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Identification and characterization of new factors regulating the phenotype of Th17 and Th22 cells

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Abstract

CD4$^+$ T helper (Th) cell subsets are crucial for efficient adaptive immune responses, but if dysregulated contribute to different autoimmune and inflammatory diseases. Distinct T cell subsets are associated with different diseases. Th17 cells represent an important mediator of the inflammatory skin disease psoriasis, while Th22 cells accumulate in affected skin areas of atopic eczema patients during the chronic phase. For a comprehensive understanding of disease pathogenesis, profound knowledge of the differentiation and regulation of the involved Th subsets in humans is a prerequisite. This work investigated a potential impact of two novel factors - Leucine-rich repeat and immunoglobulin-like domain containing nogo-receptor 4 (LINGO4) and Forkhead box protein O4 (FOXO4) - on the immunobiology of Th17 and Th22 cells.

So far, LINGO4 is a protein with unknown function in the immune system. In whole genome expression analysis elevated *LINGO4* mRNA levels were detected in human Th17 clones compared to other T cell subsets leading to the assumption that LINGO4 might play a regulatory role in this Th subset. Further, *in vitro* differentiated Th17 cells showed increased *LINGO4* expression in comparison to Th1, Th2 and Th22 differentiated cells. Kinetics of *in vitro* differentiated Th17 cells revealed a strong correlation between relative mRNA expression levels of *LINGO4* and *RORC2*, the key transcription factor of Th17 cells. While naive T cells suppressed the lentiviral-mediated overexpression of LINGO4 protein, lentiviral-mediated knockdown of LINGO4 with small hairpin (sh) RNA in naive T cells followed by Th17 differentiation resulted in decreased *RORC2* and *IL-17A* mRNA levels. While IL-17A protein production was not altered, these cells showed increased *RORA* mRNA expression suggesting a compensatory mechanism mediated by RORA that in turn maintains IL-17A protein secretion and indicating LINGO4 as a positive regulator of RORC2. Elevated *LINGO4* mRNA expression in PBMCs and lesional skin of psoriasis patients further point out to a potential regulatory role of LINGO4 in Th17 cells.

FOXO4 is a transcription factor that was found to be associated with Th22 cells when this subset was newly discovered. FOXO4 expression was induced in naive T cells already a few hours after the initiation of *in vitro* differentiation towards Th22 cells. Knockdown of FOXO4 in effector T cells with sh RNA led to reduction of secreted IL-22, whereas signature cytokines of other subsets were not altered. Lentiviral overexpression in naive T cells followed by *in vitro* Th22 differentiation significantly induced IL-22 production, but not other secreted cytokines.

In summary, this study identified a regulatory role for both LINGO4 and FOXO4 in Th17 and Th22 cells, respectively. While LINGO4 represents a potential positive regulator of RORC2, FOXO4 is involved in the regulation of IL-22 production. However, detailed mechanistic studies are needed to obtain detailed insight into the role of both factors in the complex regulation of T cell phenotypes and to understand their contribution to the pathogenesis of T cell-mediated diseases such as psoriasis and atopic eczema. Prerequisites for success, however, is the availability of a specific antibody for LINGO4 detection and the identification of a specific inducer of FOXO4 in T cells.

Zusammenfassung

CD4$^+$ T-Helferzellen (Th-Zellen) spielen eine wichtige Rolle in der adaptiven Immunantwort. Sie können bei einer Fehlregulation jedoch zur Ausbildung von Autoimmunerkrankungen oder Fehlfunktionen des Immunsystems führen. Die verschiedenen Th-Zellsubtypen sind mit verschiedenen Erkrankungen assoziiert. Th17-Zellen gelten beispielsweise als Hauptmediatoren von Psoriasis, einer chronisch-entzündlichen Hauterkrankung. Th22-Zellen sind hingegen in der chronischen Phase des atopischen Ekzems in Hautläsionen zu finden. Zum genauen Verständnis der Pathogenese dieser Erkrankungen ist es wichtig, auch die Biologie der beteiligten Th-Zellsubtypen detailliert zu verstehen. Der Fokus liegt dabei auf dem Prozess der Differenzierung und Regulation dieser relativ neu beschriebenen Th-Zellsubtypen. In dieser Arbeit wurde der Einfluss von zwei neu identifizierten Faktoren, LINGO4 (Leucine-rich repeat and Immunoglobulin-like domain containing nogo receptor 4) und FOXO4 (Forkhead Box Protein O4), in der Phänotypregulation von Th17- und Th22-Zellen untersucht.
LINGO4 ist ein Protein mit bisher unbekannter Funktion im Immunsystem. In genomweiten Genexpressionsanalysen von humanen T-Zellklonen konnte eine signifikant erhöhte Expression in Th17-Zellen gezeigt werden. Eine erhöhte *LINGO4* mRNA Expression konnte auch in *in vitro* differenzierten Th17-Zellen im Vergleich zu Th1-, Th2- und Th22-Zellen nachgewiesen werden. Des Weiteren zeigte sich während der *in vitro* Differenzierung von Th17-Zellen eine starke Korrelation zwischen den relativen Expressionen von *LINGO4* und *RORC2* mRNA, dem Haupttranskriptionsfaktor dieses Th-Zellsubtyps. Während die lentivirale Proteinüberexpression von LINGO4 in CD4$^+$ naiven T-Zellen supprimiert wurde, konnten funktionelle Schlüsse aus dem lentiviralen LINGO4-Knockdown durch small hairpin (sh) RNA gezogen werden. Naive T-Zellen wurden im Anschluss an den lentiviral vermittelten Knockdown durch sh RNA *in vitro* zu Th17-Zellen differenziert. Der LINGO4-Knockdown führte zu einer Reduktion der *RORC2* und *IL-17A* Expression auf mRNA Ebene. Während die Menge an sekretiertem IL-17A Protein unbeeinflusst blieb, wurde *RORA* mRNA hochreguliert. Dies ließ zum einen auf eine regulatorische Rolle von LINGO4 in der Th17 Differenzierung schließen, möglicherweise als positiver Regulator von RORC2, deutete gleichzeitig aber auch auf einen möglichen kompensatorischen Effekt von RORA auf die IL-17A Produktion hin. Darüber hinaus unterstreicht die erhöhte *LINGO4* mRNA Expression in Hautläsionen und PBMCs von Psoriasis Patienten eine mÃűgliche regulatorische Rolle von LINGO4 in Th17-Zellen.
FOXO4 ist ein Transkriptionsfaktor, der bereits im Rahmen der Identifizierung von Th22-Zellen mit diesen assoziiert wurde. Bereits wenige Stunden nach Beginn der Differenzierung naiver T-Zellen zu Th22-Zellen wurde die Expression von FOXO4 induziert. Der Knockdown von FOXO4 in Effektor T-Zellen durch sh RNA bewirkte eine Reduktion der IL-22 Sekretion, während die Leitzytokine anderer Th-Zellsubtypen nicht beeinflusst wurden. Wurde FOXO4 in naiven T-Zellen lentiviral überexprimiert und diese im Anschluss zu Th22-Zellen differenziert, führte dies zu einem signifikanten Anstieg der IL-22 Sekretion, nicht aber anderer sezernierter Zytokine.
Zusammenfassend konnte sowohl für LINGO4 als auch für FOXO4 eine regulatorische Rolle

