: on the one hand it might indicate a higher cell activity of iCMC T cells, on the other hand upregulated expression of the IL-2 gene could point to a higher susceptibility to cell death compared to healthy T cells.

IL-2 plays also a role in Treg development as IL-2 promotes together with TGF-β the differentiation of Tregs which express Foxp3 and the IL-2Rα (CD25) (684). Increased IL-2 expression could also imply an increased development and suppressive function of iCMC T cells accompanied by the upregulation of the Treg markers LAG3 (6-fold) and FGL2 (6-fold) (table 12).

Moreover IL-2 contributes to Th1 differentiation by promoting IFN-γ production and induction of the expression of IL-12Rβ1, IL-12Rβ2 and Tbet (678, 685). Therefore the enhanced expression of IL-2 in iCMC T cells combined with a enhanced cell activity could be linked to the also increased 8-fold IFN-γ expression (table 12) pointing to a reinforced Th1 response in iCMC.

Interestingly, IL-2 is able to diminish the development of Th17 cells that are generated in the presence of TGF-β and IL-6; and anti-IFN-γ and anti-IL-4 are added to inhibit Th1 and Th2 polarization (16, 33). IL-6 signals via IL-6Rα/gp130 and STAT3 while IL-2 signalling involves STAT5. The Th17 inhibitory effect of IL-2 is supposed to be mediated by STAT5 which competes for STAT3 binding sites in the Il17a gene locus and inhibits Il17a transcription (686). Another mechanism by which IL-2 diminishes Th17 polarization is that IL-2 inhibits the expression of the IL-6Rα/gp130 (678) which

is 2-fold downregulated in activated T cells of iCMC patients (table 13) suggesting IL-2 could play a role in Th17 inhibition in iCMC patients. But IL-2 can also lead to expansion of IL-17 producing cells once generated (687) underlining the complex roles of IL-2 in the regulation of IL-17. Already generated Th17 cells of iCMC patients might do not respond to IL-2.

In addition to the effects of IL-2 on $CD4^+$ cells, IL-2 promotes the differentiation of naive $CD8^+$ T cells into effector or memory CTLs which depends on the IL-2/IL-2R signal strength (688, 689). IL-2 is also necessary for expansion of memory $CD8^+$ T cells (690) which suggests that the activity of CTLs and $CD8^+$ T cells could be also enhanced in iCMC patients due to the higher IL-2 expression.

The involvement of IL-2 in the induction of programmed cell death (682, 683, 691) implies that the higher IL-2 expression of IL-2 could also be associated with an increased apoptosis of iCMC T cells.

Taken together, the higher expression of the IL-2 gene suggests that IL-2 might enhance cell activity favouring the development of Th1 cells, $CD8^+$ cells and CTLs and/or apoptosis while Th17 cells are inhibited.

LGALS9

Galectins comprise a family of animal lectins differing in their affinity for β-galactosides. Among the 15 members of the galectin family LGALS9 (Galectin-9, Gal-9) was reported to function as a ligand for cell immunoglobulin domain and mucin domain-3 (Tim-3) expressed on terminally differentiated Th1 cells and is known as an apoptosis inducer of activated T cells (692, 693). Moreover Gal-9 has been identified as an eosinophil chemoattractant secreted by activated T cells and can also be expressed by endothelial cells in response to IFN-γ (694-696). Sakai et al. demonstrated in a mice model that recombinant human Gal-9 inhibit mixed lymphocyte reactions in a dose-dependent manner, involving both $Ca2^+$ influx and apoptosis in T cells; and significantly inhibit the progression of GVHD in murine bone marrow transplantation models (693). Previously it has been shown that Gal-9 induces apoptosis in both activated $CD4^+$ and $CD8^+$ T cells through the $Ca2^+$-calpain-caspase-1 pathway (697). Gal-9 treatment decreased numbers of Tim-3 positive $CD4^+$ T cells and given that Tim-3 is a receptor for Gal-9 these data suggest that Gal-9 directly induces apoptosis in $CD4^+Tim\text{-}3^+$ T cells (698). In contrast, $CD4^+CD25^+$ or $CD25^+Foxp3^+$ Treg numbers were increased in re-

sponse to Gal-9 treatment (693). It has been shown that Gal-9 induces the differentiation of $CD4^+CD62L^+$ naive T cells into $Foxp3^+CD4^+CD25^+$ Treg cells and analysis of cytokine levels revealed that Gal-9 suppressed the production of IL-17, TNF-α and IFN-γ in recipient mice (699). Given that Tim-3 is expressed in Th17 cells (700) , these studies suggest that Gal-9 might induce apoptosis in Th17/Th1 cells and suppression of Th17 differentiation in iCMC patients and might redirect T cell responses to Treg differentiation.

Therefore the 3-fold upregulated Gal-9 expression in T cells of iCMC patients (table 12) could play a role in the observed IL-17/IL-22 deficiency and might be involved in *Candida*-specific Treg-biased immune responses.

MAP3K8

The serine/threonine (Ser/Thr) kinase TPL2, also known as COT and MAP3K8, belongs to the MAKP family and is hematopoetically expressed. TPL2 plays a role in oncogenesis and innate immunity as it has several functions in the regulation of TNF-α, TLR and G-coupled receptors, but its role in T cells is limited described (701). Overexpression of TPL2 in various cell types induces several molecules of the MAPK pathway including NFAT, and NFκB (701-704). Although not many data exist on the role of TPL2 in T cell function it has been shown to play a role in TCR signalling. TPL2 can be induced by TCR and IL-12 and is required by T cells for IL-12-induced IFN-γ secretion as *in vitro* stimulated $Tpl2^{-/-}$ $CD4^+$ T cells lead to an impaired IL-12- and TCR-induced IFN-γ production and impaired development of Th1 cells (701). TPL2 may not only directly influence the development of Th1 cells. It has recently been shown that TPL2 might positively regulate Th17 differentiation production as the Th17-promoting cytokine IL-23 is impaired in LPS-stimulated $Tpl2^{-/-}$ macrophages (705).