in Th17- bzw. Th22-Zellen nachgewiesen werden. Während LINGO4 möglicherweise einen positiven Regulator für RORC2 darstellt, ist FOXO4 eingebunden in die Regulation der IL-22 Expression in T-Zellen. Weiterführende mechanistische Studien sind aber nötig, um genaue Einblicke in die Rolle beider Faktoren in der komplexen Regulation von T-Zellphänotypen tiefgreifend zu evaluieren und den Beitrag zur Pathogenese T-Zell-vermittelter Erkrankungen, wie z.B. der Psoriasis oder des atopischen Ekzems, zu klären. Voraussetzung hierfür ist jedoch die Verfügbarkeit eines spezifischen Antikörpers zur Detektion von LINGO4 und die Identifizierung spezifischer FOXO4-Aktivatoren.

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List of Abbrevations

Abbrevation	**Description**
Å	angstrom
Ab	antibody
ACN	acetonitrile
AE	atopic eczema
AHR	aryl-hydrocarbon receptor
AP-1	adaptor-related protein complex I
APC	antigen-presenting cell
BATF	basic leucine zipper transcription factor
BCA	bicinchoninic acid assay
BCL-6	B cell lymphoma 6 protein
BCR	B cell receptor
bp	base pair
°C	degree celsius
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CD40L	CD40 ligand
CLA	cutaneous lymphocyte antigen
CLP	common lymphoid progenitor
c-MAF	transcription factor MAF
CMV	cytomegalovirus
CTL	cytotoxic T lymphocytes
ctrl	control
d	days
DAG	diacylglycerol
DAMP	damage-associated molecular pattern
DAPI	4’,6-Diamidin-2-phenylindol
DC	dendritic cell
DDA	data-dependent acquisition
denat	denaturation
DEPC	diethyl pyrocarbonate
DIA	data-independent acquisition

DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxid
DN	double-negative
DP	double-positive
DTT	dithiothreitol
EAACI	European Academy of Allergy and Clinical Immunology
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EF1a	elongation factor 1-alpha
e.g.	exempli gratia
ER	endoplasmatic reticulum
ERA	endoplasmatic reticulum-associated pathway
FACS	fluorescence-activated cell sorting
Fas	type-II transmembrane protein
FASP	filter-aided sample preparation
FCS	fetal calf serum
FDR	false-discovery rate
FICZ	tryptophan photoproduct 6-formylindolo[3,2-b]carbazole
FOXO	forkhead box protein 0
FOXP	forkhead box protein P
fw	forward
GATA3	GATA-binding protein 3
gDNA	genomic DNA
h	hours
HCV	hepatitis c virus
HeLa cells	human immortal cell line derived from cervical cancer cells of Henrietta Lacks
HER	human epidermal growth factor receptor 2
HET	heterozygous
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HPLC	high-performance liquid chromatography
HS	human serum
IAA	iodoacetamide
i.e.	id est
IFN	interferon
Ig	immunoglobulin
IL	interleukin

ILC	innate lymphoid cell
IL-10R	Interleukin-10 receptor
IL-17RA	Interleukin-17 receptor A
IL-22R	Interleukin-22 receptor
IP	immunoprecipitation
IP3	inositol 1,4,5-triphosphate
IRF4	interferon response factor 4
iRT	indexed retention time
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
kb	kilo base
KD	knockdown
kDa	kilo Dalton
KO	knock-out
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
lcn	long non-coding
LINGO4	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 4
LOXL3	lysyl oxidase homolog 3
LRR	leucine-rich repeat
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic-activated cell sorting
MAI	myelin-associated inhibitory proteins
MHC	major histocompatibility complex
min	minute
MG-132	*N*-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucina
mod	modified
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
m/z	mass to charge ratio
nc	non-coding
neg	negative
NFAT	nuclear factor of activating T cells
NFκB	nuclear factor of kappa light chain polypeptide gene enhancer B cells 1
NK cell	natural killer cell
NKT cell	natural killer T cell

NLK	nemo-like kinase
NLRP3	nucleotide-binding domain, leucine-rich repeat containing protein family, pyrin domain containing
OE	overexpression
pAb	polyclonal antibody
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS^{def}	phosphate buffered saline without Ca^{2+} and Mg^{2+}
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGRN	progranulin
PHA	phythemagglutinin
PIP2	phosphatidylinositol 4,5-biphosphate
PKB	protein kinase B
PLC-γ	phospholipase C-gamma
PMSF	phenylmethylsulfonylfluorid
pos	positive
PRR	pathogen-recognition receptor
PU.1	transcription factor, also called spleen focus forming virus proviral integration protein (Spi1)
P/S	penicillin/streptomycin
PVDF	polyvinylidene fluoride
RA	rheumatoid arthritis
rev	reverse
rIL17A	recombinant IL-17A
RIPA	radioimmunoprecipitation assay
ROR	retinoic acid-related orphan nuclear receptor
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (Medium)
RT	room temperature
RSLC	rapid separation liquid chromatography
RUNX1	Runt-related transcription factor
Rxn	reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis

SI	small intestine
SNP	single nucleotide polymorphism
SP	single-positive
STAT	signal transducer and activator of transcription
TBET	T-box transcription factor TBX21
TBST	Tris-buffered saline with Tween20
TCR	T cell receptor
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TFA	triflouroacetic acid
Tfh cell	follicular helper T cell
Th cell	T helper cell
TMB	3,3‘,5,5‘-Tetramethylbenzidine
TNF	tumor necrosis factor
Treg cell	regulatory T cell
U	enzyme unit
UPR	unfolded protein response
V	volt
w/o	without
WB	western blot
XIC	extracted ion chromatogram

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1. Introduction

1.1 The immune system - an overview

The human immune system protects the body against infections caused by viruses, bacteria, funghi and parasites but also environmental harmful factors like toxins. The three main functions of the immune system are: 1. Recognition of invading pathogens; 2. Raising the most efficient and appropriate immune responses for pathogen elimination; and 3. Developing an immunological memory to establish faster immune responses to the same antigen at repeated exposure [Kabelitz, 2011, Abbas et al., 2014, Murphy and Weaver, 2014, Atenhan, 2014]. The immune system is divided into the innate and the adaptive branch (Fig 1). Innate and adaptive immune responses are distinguished based on involved cells and the velocity of an immune response [Abbas et al., 2014, Murphy and Weaver, 2014, Atenhan, 2014].

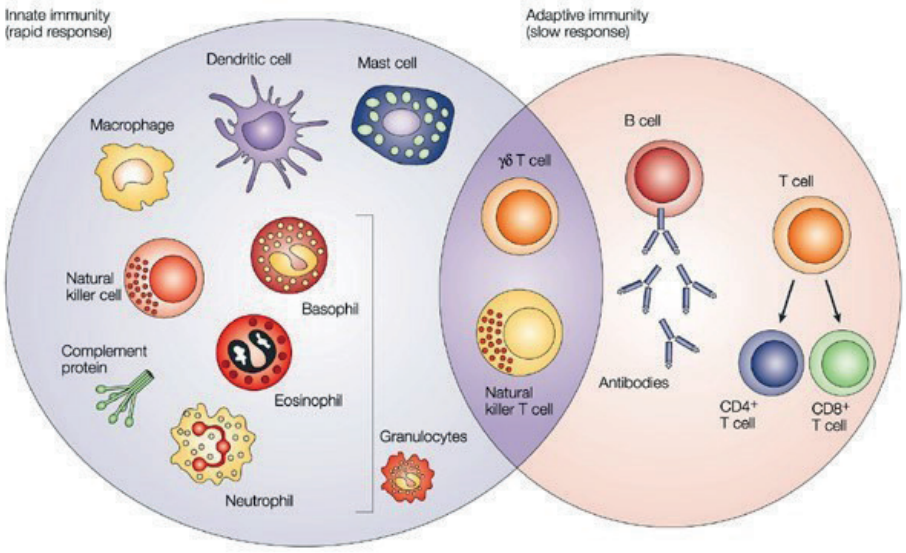


Figure 1 Different cell types of the immune system [Dranoff, 2004].

1.1.1 The innate immune system

The innate immune system provides the first and immediate response to external harm [Janeway and Medzhitov, 2002]. It recognizes pathogens or harmful substances, eliminates them and recruits other immune cells to the site of infection. Other immune cells are attracted by mediators (cytokines and chemokines) and specialized cells that present the antigen activate the adaptive immune system.

The innate immune system consists of cellular and non-cellular, so-called humoral components. The epithelium is the first barrier for pathogens that enter the body and represents

not only a physical barrier but also a chemical one by secreting anti-microbial enzymes and peptides (e.g., defensins). Another humoral component is the complement system consisting of a collection of soluble proteins present in the blood and other body fluids. The main function of the complement is the opsonization of pathogens. It facilitates phagocytic uptake and therefore elimination of pathogens or toxins. Three different complement pathways are distinguished according to their mode of activation: The classic, alternative and the lectin pathway - all resulting in pathogen elimination [Nesargikar et al., 2012, Murphy and Weaver, 2014].

The cellular response of the innate immune system is mediated by phagocytes that include different cell types like macrophages, granulocytes, mast cells and dendritic cells (DCs) (Fig 1). When pathogens pass the physical barrier, cells present in the surrounding tissue are activated. However, the reaction is limited to distinct structures recognized by receptors called PRRs (Pathogen-Recognition Receptors), expressed extra- or intracellularly by innate immune cells. These receptors recognize only general common features of pathogens summarized as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). These include microbial nucleic acids, carbohydrates or lipoproteins and are released by damaged cells [Janeway and Medzhitov, 2002]. Therefore, contrary to the adaptive immune system, innate immune responses are not specific to pathogens, although in most cases they are sufficient for their elimination; if not, the adaptive immune system is activated and takes over.

The cellular component of the innate immune system consists of different cell types. Macrophages, granulocytes and dendritic cells share the important feature of phagocytosis to eliminate pathogens, but differ in their specific function and ability. Macrophages are matured tissue-resident monocytes and engulf and kill pathogens by phagocytosis [Aderem and Underhill, 1999]. Further, they activate other immune cells by cytokine secretion. Granulocytes, among them neutrophils, eosinophils and basophils, destroy microorganisms in intracellular vesicles with degradative enzymes and antibacterial substances. Dendritic cells (DCs) use, aside from phagocytosis, also macropinocytosis to search for infectious agents in extracellular fluids. They are specialized in antigen presentation and therefore represent the most important antigen-presenting cell (APC) for T cell activation. DCs process ingested antigens and present the processed peptides on their surface bound to major histocompatibility complex (MHC) molecules that can bind to corresponding antigen-specific T cell receptors. This interaction results in T cell activation and therefore initiation of adaptive immunity. Consequently, DCs represent a crucial link between innate and adaptive immunity. Contrary to this, mast cells eliminate pathogens by releasing cytotoxic substances from their large granules into the environment. Furthermore, they secrete cytokines to attract more immune cells and start an inflammatory cascade.