TPL2 could also play a role in iCMC as it is 3-fold upregulated in activated T cells of iCMC patients (table 12). The hypothesis of the involvement of TPL2 in Th1 development corresponds to several other upregulated Th1-associated genes and supports an impaired Th1 response that might be responsible for the observed Th17 defects. The suggested role of TPL2 in Th17 cells provides an interesting aspect that needs further investigation concerning the pathogenesis of iCMC.

TNF-α

TNF-α as a member of the TNF superfamily plays an important role in innate and adaptive immunity and is produced by several cell types including immune cells (B cells, basophils, eosinophils, neutrophils, mastcells, DCs, B cells, NK cells and T cells), but also by non-immune cells e.g. keratinocytes, fibroplasts. TNF-α modulates cell growth and differentiation of various cell types but also induces inflammation and apoptosis. Cytokines induced by TNF-α are e.g. IL-1β, IL-1R, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, IFN-γ and TGF-β (706). Therefore the upregulation of the genes IL-2, IL-1β and IFN-γ in activated T cells of CMC patients could be associated with the increased expression of TNF-α. IL-12 and IL-18 induced by TNF-α lead to IFN-γ production supporting the role of TNF-α in amplifying Th1 immune responses and increasing $CD4^+$ T cell activation and IFN-γ production (706). The 3-fold increased expression of TNF-α in combination with the 8-fold upregulation of IFN-γ in activated iCMC T cells (table 12) points to an enhanced immunosuppressive activity of TNF-α and IFN-γ and an increased inflammatory potential of iCMC T cells. This hypothesis is supported by the fact that secretion of TNF-α and IFN-γ can be induced by IL-2 which is also 4-fold upregulated. TNF-α also mediates apoptosis (706, 707) that correlates with several upregulated genes in iCMC T cells which are involved in apoptosis and suggests a higher susceptibility of iCMC T cells to cell death compared to healthy controls.

Interestingly, boosting of Tregs by effector T cells and TNF-α has been recently discussed (708). Approximately 40 % of peripheral Tregs express the TNFR2 which makes TNF-α able to induce together with IL-2 activation, proliferation, Foxp3-upregulation and increased suppressive activity of Tregs overcoming the anergic state of Tregs (709). These data suggest that increased expression of TNF-α in T cells of iCMC patients could be involved in an enhanced Treg activity in iCMC patients that correlates with several upregulated Treg-associated genes analyzed in the gene array of the present study.

CDKN2A

The cyclin-dependent kinase inhibitor 2A (CDKN2A), also known as ARF, MLM, P14, P16, P19, CMM2, INK4 is a cyclin-dependent kinase which inhibits cell cycle processes and has been suggested to be involved in T cell apoptosis and controlling T cell populations (710-714). A number of cell cycle activators including cyclin-dependent kinases are involved in the entry of quiescent (G0) cells into G1 phases of the cell cycle.

The CDKs are a family of Ser/Thr protein kinases, which are activated by phosphorylation/dephosphorylation binding to regulatory subunits or cyclins and are regulated by small polypeptides, the CDK-inhibitors (CKIs) (713, 715). In T cells, activation via TCR and CD28 costimulation or growth factor stimulations leads to induction of CDK4/6 and D-type (711, 712, 716).

The 3-fold higher expression of the CDKN2A gene (table 12) might indicate a higher activity of cyclin-dependent kinases in iCMC T cells which lead to inhibiton of cell cycle supporting a higher susceptibility for apoptosis in iCMC.

PDCD1

The gene Programmed Cell Death PDCD1, also known as PD1, PD-1, SLEB2, hPD-1, hPD-lor CD279 is a receptor inducibly expressed on activated $CD4^+$ T cells, $CD8^+$ T cells, NK T cells, B cells and monocytes (717). The ligands of PD1 are PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273) which are expressed on B cells, DCs, macrophages, mast cells and T cells, as well as on non-hematopoetic cell types and are further upregulated in response to activation. The PD-1-PD-L pathway is a key regulator of the balance between stimulatory and inhibitory signals for effective immune responses. The role of PD1 ligands concerning the induction or inhibition of T cell proliferation are controversial discussed. Resting DCs have been described to induce peripheral $CD8^+$ T cell tolerance by PD1 and CTLA-4 (718). The expression of PD-L on DCs can also diminish the effects of pathogenic $CD4^+$ T cells in experimental autoimmune encephalitis (EAE) (719). PD-L1 and PD-L2 are involved in the regulation of T cell activation and tolerance (717, 720).

The 2-fold upregulated PDCD1 expression in activated T cell of iCMC patients (table 12) might indicate in impaired T cell activity in iCMC T cells compared to healthy controls.

IL-15

IL-15 is a member of the γc family including IL-2, IL-7, IL-9, IL-21, and the IL-7-related cytokine TSLP. IL-15 plays an important role in the development of NK cells and $CD8^+$ T cell homeostasis as they are the main consumers of IL-15 (721-724). IL-15 has a key function in the homeostastic expansion of effector and memory T cells as most T cells downregulate IL-7Rα immediately after TCR activation followed by

upregulation of IL-2Rα, IL-15Rα, and IL-2Rβ. Therefore IL-2 and/or IL-15 may increase the proliferative rate of effector T cells (725-727).

The increased 2-fold upregulation of IL-15 in activated T cells of iCMC patients (table 12) might indicate an enhanced proliferation or cell activity of effector T cells in iCMC patients. On the other hand it might point to an increased activity of CTLs as IL-15 has strong effects on NK cell and CTL activity (728).

RIPK2

The receptor-interacting serine-threonine kinase 2 (RIPK2), also known as CCK, RICK, RIP2, CARD3, GIG30 or CARDIAK is a member of the receptor-interacting protein (RIP) family of serine/threonine protein kinases intermediating with the death receptors Fas and tumor necrosis factor receptor 1 (TNFR1) (729, 730). Fas and TNFR1 are cytokine receptors activating cell death pathways through the intracellular region called death domain whereas TNFR1 activation also can lead to a protective pathway mediated by NFκB activation (729, 731). Overexpressed RIP has been described to induce NFκB activation, stress activated protein kinase/c-Jun N terminal kinase (SAPK/JNK) activation and apoptosis (730, 732-734).

The 2-fold upregulated expression of RIPK2 in activated T cells of iCMC patients (table 12) supposes an enhanced signalling of the the death receptors Fas and TNFR1 and might indicate an enhanced activity of cell death in iCMC T cells.