Innate lymphoid cells (ILCs) are a new class of innate immune cells that were recently discovered; they mirror T cell functions without being antigen-specific [Neill et al., 2010, Mjoesberg

et al., 2011]. ILCs are classified into different subtypes based on their cytokine and transcription factor profiles that resemble the classification of T helper cells. They were shown to play a role in lymphoid organogenesis, inflammation and moreover also tissue remodelling [Yang et al., 2017]. One example of ILCs are natural killer (NK) cells that are important for elimination of abnormal cells, e.g., tumor cells [Robinette et al., 2015].

Natural killer T (NKT) cells and $\gamma\delta$ T cells are classified at the interface between innate and adaptive immunity [Murphy and Weaver, 2014, Atenhan, 2014]. $\gamma\delta$ T cells represent a minority of T cells, which classically are part of the adaptive immune system, and express a $\gamma\delta$ T cell receptor with limited antigen-specificity. Contrary to $\alpha\beta$ T cells, $\gamma\delta$ T cells mainly recognize lipid antigens with their invariant receptor, a reason why they are functionally positioned at the interface between innate and adaptive immunity [Murphy and Weaver, 2014, Atenhan, 2014, Morita et al., 1995].

1.1.2 The adaptive immune system

The adaptive immune system is the second line of defense. Even though it is slower, it is a more specific and efficient immune response to invading pathogens. It is based on antigen-specific receptors of lymphocytes and initiated by the interaction with antigen-presenting cells. Adaptive immunity is divided into humoral and cellular mediated responses, represented by B and T cells as cellular components and antibodies as humoral factors. Both cell lineages originate from hematopoietic stem cells in the bone marrow and express specific B cell receptors (BCRs) and T cell receptors (TCRs), respectively, on their surface. BCRs and TCRs consist of a constant and a variable domain, which are encoded by different gene segments. During the recombination process different gene segments are joint leading to the high diversity of around 10^6 different antigen-specific receptors [Boehm and Swann, 2014]. This process is known as V(D)J-rearrangement and assures the high diversity in receptor specificity. Receptors against self-peptides are formed as well during this process and cells carrying a receptor recognizing those auto-antigens have to be excluded in a selection process during maturation and differentiation. However, failures in this selection process can result in autoimmune diseases or immune disorders, as cells with receptors directed against self-peptides or harmless structures cause misdirected immune reactions [Klein et al., 2014].

Humoral immune responses involve the secretion of antibodies by B cells. Upon activation by specific antigens, B cells undergo maturation into plasma cells. The maturation process includes clonal expansion and the secretion of the previously surface-bound antigen-specific receptors as antibodies. The pathogen or substance responsible for the induction is eliminated by neutralization, opsonization or complement activation. Different isotypes of antibodies exist fulfilling distinct functions. B cells can undergo class switch, meaning they change the isotype of immunoglobulins (Igs) expressed, depending on the elimination task. This switch only happens in the constant region, leaving the antibody specificity unaffected [Rudin and Thompson, 1998]. Cellular mediated immune responses mainly rely on cell-bound TCRs

on T cells. T cell receptors cannot directly recognize antigens, they depend on APCs. The antigen must be presented bound to MHCs on the surface by APCs. Two different antigen-presenting MHC classes are distinguished, MHC I and MHC II, activating different subtypes of T cells, $CD4^+$ or $CD8^+$ T cells, respectively [Smith-Garvin and Koretzky, 2009, Murphy and Weaver, 2014].

One of the most important feature of the adaptive immune system is the formation of an immunological memory, enabling a quick immune response at repeated exposure to the same pathogen. Immunological memory is mediated by memory B and T cells. These are long-lasting cells that do not die after a successful immune response but remain in the body for several years up to a lifetime, providing a fast and efficient secondary immune response [Farber et al., 2016].

1.2 T cells

As previously mentioned, T cells represent one cellular component of adaptive immunity. They originate from hematopoietic stem cells in the bone marrow but mature in the thymus. In general, all T cells express the surface marker CD3 in addition to the antigen-specific TCR. Based on the expression of their co-receptor, they are classified into $CD4^+$ T helper (Th) cells or $CD8^+$ cytotoxic T cells (CTLs). These T cell types differ not only in expression of the co-receptors CD4 and CD8, but also in mediated immune responses [Klein et al., 2014, Abbas et al., 2014].

The class of $CD8^+$ cytotoxic T cells possesses the ability to directly kill virus infected or tumor cells. Upon activation, they release the cytotoxic molecules granzyme B and perforin and can induce apoptosis by the interaction of Fas with Fas ligand. Cytotoxic T cells interact via their CD8 receptor only with APCs and tissue cells presenting antigens bound to MHC class I molecules. Peptides presented on MHC class I molecules are mainly of intracellular origin, like viruses and own peptides, explaining the function of $CD8^+$ T cells in virus and cancer defense [Andersen et al., 2006].

The class of $CD4^+$ T helper cells plays a more indirect role by mediating activation and recruitment of innate and adaptive immune cells to clear infections. They recognize antigens bound to MHC class II molecules on APCs which mainly originate from extracellular pathogens [Murphy and Weaver, 2014, Abbas et al., 2014].

1.2.1 Maturation of T cells and induction of immunological tolerance

Immunological tolerance is an important feature of the immune system. It prevents formation of self-specific T and B cells and, therefore, over-reactive immune responses against self-antigens, the cause of autoimmune diseases. Two types of tolerance are distinguished based

on the site they are acquired: central and peripheral. Central tolerance is formed in the primary lymphoid tissues of bone marrow and thymus during maturation, while acquisition of peripheral tolerance takes place in secondary lymphoid organs like lymph nodes, spleen and other organs [Abbas et al., 2014, Murphy and Weaver, 2014].

T cells develop from hematopoietic stem cells that evolve to common lymphoid progenitors (CLPs), giving rise to B and T cells. While B cells arise from CLPs in the bone marrow, T cells develop from T cell progenitors in the thymus, the site where central T cell tolerance is induced. This process is regulated by the expression of the surface marker Notch1 and the transcription factor GATA3, determining the fate of the T cell precursor [Koch and Radtke, 2011, Haller et al., 2012]. T cell precursors arriving in the thymus are called thymocytes and are double-negative (DN), neither expressing CD3, CD4 nor CD8. Those pro-T cells further show expression of either $\alpha\beta$ or $\gamma\delta$ TCRs after TCR rearrangement, a process controlled by RAG-1 and RAG-2 proteins. $\alpha\beta$ T cells mature into pre-T cells with a full T cell receptor complex, including CD3 and double-positive (DP) expression of CD4 and CD8. DP T cells then pass a first selection process to induce central tolerance. Epithelial cells present self-antigens via MHC molecules. Pre-T cells recognizing them with low affinity are positively selected, surviving due to the resulting TCR signaling. They mature to single-positive (SP) $CD4^+$ or $CD8^+$ T cells, depending on which MHC molecule they bound to. At the same time, pre-T cells that bind self-peptides with a high affinity are depleted by negative selection as they undergo TCR-induced apoptosis to prevent autoimmune reactions [Sprent and Kishimoto, 2001, Ciofani and Zúñiga-Pflücker, 2007].

Peripheral tolerance is necessary because not all self-peptides are presented in the thymus. T cells that passed this selection process leave the thymus as naive, antigen-unexperienced T cells. Naive T cells circulate through the secondary lymphoid organs where they are exposed to other self-components they might mistakingly take as harmful. Different mechanisms mediate peripheral tolerance like anergy and T cell mediated tolerance. If T cells are activated by their TCR but without the required co-stimulatroy signals they get unreactive (anergic) and die [Schwartz, 1996]. Another mechanism is the induction of apoptosis mediated by Fas-signaling. This occurs when T cells are stimulated by their TCR over a long time to protect against an over-reactive immune response [Singer and Abbas, 1994].

Other components that keep the immune system in balance are $CD4^+$ regulatory T cells (Tregs). Those T cells are also educated in the thymus and recognize self-antigens. However, instead of reacting to them with initiating an immune response, they dampen down the reaction by secretion of regulatory cytokines like IL-10 or TGF-β [Sakaguchi et al., 2006].

1.2.2 T cell activation

In general, naive T cells require three signals for activation: the TCR-specific signal, a co-stimulatroy signal and signals mediated by the cytokine milieu [Goral, 2011]. After maturation

in the thymus, naive T cells (i.e., T cells without antigen-experience) circulate through the blood stream and secondary lymphoid organs. In lymph nodes, naive T cells meet APCs, in particular activated DCs that migrate from the peripheral tissues into the draining lymph node after antigen uptake, and get activated upon specific antigen recognition [Abbas et al., 2014, Murphy and Weaver, 2014, Croft, 1994].

The first activation step is based on the antigen-specificity. The TCR recognizes specifically the antigen-peptide that is processed and presented by APCs. TCRs form complexes with CD3, a receptor also used as common T cell marker. The CD3-TCR complex binds specifically to the MHC class-bound antigens, but with relatively low affinity [Smith-Garvin and Koretzky, 2009]. Affinity is increased by binding of the co-receptors CD4 and CD8 to MHC class II and MHC class I proteins, respectively, strengthening the interaction of APC and T cell [Koretzky, 2010]. However, TCR-binding to antigen is not sufficient for a proper T cell activation, co-stimulatroy signals are needed. Otherwise, T cells undergo anergy, a mechanism to prevent responses to self-antigens [Schwartz et al., 1989, Chen and Flies, 2013].

The second activation step depends on co-stimulatory signals. They are mainly mediated by CD28 on the surface of T cells binding to B7-family proteins like CD80 and CD86 expressed on APCs. Through T cell activation, the expression of CD40 ligand (CD40L) on T cells is induced, interacting with CD40 on APCs and further upregulating CD28 expression to increase T cell activation signaling [Murphy and Weaver, 2014, Chen and Flies, 2013].

The third activation step is mediated by the microenvironment, where the cytokine milieu is particularly important [Curtsinger and Mescher, 2011]. Upon recognition of MHC-bound antigen by a TCR, an immunological synapse forms between APC and T cell. The immunological synapse includes all important receptors and adhesion molecules participating in a successful T cell activation and provides an efficient interaction between these cells [Fooksman et al., 2010]. Effector T helper cells use the same principle to activate target cells [Grakoui et al., 1999].

Only when all these extracellular activation signals are present, signaling pathways in T cells are switched on. Signal transduction is mediated by the intracellular part of the TCR-CD3 complex via the sequence motifs of immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of their tyrosines by lymphocyte-specific protein tyrosine kinase (LCK) leads to further recruitment and activation of enzymes and proteins, resulting in phospholipase C-γ (PLC-γ) activation [Iwashima et al., 1994]. This enzyme cleaves phosphatidylinositol 4,5-biphosphate (PIP2) into inositol 1,4,5-triphospate (IP_3) and diacylglycerol (DAG). IP_3 and DAG lead to expression and activation of transcription factors like nuclear factor of kappa light chain polypeptide gene enhancer in B cells 1 (NF-κB) , adaptor-related protein complex I (AP-1) and nuclear factor of activated T cells (NFAT) [Crabtree and Olson, 2002, Johnson, 2002, Vallabhapurapu and Karin, 2009, Jordan et al., 2003]. Activation of these transcription factors further induces target genes for the expression of cytokines like IL-2, which is important for

T cell proliferation. Specific transcription factors are activated leading to differentiation of naive T cells into effector cells. For an efficient immune response, effector T cells undergo clonal expansion to fight invading pathogens [Malherbe et al., 2004, Murphy and Weaver, 2014]. Within this process, the signals of the cytokine milieu influence the differentiation fate of a T cell. These signals activate specific transcription factors that further induce according target genes, characterizing the subset that the T cell differentiates into [Murphy and Weaver, 2014].