TRIM69

TRIM69 also known as ring finger protein (RNF)36 or Trif is a member of the RING zinc finger protein family. In COS-7 and HEK-293 cell lines it was shown that overexpression of RNF36 induced about half of transfected cells to undergo cell death which was associated with elevated expression of Bax, caspase-2 and receptor-interacting protein. Moreover it was suggested that RNF36 colocalizes and interacts with another RING finger protein promyelocytic leukemia (PML) (735).

The 2-fold upregulated expression of TRIM69 (table 12) suggests an increased apoptosis activity in iCMC T cells.

SOCS3

Members of the SOCS (suppressor of cytokine signaling) family modulate cell responses by the negative regulation of cytokine signal transduction. These proteins in-

hibit the cytokine-activated Janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling pathway (736). SOCS3 is a major feedback regulator of signal transducer and activator of transcription 3-activating cytokines. IL-6, IL-23 and IL-21 promote Th17 development (16) and induce STAT3 activation as well as the expression of SOCS3 which acts as a negative feedback regulator of these STAT3-activating cytokines followed by negative regulation of Th17 cells (737). IL-6 binds to the receptor chain gp130 in combination with either a membrane bound or secreted version of the IL-6Rα chain (738) which results in STAT3 and SOCS3 activation. Therefore the 2-fold upregulated SOCS3 expression in activated iCMC T cells (table 12) might lead to an inhibition of gp130 - which is 2-fold downregulated in iCMC T cells - and STAT3 signalling. These speculations would be in accordance with the decreased DNA-binding activity of STAT3 analyzed in PBMCs of iCMC patients. The increased SOCS3 expression could play a role in the observed impaired Th17 response in iCMC patients as it inhibits signalling pathways including STAT3 of Th17 differentiating cytokines.

BCL2, BAX, BCL2L1 (Bcl-X), BCL2L11 (Bim), CYCS

Members of the Bcl-2 family are proteins sharing at least one of four Bcl-2 homology (BH) domains with the protein Bcl-2 and modulate the intrinsic pathway of apoptosis being either pro-apoptotic or anti-apoptotic. The pro-apoptotic members can be divided into two groups: the multidomain pro-apoptotic proteins and the BH3-only proteins regulating T cell survival (739). The multidomain Bcl-2 family member Bax is required for apoptosis through the mitochondrial pathway by induction of cytochrome c into the cytosol followed by the activation of caspases and cell apoptosis (739-741). The BH3-only protein BCL2L11 (Bim) responds to different stimuli and acts an iniator of apoptosis by interactions with other Bcl-2 family members. Their direct activation by Bax or indirect activation by neutralization of the anti-apoptotic proteins has been discussed by several studies (742-745). Anti-apoptotic proteins members of the Bcl-2 family are Bcl-2 and BCL2L1 (Bcl-x) which are identified as a key inhibitor of apoptosis by binding Bak and/or Bax and BH3-only proteins (745-748). Bcl-2 is indicated to be required for the maintenance of mature T cells as Bcl-2 has been described to be expressed in naïve T cells, downregulated in effector cells and highly expressed in memory cells. Bcl-x is most highly expressed in activated T cells as it can be upregulated in response to activation through the T cell receptor (TCR) and the coreceptor CD28 (739, 749, 750).

The expression of the apoptose-related genes BCL2 (2-fold), BAX (2-fold), BCL2L1 (Bcl-X, 2-fold), BCL2L11 (Bim, 2-fold) and CYCS (2-fold) is upregulated in activated T cells of iCMC patients compared to healthy controls (table 12) that might be due T cell activation and could also point to an increased regulation of cell death in iCMC T cells.

CREM

The transcriptional repressor cAMP response element modulator (CREM) which is 2-fold upregulated in activated T cells of iCMC patients (table 12) has been described to have an important role in T cells: Rauen et al. described that CD3/CD28-stimulation enhances CREM promoter activity in healthy subjects while patients with systemic lupus erythematosus showed no increase of their basal CREM levels upon T cell activation (751). Another study showed that CREM is early induced in $CD4^+CD25^+$ Tregs and Foxp3 expression in Tregs promotes suppression by induction of CREM expression in activated $CD25^+Foxp3^+$ effector T cells thus preventing them from IL-2 production (752, 753).

Therefore CREM could be upregulated in T cells of iCMC patients due to the anti-CD3/anti-CD28 stimulation or might indicate an increased suppression activity of Tregs in iCMC patients.

SLAM6

SLAM6, also known as CD352, NTBA or Ly108 is a member of the SLAM family of hematopetic-surface receptors which are expressed on thymocytes, NK-, T- and B cells, low on DCs, eosinophils and neutrophils (754-758). Ly108 binds to the single SH2-domain adapters SAP and EAT-2 which recruit and activate the Src protein tyrosin kinase Fyn (759, 760). The SLAM/SAP/Fyn signalling pathway plays an important role in development, differentiation and effector function of several leukocyte lineages of the innate and adaptive immune system (761). SLAM related receptors act as costimulatory molecules and the following SLAM/SAP/Fyn pathway increases the TCR-mediated NFκB activation during the activation of T cells (754, 760). It has been reported that engagement of Ly108 promotes SAP dependent the activity of cytotoxicity of human NK cells (762). Stimulation of human $CD4^+$ T cells with Ly108-antibodies leads to an increased IFN-γ production speculating the involvement of Ly108 in the regulation of Th1 cells (763, 764).

The 2-fold upregulation of SLAM6 in activated T cells of iCMC patients (table 12) suggests an enhanced SLAM6 signalling and NFκB activation in iCMC T cells or might indicate an increased cell activity including Th1 cells which is in concordance with several upregulated inflammatory genes such as IFN-γ (8-fold).