1.2.3 T helper cell subsets

T helper cells are classified by expression of co-receptor CD4, recognize antigens bound to MHC class II and direct immune responses against extracellular pathogens and parasites. They represent a central cell part of the adaptive immunity as they are responsible for B cell activation (i.e., antibody production) and for recruitment and activation of innate immune cells (e.g., macrophages and neutrophils) to establish an efficient immune response [Murphy and Weaver, 2014, Abbas et al., 2014, Zhu and Paul, 2009]. However, dysregulation in the T helper cell compartment can lead to autoimmune and inflammatory diseases. These immune disorders develop when T cell specificity is directed against self-antigens or harmless environmental antigens and activation cannot be controlled anymore. Examples of T helper cell mediated diseases are: allergic asthma, psoriasis and multiple sclerosis [Lafaille, 1998, Pène et al., 2008].

T helper cells are further classified into subsets with different functions based on transcription factor, chemokine and cytokine expression [Mosmann et al., 1986, Zhou et al., 2009a]. The fate of a naive $CD4^+$ T cell after specific antigen recognition is strongly dependent on the cytokine microenvironment that decides into which Th subset the cell differentiates [Ashkar, 2000, Zhu and Paul, 2010, Tao et al., 1997]. In 1978, Th1 and Th2 were the first T helper subsets described [Tada et al., 1978]. However, Th1 and Th2 cells cannot explain all observed phenotypes and functions of the T helper cell population and the discovery of more T helper subsets overcame the paradigm of Th1/Th2 cells. Additional subsets have been identified named as: Th17, Th22, Th9, follicular helper T cells (Tfh) and regulatory T cells (Tregs) (Fig 2). Their detailed differentiation process and function will be described in the following.

Th1 cells

Th1 cells are important for immune responses against intracellular pathogens; they are mainly characterized by production of IFN-γ and to a lesser extend TNF-α and IL-2 [Romagnani, 1999, Nagarkatti et al., 1990]. The differentiation of naive T cells into Th1 cells is induced by a strong TCR stimulus and the cytokines IL-12 and IFN-γ [Manetti et al., 1993, Hsieh et al., 1993, Wenner et al., 1996]. Key transcription factors are STAT1 and TBET. In addition, STAT4 is involved in the differentiation process [Afkarian et al., 2002, Ma et al., 2010, Szabo et al., 2000]. STAT1 is activated by IFN-γ receptor and induces TBET which subsequently activates IFN-γ signaling and IL-12 receptor (IL-12R) expression, while STAT4 is induced by

IL-12 [Afkarian et al., 2002, Ma et al., 2010, Szabo et al., 2000]. In the last years, further factors participating and controlling Th1 differentiation were identified. Examples are EGR1 and EGR2 that induce TBET expression as well as EOMES, RUNX3 and HLX that regulate IFN-γ production and prevent production of IL-4 and therefore differentiation into Th2 cells [Du et al., 2014, Shin et al., 2009, Intlekofer et al., 2010, Zhu and Paul, 2011].

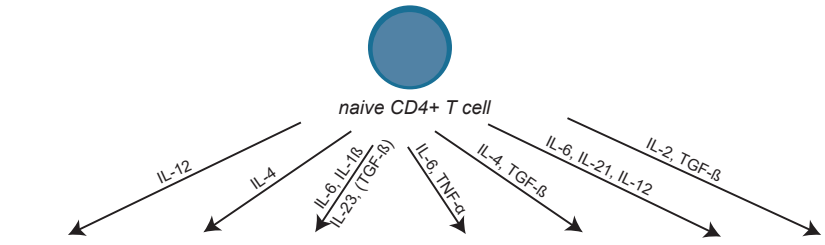
Th2 cells

Th2 cells are important for elimination of extracellular pathogens, especially parasites like helminths. Also, they are strongly associated with allergy, asthma and atopic dermatitis [Lafaille, 1998, Druet et al., 1995, Robinson et al., 1992]. Th2 cells mainly induce a humoral immune response by activating B cells to secrete IgM, IgA or IgE instead of IgG antibodies [Lafaille, 1998, Kopf et al., 1993]. They further recruit eosinophils and lead to alternative macrophage activation [Coffman et al., 1989, Anthony et al., 2006]. They are characterized by secretion of IL-4, IL-5 and IL-13 and expression of the master transcription factors GATA3 and STAT6 [Mosmann et al., 1986, Zheng and Flavell, 1997, Kurata et al., 1999, Kopf et al., 1993]. Similar to the induction of Th1 cells by STAT1 and TBET, STAT6 acts upstream of GATA3 and induces its expression upon activation by IL-4 [Lederer et al., 1996]. GATA3 induces IL-4 expression, forming a positive feedback loop [Zheng and Flavell, 1997]. Further factors participating and regulating Th2 differentiation were recently described: DEC2, MAF, SATB1 and NLRP3 [Liu et al., 2009, Ho et al., 1998, Ahlfors et al., 2010, Bruchard et al., 2015]. During the differentiation into Th1 or Th2 cells formation of the other subset is inhibited. IL-12 prevents T cells from differentiating into IL-4 producing Th2 cells, while GATA3 inhibits the induction of IFN-γ and thereby induction of the Th1 phenotype [Manetti et al., 1993, Zhu et al., 2006].

Th17 cells

IL-17 producing Th cells were first described in 2003 and further defined as a new subset in 2005 [Aggarwal et al., 2003, Murphy et al., 2003, Park et al., 2005, Harrington et al., 2005]. While they play a protective role against extracellular pathogens, they also contribute to several inflammatory diseases like psoriasis, rheumatoid arthritis and inflammatory bowl disease. They accumulate in affected tissues and induce strong inflammatory reactions by cytokine secretion and immune cell recruitment, particularly neutrophils [Pène et al., 2008, Korn et al., 2009].