Foxd2

Foxd2 is a member of the forkhead (Fox) gene family which comprises a diverse group of "winged-helix" transcription factors that play an important role in multiple biological processes including development, metabolism, aging and cancer (765-768). Foxd2, expressed in multiple mesodermal lineages was initial suggest to play an important role in the kidney but Foxd2$^{-/-}$ mice showed only mild renal abnormalities underlining the unkown biological and immunological function of Foxd2 (765, 769). Rare data indicate the expression of Foxd2 in T cells and monocytes, but not in B cells (770). T cells deficient in Foxd2 have been described to be less sensitive to cAMP-mediated inhibition of proliferation which was correlated with a deficient cAMP-dependent protein kinase type I (PKA type I) (770). These data suggest Foxd2 as a reguator of cell activation by modulation of sensitivity to cAMP (765, 770). Moreover increased PKA activity has been described to associated with the anergy and hypresponsivness of T cells in HIV infection while a defective PKA activity has been discussed to be associated with a T cell hyperactivity in lupus (771, 772).

The 2-fold upregulation of the gene for Foxd2 in activated T cells of iCMC patients (figure 12) might indicate that T cells of iCMC patients show an impaired cAMP signalling and are more sensitive to inhibition of proliferation which can result in anergy and hyporesponsivness.

HRH4

Histamine H4 receptor is a pertussis-toxin-sensitive G-protein coupled receptor expressed on macrophages, monocytes, eosinophils, DCs, NK cells and T cells (773-778). HR4 has been described to have a higher affinity for histamine compared to the HR1 and HR4 activation results in leukocyte chemotaxis to sites of inflammation and leads to increased intracellular $Ca2^{+}$ concentration (778, 779). The H4 receptor expressed on DCs, $CD4^{+}$ and $CD8^{+}$ T cells controls the secretion of cytokines and chemokines during the integration of Th1/Th2 differentiation (780). Engagement of the H4 receptor by histamine has been described to suppress *ex vivo* the mitogen-induced STAT1 phosphorylation and its specific interaction with DNA in PBMCs from non-atopic individuals

(781), while the H4R antagonist JNJ7777120 has been reported to inhibit STAT6 DNA binding in cells derived from atopic subjects (782). H4R is higher expressed in resting than in activated $CD4^+$ and $CD8^+$ T cells and is involved in the release of the $CD4^+$ cell chemoattractant IL-16 from human $CD8^+$ T cells *in vitro* underlining its role in immuneregulation (783, 784).

The 3-fold downregulation of the HR4 gene in activated iCMC T cells (table 13) could be due anti-CD3/anti-CD28 stimulation because only resting $CD4^+$ and $CD8^+$ T cells express the H4R or might be associated with an increased activity of $CD8^+$ T cells.

IGF1R

The Insulin-Like Growth Factor-I Receptor (IGFR1) is expressed on B- and T cells and binds to Insulin-Like Growth Factor(IGF)-I and -II and insulin (785, 786). Saturable binding of IGF-1 was reported on human resting and activated T cells leading to proliferation and chemotaxis (787). Yang et al. found that IGFR abundance was enhanced by cell activation and reduced IGFR lead to a reduced proliferation and production of cytokines and IgM in human cord blood lymphocytes (788). IGF-1 and IGF-2 have been described to play an important role in T cells as they activate T cell Akt and increase lymphocyte survival. But considering IGF1 and insulin as generally increasing growth and function of lymphocytes are also able to inhibit IL-2 dependent the proliferation of lymphocytes (789, 790). Naïve $CD4^+$ and $CD8^+$ cells were described to show a higher expression of the IGFR compared to memory $CD4^+$ and $CD8^+$ cells and IGFR is downregulated upon differentiation as levels of IGFR were markedly lower in activated T cells *in vitro* and *in vivo*. $CD4^+CD45RO^+$ cells activated by PMA *in vitro* express considerably lower levels of IGFR compared to inactive lymphocytes (786, 791-794). Another study reported that human PBMCs that were activated with anti-CD3 show an upregulated expression of IGFR and proliferated in response to IGF-1 and IGF-2 (795). Moreover IGFR expression can be upregulated in T cells by CD28 receptor cross-linking or by activating the CD80/CD86 pathway while inhibition of IGFR on TCR- and CD28-engaged T cells leads to a reduced lymphocyte survival in the presence of IL-2 (796).

Therefore the 2-fold downregulation of the IGFR in activated T cells of iCMC patients compared to healthy controls (table 12) is difficult to discuss. On the one hand this observed downregulation might be the response to anti-CD3/anti-CD28 stimulation indicating a higher activity of iCMC T cells. One the other hand IGFR expression might be

downregulated because iCMC T cells have a lower activity and reduced proliferative capacity. Thus it needs further investigation how the IGFR is involved in the T cell mediated pathogenesis of iCMC.

gp130

gp130 is a receptor subunit utilized by several cytokines including IL-6, IL-11, IL-27, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin 1 and cardiotrophin-like cytokine.These cytokines exert similar functions including the induction of acute phase proteins, stimulation of hematopoiesis and promotion of B cell development and antibody production (797-801). But these cytokines also exhibit distinct functions, by using unique receptor alpha chains that pair with gp130 to form functional receptor complexes. The single subunit cytokine IL-6 binds gp130 in combination with either a membrane bound or secreted version of the IL-6Rα chain (738, 802). IL-27 consisting of the two subunits p28 and EBI3, binds to the unique receptor subunit IL-27Rα that pairs with gp130 and IL-27p28 has been described to inhibit IL-6 mediated Th17 differentiation EBI3 independent and is able to antagonize gp130 signalling mediated by IL-6, IL-11 and IL-27 (801, 803).

The 2-fold decreased expression of gp130 in activation in activated iCMC T cells might be associated with the peviously described 4-fold upregulation of IL-2 which has been described to inhibit gp130 (678). The IL-6-gp130-STAT3 pathway has been described to directly promote the development of Th17 cells (804) and the downregulated gp130 expression in iCMC T cells together with the analyzed impaired STAT3 activity might indicate a disturbed IL-6 signalling in iCMC T cells and could be one explanation for the impaired Th17 responses described in iCMC patients. As cytokines including IL-6, IL-11 and IL-27 signal via the gp130 subunit and gp130 mediated signalling by these cytokines might be impaired in T cells of iCMC patients compared to healthy controls. Moreover IL-27 antagonizes IL-6 signalling by gp130 suggesting a possible role of IL-27 that needs further investigation. SOCS3 is known to inhibit gp130-STAT3 signalling and the 2-fold upregulation of SOCS3 in iCMC T cells could be in association with the decreased gp130 expression observed in iCMC patients.

PPARG

The transcription factor PPARγ is a member of the nuclear receptor superfamily was originally characterized as a key regulator of adipocyte differentiation and lipid metabo-

lism (805-809). PPARγ is predominantly expressed in adipocytes, but also found in other cell types such as fibroplasts, myocytes, monocytes, macrophages, endothelial cells, B cells and T cells (810-815). PPARγ plays a role in T cell immunity as PPARγ has been described to be upregulated in activated T cells and PPARγ ligands have been shown to inhibit T cell proliferation (812, 814-816). Activated PPARγ inhibits DNA binding of the transcription factor nuclear factor of activated T cells (NFAT) followed by blocking the transcriptional activation of the IL-2 promoter (815). Another study by Harris and Phipps suggests PPARγ mediates apoptosis in T cells (816). Moreover several studies reported suppression of IFN-γ by PPARγ ligands in activated T cells (812, 817, 818). PPARγ was also identified as negative key regulator of Th17 differentiation in mice and human as PPARγ activation in $CD4^+$ T cells selectively inhibits Th17 differentiation, but not polarization to Th1, Th2, or regulatory T cells. PPARγ controls Th17 differentiation by the inhibition of TGF-β/IL-6-induced expression of RORγt in T cells (819).

The 2-fold downregulation of PPARγ expression in activated T cells of iCMC patients (table 13) might represent a decreased cell activity of iCMC T cells. The specific association between the downregulated PPARγ expression and Th17 defect observed in iCMC patients needs further investigation.

The analyzed data of the gene expression array lead to the assumption that many genes that are upregulated compared to healthy controls seem to be involved in Th1- and Treg-mediated responses. Moreover genes which are associated with mechanisms of cell activation and cell death are more regulated in activated T cells of iCMC patients compared to healthy subjects. Both patients analyzed in the array have isolated CMC with a known *STAT1* mutation and the gene expression profile of activated T cells of these patients which is dominated by inflammatory Th1-associated genes is in line with the gain-of-function *STAT1* mutations and might result in impaired Th17 responses followed by an insufficient *Candida* defence. Future studies e.g. using Real-time PCR will clarify the differential expression of these genes between iCMC patients and healthy controls. Moreover regarding CMC as very heterogeneous disease a larger collective of patients has to be involved to draw any general conclusions.

Taken together, the present study shows that in case of the chronic mucocutaneous candidiasis including autoimmune polyendocrine type I syndrome innate immune mechanisms are disturbed resulting in dysregulated adaptive immune responses. The results of

the present study suggest that in particular the T cell compartment is more affected since CMC patients are not able to produce IL-17 and IL-22. Consequently T cells fail to differentiate to Th17 cells that are important defence mechanisms against *Candida.* These data show that patients of both analyzed CMC subgroups isolated CMC and APS1 suffer from chronic *Candida*-infections and have deregulated Th17 responses in common but they have to be immunologically and genetically considered as two distinct subgroups of CMC as they show different impaired immune responses on the level of PBMCs, DCs and T cell differentiation. Compelling evidence suggests that isolated CMC arises from genetic defects since it has been shown that some iCMC patients are characterized by mutations in the IL-17R or IL-17F. Now gain-of-function *STAT1* mutations were identified as a new genetic etiology of iCMC in the present study. Further investigation on these immunological and genetic failures is needed to provide new therapeutic strategies.

An alternative method to discuss is whether an additional treatment with probiotics would be helpful to treat or dampen *Candida*-infections as a number of bacterial species e.g. *S.boulardii* or *L.acidophilus* exert the ability to inhibit the growth of *Candida* species (820-822). Several studies focused on the involvement of bacteria in *Candida*-infections suggesting that probiotics could provide new possibilities in antifungal therapy (467, 473, 475). It has been reported that lactic acid bacteria (LAB) have the capacity to protect immunedeficient mice from Candidiasis presenting a key first-line defence against mucosal and systemic *Candida*-infections (468, 469). Furthermore *L.helveticus* has been described to prevent E.coli-mediated STAT1 inhibition proposing that LAB are capable to intervene signalling pathways and also might have an influence on gain-of-function *STAT1* mutations in iCMC patients (823).

To investigate the use of probiotic bacteria for prophylaxis and therapy of Candidiasis basic immunological science on the function of bacteria - in particular LAB, in the human immune system is required as presented in the following second part of this study.

5.12 LAB strains differently induce cytokine production in human MoDC

The innate immune system plays an essential role in activation and modulation of subsequent adaptive immune responses. After activation of distinct PRRs DCs produce a certain cytokine profile that determines the polarization of Th1, Th2, Th17 or regulatory T cells (403, 404, 824-827). Therefore the cytokine response of human MoDC after exposure to the different bacterial strains IMS1 and IMS2 was investigated. One important cytokine released by DCs is IL-12 that leads to Th1 polarization and IFN-γ production by these T cells via STAT4 (6, 826, 828). IMS2 from the mid-log growth phase triggered IL-12 production in human MoDC whereas IMS1 from the mid-log growth phase instructed MoDC to secrete remarkable low levels of IL-12. The differences in IL-12 induction between both strains were less prominent in the stationary growth phase cultures where both strains induced IL-12 in MoDC. The capacity of IMS2 to elicit high amounts of IL-12 in DCs compared to IMS1 suggests that IMS2 might be more potent in priming DCs to drive the development of Th1 cells. In contrast, IL-10 as a key factor of probiotic bacteria has anti-inflammatory effects by affecting IL-12 and following IFN-γ production that leads to differentiation of Th2 or regulatory T cells (829, 830). Both strains obtained from the mid-log growth phase promoted IL-10 production in MoDC at the highest concentration of 10^7 cfu. IMS1 was a more potent to induce IL-10 at concentrations of 10^6-10^5 cfu compared to IMS2. Also among the stationary phase cultures IMS1-stimulation of MoDC revealed strong IL-10 responses. Growth phase/cultivation period, culture medium and cell density can influence cytokine production (460, 461, 831-833), that could be confirmed by the observed differences in cytokine induction between the different bacterial concentrations in this study indicating that different growth phase conditions and DC:bacteria ratios lead to different activation programs in DCs.