Th17 cells are characterized by secretion of the key cytokines IL-17A and IL-17F as well as IL-21, IL-22 and IL-23, with IL-21 representing an autocrine factor that regulate Th17 differentiation by STAT3 [Aggarwal et al., 2003, Park et al., 2005, Murphy et al., 2003, Harrington et al., 2005, Wei et al., 2007]. Differentiation of naive T cells into the Th17 phenotype is initiated by IL-6 and IL-1β [Yang et al., 2008b]. TGF-β is also discussed to be important for differentiation, however controversial data exist [Yang et al., 2008a, Ghoreschi et al., 2010]. IL-23 is less important for differentiation initiation but crucial for maintenance of Th17 cells [Stritesky et al., 2008]. Th17 differentiation starts with the activation of STAT3 that is induced by IL-6



	Th1	Th2	Th17	Th22	Th9	Tfh	Treg
Key effector cytokines	IFN-γ	IL-4 IL-5 IL-13	IL-17A/F IL-21 IL-22	IL-22 TNF-α	IL-9	IL-21	IL-10 TGF-ß
Surface marker	CXCR3	CRTH2	CCR6	CCR4 CCR10	?	CXCR5	CD25
Function	defense against intracelullar pathogens	defense against extracellular pathogens/ helminths	defense against extracelullar pathogens	wound healing/ tissue homeostasis	defense against extracellular pathogens/ helminths	B cell activation	tolerance/ immune suppression
Diseases involvement	autoimmunity	allergy and asthma	autoimmunity	tissue remodeling	atopic diseases?	autoimmunity	autoimmunity

Figure 2 The different T helper cell subsets shown with their key effector cytokines, function and contribution to diseases.
Naive T cells get activated by DCs presenting MHC class II-bound antigens and differentiate, depending on further cytokine signals, into the different Th subsets Th1, Th2, Th17, Th22, Th9, Thf or Tregs. These subsets differ in their transcription factor and key cytokine expression. Represented surface makers are further used for identification and characterization. Th subsets have different functions and contributions in autoimmunity/inflammatory conditions.

and, at least in mice, TGF-β. STAT3 then induces RORC2 (RORγt) expression, the master transcription factor being also important during thymopoiesis. Besides RORC2, also IL-23R and IL-21 expression is induced by STAT3 establishing a positive feedback-loop [Ivanov et al., 2006, I et al., 1999, Zhou et al., 2007, Wei et al., 2007]. RORγt subsequently activates IL-17A and IL-17F expression by binding to the promotor regions of their gene loci [Ivanov et al., 2006, Durant et al., 2011]. Besides STAT3 and RORC2/RORγt, a set of other factors were identified to be involved in the regulation of Th17 differentiation. The transcription factors BATF, RUNX1, IRF4, IκBζ, c-MAF, AHR and DDX are also required for RORγt induction and IL-17 expression [Schraml et al., 2009, Zhang et al., 2008, Huber et al., 2008, Okamoto et al., 2010, Bauquet et al., 2009, Veldhoen et al., 2008a, Huang et al., 2015]. In addition, RORA (RORα) was described to participate in Th17 differentiation, leading to IL-17 expression even in the absence of RORγt [Yang et al., 2008b]. Though more transcription factors and RORγt interaction partners are identified, questions regarding the regulation of Th17 differentiation still remain open for a thorough understanding of the entire process, particularly in humans [McGeachy and Cua, 2008].

Th22 cells

Th22 cells were first described in 2009 as new T helper cell subset expressing IL-22, but no IL-17A or IL-17F [Eyerich et al., 2009, Duhen et al., 2009, Trifari et al., 2009]. In addition to IL-22, they also secrete TNFα and/or IL-10. To date, AHR is described as key transcription factor, while RORC2 - the key transcription factor of Th17 cells - did not play a role; further, TBET was observed to be upregulated [Trifari et al., 2009]. Differentiation of naive T cells into the Th22 phenotype strongly depends on IL-6 and TNF-α [Basu et al., 2012, Duhen et al., 2009]. Although these factors were identified, the exact differentiation process of Th22 cells remains elusive and Th22 enriched *in vitro* cultures are difficult to obtain [Plank et al., 2017]. However, transcriptome analysis of Th22 clones revealed exclusive expression of human forkhead box protein O4 (FOXO4) compared to clones of other T cells subsets, suggesting a potential participation of this transcription factor in generating the Th22 phenotype [Eyerich et al., 2009].

IL-22, the key cytokine of Th22 cells, belongs to the IL-10 family and is further expressed by ILCs and NK cells. It has ambivalent functions with both anti- and pro-inflammatory properties [Liang et al., 2006, Kreymborg et al., 2007, Cupedo et al., 2009]. The receptor for IL-22, a heterodimer of IL-10 receptor (IL-10R) β-chain and IL-22 receptor (IL-22RA1) is mainly expressed on tissue cells. Therefore, Th22 cells mainly act on non-immune cells like epithelial cells and induce innate immune responses [Xie et al., 2000, Wolk et al., 2004]. Pro-inflammatory effects of IL-22 were described in psoriasis as well as human bronchial epithelial cells and colon tissue, while a protective role was observed in human keratinocytes resembling wound healing effects *in vitro* and induction of anti-microbial peptide expression [Eyerich et al., 2009, Aujla et al., 2008, Boniface et al., 2005, Andoh et al., 2005, Liang et al., 2006]. Most of these functions were later attributed to Th22 cells, for instance, Eyerich *et al.* showed wound healing effects *in vitro* on keratinocytes incubated with supernatant of Th22 cells [Eyerich et al., 2009]. Further, Th22 cells were shown to participate in (auto-)immune disorders like psoriasis, atopic eczema, multiple sclerosis and several other diseases [Azizi et al., 2015, Rolla et al., 2014].

Th9 cells

Th9 cells need TGF-β and IL-4 for differentiation. Like Th2 cells, the expression of transcription factor GATA3 and STAT6 is required; the Th9 master transcription factor PU.1 is induced in a later state by IRF4 to secrete IL-9 and IL-10 [Angkasekwinai et al., 2010, Gerlach et al., 2014, Staudt et al., 2010]. Signaling induced by TGF-β prevents differentiation into Th2 cells, however, it is not clear if also other factors are involved [Veldhoen et al., 2008b]. The function is still not completely understood, but the subset is associated with different autoimmune diseases and responses against parasites and gut inflammation [Veldhoen et al., 2008b, Kaplan, 2013].