The differing capacity of both strains concerning IL-10 induction got more evident analyzing the IL10/IL-12 ratio showing that IMS1 is charcaterized by an enhanced IL-10/IL-12 ratio in comparison to IMS2. The increased IL-10/IL-12 ratio observed in IMS1-triggered MoDC might propose that IMS1 is more anti-inflammtory. Furthermore IMS1 could be predictive for priming naïve T cells to IL-10 producers. In contrast, IMS2 may exhibit pro-inflammatory potentials and drives the differentiation of Th1 cells. These findings confirm that LABs differ in their ability to induce IL-12 and IL-10 in human MoDC. These strain-specific effects are in agreement with previous observa-

tions that LAB regulate DC function in a strain-dependent manner (834). Several studies described strain-specific effects of IL-10/IL-12 induction in DCs (406, 408, 416, 418) and a theory of dividing LAB into two groups due to their ability of IL-12 induction has been discussed (409). The mechanisms underlying the different DC responses instructed by IMS1 and IMS2 are unknown so far. On the one hand receptor mechanisms by which IMS1 and IMS2 activate DCs have to be further evaluated as it is known that LABs are able to signal via a variety of PRRs including TLR2, NOD and DC-SIGN (118, 425-427, 835-837). NOD 1 and NOD2 receptors play an essential role in LAB recognition as they recognize intracellularly peptidoglycan-derived muramyl dipeptides of the bacterial cell wall of gram-positive bacteria (430, 838, 839). The two strains tested in this study are gram positive bacteria indicating IMS1 and IMS2 might mediate their effects via NOD. The analysis of cytokine secretion, in particular IL-10 by certain TLR antagonists would represent one possibility to explore the receptor pathways IMS1 and IMS2 are involved in.

Another interesting aspect would be to examine the expression of the AhR in IMS1- and IMS2-exposed MoDC which is involved in the differentiation of Tregs and Th17 cells (840). A previous work could show that *L.bulgaricus* is potent in activating the Ahr pathway and thereby suppressing dextran-sodium sulfat (DSS)-induced colitis in mice (437).

On the other hand futher mechanisms have to be taken into consideration. The capacity of probiotic bacteria to trigger distinct cytokine profiles in DCs in a strain-dependent manner could also be due to certain surface-associated compounds or soluble mediators (421-423). Findings of a prolonged DC survival through TLR2 activation and enhanced amounts of IL-10 induced by the supernatant of *B.breve* highlight a potential role of soluble compounds (422). Many studies focused on the mechanisms attributed to the probiotic function of LAB describing that polysaccharide A (PSA), a microbial molecule produced by *Bacteroides fragilis* promotes TLR2-dependent the development of IL-10 producing inducible Tregs in *B.fragilis*-colonized mice and protects from colitis (446, 447). For this purpose the effects of bacterial supernatants on DC function and the precise molecular mechanism of the responsible mediators have to be further examined. This in turn will contribute to the understanding how IMS1 and IMS2 exert their beneficial effects. Also of interest is whether both strains tested in this study have the same effect on human DCs when they are heat-inactivated or cell wall structures are fixed. Compounds of the cell wall of gram-positive bacteria including peptidoglycane and

lipoteichoic acid (LTA) that signal via TLR2 have been shown to affect cytokine secretion (428, 837, 841). In particular the content of D-Alanine of LTA has been described as a key mediator in modulating immune responses as a Dlt-mutant of *L. plantarum* results TLR2-dependent in an increase of IL-10 and was more protective in a murine colitis model (428, 433). Because both strains tested in this study are gram-positive bacteria that do not contain LTA in their cell wall excludes possible effects of alanin-compounds in the cell wall.

5.13 LAB strains differently upregulate maturation markers in human MoDC

To investigate the maturation patterns of MoDC exposed to IMS1 and IMS2, the expression of CD80, CD83, CD86, CD40 and HLA-DR was determined by flow cytometry. Immature DCs upregulate these costimulatory and activation markers upon stimulation (842-844). Triggering by both strains resulted in a maturation program in immature MoDC that is in line with several studies reporting an increase of maturation marker expression in LAB-exposed DCs (406, 408, 409, 416, 458, 845, 846). A previous report described a correlation between a strong upregulation of surface molecules and a strong IL-12 induction in DCs that is not in concordance with the results of mid-log phase cultures of this study. Although IMS1 tended to induce a stronger DC activation, this strain led to a weak IL-12 secretion in human MoDC. Among the stationary phase cultures IMS2-stimulation resulted in a more effective activation program that would correlate with its capacity of a more potent IL-12 induction in MoDC. These discrepancies could be explained by the use of different bacterial species or culture methods. The data of the present study further underline that different bacterial strains induce a different maturation pattern in human MoDC followed by different cytokine responses.

5.14 Different T cell responses induced by LAB

DCs receive distinct T cell-instructive signals and promote the development of Th1, Th2, Th17 or Treg cells (403, 404, 824-827). Concerning the differential IL-10/IL-12 cytokine ratio elicited by IMS1 and IMS2 the effects of these both strains on DC-dependent T cell responses were investigated *in vitro.* IMS1-treated MoDC orchestrated a T cell response dominated by enhanced amounts of IL-10 and low levels of IFN-γ. Effector T cells generated in response to IMS2-triggered MoDC secreted predominantly IFN-γ. Of note, IL-10 induction in naïve T cells by IMS1 was even higher than the produced IL-10 of generated Tregs. These IL-10-inducing effects were less pronounced in the stationary phase compared to mid-log phase cultures. MoDC triggered with IMS2 from the stationary phase still promoted strong IFN-γ secretion. These results suggest IMS1 as an imunoregulatory strain that is able to instruct a regulatory T cell phenotype underlined by the enhanced IL-10/IL-12 ratio. This effect is more prominent with bacterial cultures from the mid-log growth phase. In contrast, IMS2 is capable of inducing a more inflammatory Th1 response that is attributed to its strong IL-12 induction in DCs. The production of effector cytokines from Th17 and Th2 cells were also assessed but there were no amounts of IL-17 and IL-4 detectable in cocultures. This suggests that IMS1 and IMS2 do not promote the conversion of naïve T cells into Th17 or Th2 cells.

Regulatory T cells are suppressive and important for immune homeostasis controlling adaptive immune responses. Several functional phenotypes of Tregs have been described including $CD4^+CD25^+$high $Foxp3^+$ cells, Th3 and Tr1 cells (47, 51, 58, 100). Foxp3 as key marker for regulatory T cells is not sufficient enough as Foxp3 is also upregulated in TCR-activated $CD4^+CD25^-$ cells without suppressive function (847, 848). So the expression of further Treg marker such as CD4 and CD25 were investigated. The regulatory T cell type driven by IMS1-matured MoDC could be further confirmed by an increased percentage of IL-10 producing T cells expressing CD4, CD25 and Foxp3 in comparison to IMS2-promoted T cells. Other surface markers induced by these bacterial strains that are characteristic for Tregs such as CD39, CTLA4, CD127 and GITR (64, 65, 68, 81) could provide more insight into the developing Tregs in response to these bacterial strains. Also of interest is the analysis of TGF-β production by bacteria-polarized T cells as one direct mechanism by which Tregs exert their suppression is the release of IL-10 and TGF-β (71). But amounts of TGF-β in the coculture

couldn't be investigated so far due to technical problems with the commercial available ELISA.

Again, these date underline the strain-specific effects of different LAB concerning Th1 and Treg induction that is in concordance with numerous studies showing that LAB are able to promote Th1 functions (408, 418, 420, 849, 850) or Tregs (118, 454). It has also been reported that LAB induce a hyporesponsivness in $CD4^+$ Tcells displaying suppressor function (405, 458). To proof the suppressive functionality of differentiated Tregs in response to IMS1 and IMS2 *in vitro* suppression assays were established showing that T cells induced by IMS1 displayed suppressive functions by an enhanced inhibition of the proliferation of $CD4^+$ responder T cells compared to IMS2-promoted T cells. It remains to be identified how these newly generated suppressor T cells exert their suppression. In the suppression assay the supernatant as well as the cell suspension of the ASA were tested in the suppression assay. Interestingly, analysis of IL-10 containing supernatant concerning its capacity to suppress the proliferation of $CD4^+$ responder cells did not yield acceptable results (data not shown) suggesting that bacteria-primed T cells suppress rather in a cell-cell dependent manner than IL-10 dependent. It remains to be explored if neutralizing IL-10 in the suppression assay would restore the proliferative capacity of responder cells coincubated with bacteria-primed T cells.

In summary IMS1 induces high amounts of IL-10 and decreased levels of IL-12p70 in human healthy MoDC whereas IMS2 is more potent to promote high amounts of IL-12p70 in MoDC. IMS1-primed MoDC lead to the differentiation of phenotypically and functionally tested Tregs that produce high amounts of IL-10 and decreased amounts of IFN-γ and were able to inhibit the proliferation of $CD4^+$ T cells. These immunoregulatory characteristics of IMS1, in particular its mechanism of action, have to be further investigated *in vitro* and *in vivo*. To explore the effects of IMS1 on innate and adaptive immunity in an intestinal environment experiments with human and mice epithelium models need to be performed. Moreover it is of interest if IMS1 is capable of inducing mucosal tolerance in mice models and can promote protection against colitis or allergy. Regarding the influence of IMS1 and IMS2 on DC function in allergy DC stimulation experiments with these bacteria in allergic subjects would provide useful information.

A further aspect is the combination of both strains with prebiotics that are defined as a selectively fermented ingredient that allows specific changes, both in the composition

and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health (851). Here, it needs to be investigated if IMS1 and IMS2 could have additive or synergistic effects in combination with oligosaccharides as prebiotics. Another point would be the role of IMS1 and IMS2 in affecting skin immunity, therefore *in vitro* studies with human keratinocytes and *in vivo* skinprick test studies with healthy, allergic and *Candida*-infected subjects would provide more insight into immunological effects of these bacterial strains. The anti-inflammatory characteristics of IMS1 and the pro-inflammatory effects of IMS1 present new strageties for the therapeutical treatment of many diseases including skin diseases, *Candida*-infections or allergy and open new aspects for the application of these strains in nutrition. Finally for this purpose studies on fulfilling the criteria of probiotics for example antibiotic or gastric acid resistance remain to be performed.

6 Zusammenfassung

Diese Arbeit charakterisiert T-Zellantworten, die durch Mikroorganismen wie Hefepilze oder Milchsäurebakterien induziert werden. Diese Mikroorganismen aktivieren das innate Immunsystem des Wirts, gefolgt von Reaktionen des adaptiven Immunsystems einschließlich T-Zellen, die entweder Resistenz oder Toleranz zur Folge haben. Sind diese Mechanismen dereguliert, können Infektionen entstehen. T-Zellantworten, die durch Hefepilze wie *Candida albicans* ausgelöst werden, wurden anhand eines komplexen Krankheitsmodells wie der chronisch mukokutanen Candidose (CMC) untersucht. Ebenso wurden T-Zellen, die durch Milchsäurebakterien aktiviert werden, in gesunden Probanden näher beleuchtet.

Die Untersuchung von CMC Patienten einschließlich Patienten mit isolierter CMC (iCMC) und Patienten mit dem Autoimmun-Polyendokrinopathie-Syndrom Typ I (APS1), die an chronischen oder immer wiederkehrenden *Candida*-Infektionen leiden, ergab, dass diese Patienten einen Th17-Defekt aufweisen, der anhand einer IL-17/IL-22 Defizienz in peripher mononuklearen Zellen (PBMCs) nachgewiesen wurde. Auf Antigen-präsentierender Seite konnte gezeigt werden, dass dendritische Zellen (DCs) von Patienten mit iCMC nach Stimulation mit *Candida*-Zellwandbestandteilen ein verändertes Zytokin-Profil aufweisen und diese DCs vermindert IL-17 und IL-22 in naiven T-Zellen induzieren. Auf T-Zell-Ebene stellte sich ebenso heraus, dass eine Th17-Differenzierung aus naiven T-Zellen sowohl bei iCMC als auch bei APS1 Patienten nicht möglich war, während eine Th1- und Th2-Polarisierung festgestellt werden konnte. Zusätzlich konnte eine „gain-of-function“ Mutation des signal transducer and activator of transcription (*STAT*)*1* Gens bei iCMC Patienten aufgezeigt werden, das mit einer verstärkten DNA-Bindekapizät einherging und mit der defekten Generierung von Th17 Zellen in Zusammenhang stehen kann. Eine Genom-Analyse von stimulierten T-Zellen ergab, dass einige STAT1-assoziierten Gene bei iCMC Patienten verstärkt exprimiert waren. Diese beobachteten Defekte, die bei einer fehlregulierten Immunantwort gegen Hefepilze wie *Candida albicans* auftreten können, spielen eine wichtige Rolle bei der Pathogenese von CMC und können zur Entwicklung neuer Behandlungsstrategien beitragen.

Die Analyse der innaten Immunantwort in gesunden Probanden, ausgelöst durch zwei verschiedene Milchsäurebakterien-Stämme IMS1 und IMS2 (IMS=immune-modulatory

strain) ergab, dass DCs, die mit IMS1 stimuliert wurden, im Vergleich zu IMS2 vermehrt IL-10 und vermindert IL-12p70 freisetzen. Von Interesse war daher, welche T-Zellantworten durch diese beiden Stämme IMS1, der sich durch einen erhöhten IL-10/IL-12 Quotienten auszeichnet, und IMS2, der eher proinflammtorische Kapaziäten aufweist, induziert werden. Die Kokultivierung von naiven T-Zellen mit IMS1-stimulierten MoDC führte im Vergleich zu IMS2-gereiften MoDC zu einem erhöhten Anstieg der IL-10 Freisetzung und einer erniedrigten IFN-γ Produktion. Diese IMS1-induzierten T-Zellen konnten dann anhand eines erhöhten Anteils an IL-10 positiven $CD4^+CD25^+Foxp3^+$ Zellen eher einem regulatorischen T-Zelltyp zugeordnet werden im Vergleich zu IMS2-instruierten T-Zellen, die durch eine vermehrte IFN-γ-Sekretion charakterisiert waren. Somit zeigte IMS1 immun-modulatorische Kapazitäten *in vitro* und kann als möglicher gesundheitsfördernder Bakterienstamm bezeichnet werden. Weitere *in vitro* Untersuchungen sind nötig, ebenso eine Analyse von *in vivo* Effekten.

In dieser Arbeit konnte gezeigt werden, dass neben einem defekten innaten Immunsystem Th17-Zellen bei deregulierten Pilzinfektionen wie der chronisch mukokutanen Candidose, eine essentielle Rolle spielen und ein Defekt dieses T-Zelltyps dazu beitragen kann, dass Pilzinfektionen wie *Candida albicans* nicht mehr ausreichend abgewehrt und chronisch werden. Ebenso wurde nachgewiesen, dass ausgewählte Milchsäurebakterien-Stämme unterschiedliche T-Zelltypen induzieren, wie die eines regulatorischen T-Zelltyps, der hinsichtlich verschiedener Krankheiten wie Allergien und Hauterkrankungen sowie der Verarbeitung in probiotischen Produkten von besonderem Interesse ist.

7 Summary

This work characterizes T cell responses induced by microorganisms such as fungi or lactic acid bacteria. The human innate immune system is activated by these microorganisms and leads to activation of the adaptive immune system including T cells followed by resistance or tolerance. In the case of deregulation of these mechanisms infections develop. T cell responses promoted by pathogenic yeast such as *Candida albicans* were demonstrated in the complex and heterogeneous disease model of chronic mucocutaneus candidiasis (CMC). Moreover T cells induced by lactic acid bacteria were characterized in healthy volunteers.

The analysis of CMC patients including patients with isolated CMC (iCMC) and patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APS1), suffering from chronic or recurrent infections with *Candida albicans* revealed that these patients are characterized by defects of Th17 cells due to the observed IL-17/IL-22 deficiency in PBMCs of these patients. On the level of antigen presenting cells dendritic cells (DCs) of iCMC patients showed an impaired cytokine profile after stimulation with *Candida* cell wall compounds followed by an insufficient IL-17/IL-22 induction in naïve T cells by these DCs. On the T cell level it could be shown that naïve T cells of both iCMC and APS1 patients were not able to differentiate to Th17 cells while a Th1 and Th2 polarization was possible. Moreover "gain-of-function" mutations of the signal transducer and activator of transcription (*STAT*)*1* gene were identified in iCMC patients accompanied by an enhanced DNA binding activity of STAT1 and these observed effects are proposed to be associated with the analyzed Th17 defect. Whole genome analysis of stimulated T cells revealed an enhanced upregulation of several STAT1-assoiated genes in T cells of iCMC patients. Further investigation is necessary to find the association between the observed immunological and molecular defects but the data of CMC patients in this work contribute to a better understanding of this complex and heterogeneous disease and may offer insights in new therapy strategies.

The analysis of innate immune response in healthy volunteers induced by the two lactic acid bateria strains IMS1 and IMS2 revealed that dendritic cells (DCs) stimulated with IMS2 produced enhanced amounts of IL-10 compared to IMS2 whereas IMS2-triggered DCs were characterized by an increased IL-12 secretion. Therefore T cell responses promoted by IMS1-characterized by a high IL.10/IL-12 ratio and IMS2- characterized

by pro-inflammatory capacities were of interest. Cocultivation of naïve T cells with IMS1-matured DCs resulted in increased amounts of IL-10 and decreased levels of IFN-γ whereas T cells promoted by IMS2-matured DCs secreted enhanced amounts of IFN-γ. T cells induced by IMS1 could be further characterized as regulatory T cells due to the increased percentage of IL-10 producing $CD4^+CD25^+Foxp3^+$ cells that were functional suppressive compared to IMS2-instructed T cells. Thus, IMS1 shows immunemodulatory capacities *in vitro* and might be considered as health-promoting probiotic strain for the use in food or treatment of different diseases including skin diseases, *Candida*-infections or allergy. Further *in vitro* and *in vivo* studies are necessary to support these data as well as detailed analysis of the different strains to better understand the observed immune regulations of human DCs and T cells.

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