Follicular helper T cells

Follicular helper T cells (Tfh) are found in close proximity to B cells in secondary lymphoid

tissues. They are responsible for activation, expansion and differentiation of B cells [Ma and Deenick, 2014]. Key cytokines expressed by Tfh cells are IL-21, low levels of IFN-γ, IL-4 and IL-17. As transcription factor, BCL-6 was identified as well as STAT3 and STAT5, IRF4 and BATF [Liu et al., 2013].

Regulatory T cells
Regulatory T cells (Tregs) represent another subset of T helper cells with a special function. They suppress immune responses and avoid reactions to self-antigens and autoimmune diseases, representing an important part of the immunological tolerance [Sakaguchi et al., 2006]. Tregs are characterized by expression of the surface marker CD25 and key transcription factor FOXP3, while RUNX1 or SMAD3 are also involved in the differentiation process [Hori et al., 2003, Ruan et al., 2009]. The secretion of IL-10 and TGF-β suppresses activation of macrophages and proliferation of B and T cells by inhibiting GATA3 and TBET expression [Gorelik et al., 2000, Gorelik et al., 2002]. As both Tregs and Th17 cells are induced by TGF-β, a tight regulation of the differentiation processes is necessary. It was found that STAT3, an important transcription factor for Th17 cells, suppresses FOXP3 expression and therefore differentiation into the Treg phenotype. Further, Treg and Th17 differentiation depend on different additional cytokines to regulate this process: IL-6 and IL-1β are necessary for Th17 cells, while Tregs require IL-2 for differentiation [Laurence et al., 2012, Zheng et al., 2007, Yang et al., 2008a].

The plasticity of T helper cell subsets

Despite the clear classification of T helper cells based on expression of different cytokines, chemokines, transcription factors and functions to protect against diverse pathogens or threats, by now these subsets were shown to be plastic [Bluestone et al., 2009]. Under the right conditions, different T helper cell subsets have the ability to convert into other subsets or at least resemble them in terms of cytokine expression and transcription factors, keeping the flexibility to react fast with a proper defense mechanism [Bluestone et al., 2009].

It was found that Th17 cells can convert into Th1 or “Th1-like” cells. Transfer of Th17 cells in a diabetes mouse model showed that these cells rapidly upregulate the Th1 transcription factor TBET and the production of IFN-γ, thus, converting into Th1 cells [Bending et al., 2009]. Further, Th17 cells that tend to produce IFN-γ are handled as pathogenic Th1/Th17 cells during inflammation/autoimmune disorders [Lee et al., 2009, Hwang et al., 2004, Komiyama et al., 2006, Harbour et al., 2015, Leung et al., 2010]. A similar plasticity was described for Tregs and Th17 cells. Tregs convert into “Th17-like” cells when cultured under Th17-polarizing conditions with IL-1β and IL-2, also resulting in downregulation of Treg transcription factor FOXP3 [Deknuydt et al., 2009]. In turn, Th17 cells can transdifferentiate into Tregs [Gagliani et al., 2015]. Th2 cells, originally mistaken to be a stable T cell subset, can co-produce IFN-γ and IL-4 as well as TBET in addition to GATA3, thereby turning into stable “Th1-Th2 like” cells during virus infection [Hegazy et al., 2010]. In addition, they can transdifferentiate into Th9 cells when stimulated with TGF-β and adopt a Tfh phenotype during helminth infection [Veld-

hoen et al., 2008b, Zaretsky et al., 2009]. Those, in turn, can express IL-4 upon helminth infection, but also IFN-γ like Th1 cells in viral infections, making them a very plastic T cell subset [Zaretsky et al., 2009, Reinhardt et al., 2009].

Dysregulated T helper cell responses are associated with various disease patterns in human skin

Autoimmune or inflammatory diseases are misregulated reactions of the immune system to self-antigens or harmless substances misclassified as dangerous. They are often mediated by misdirected T and B lymphocytes that failed to be depleted during central tolerance induction and cannot be controlled by peripheral tolerance any more. These diseases can be limited to certain tissues like lung or skin but can also have systemic impact [Devarajan and Chen, 2013, Chen and Flies, 2013]. About 5 - 10 % of the world-wide population suffers from autoimmune diseases with increasing tendency. Reasons for increasing numbers are widely unknown, although environmental changes as industrialization might be involved [Devarajan and Chen, 2013, Chen and Flies, 2013]. In this context, the hygiene hypothesis is discussed. It states that as bodies in industrialized countries are less exposed to parasites and other pathogens (in early childhood), they are more prone to develop allergies, inflammatory diseases and autoimmune diseases [Strachan, 1989, Bloomfiled et al., 2006].

T helper cells are identified as cause of many autoimmune and chronic inflammatory diseases like asthma, multiple sclerosis (MS), type 1 diabetes, rheumatoid arthritis (RA) as well as the skin affecting inflammatory diseases atopic eczema (AE) and psoriasis [Raphael et al., 2015]. Other skin diseases are mediated by auto-antibodies produced by B cells like pemphigus vulgaris, while lupus erythematosus is triggered by both B and T cells [Amagai et al., 1991, Fattal et al., 2010, Raphael et al., 2015]. However, understanding of the exact pathogenesis mechanism is often amiss [Raphael et al., 2015].

Psoriasis is a Th17-mediated inflammatory skin disease

Psoriasis is a chronic inflammatory skin disease that affects about 1 - 3 % of the worldwide population [Boehncke and Schön, 2015, Nestle et al., 2009]. It manifests in abnormal proliferation of keratinocytes as well as T cell and neutrophil infiltration in the skin, leading to characteristics like dry, red raised plaques and adherent silvery scales in the most common form psoriasis vulgaris [Boehncke and Schön, 2015, Cai et al., 2012]. Histological features are keratinocyte hyperproliferation and aberrant differentiation, dilated blood vessels and inflammatory infiltration of leukocytes into the skin [Boehncke and Schön, 2015]. The pathogenesis of psoriasis is complex and involves a genetic predisposition, environmental factors and a dysregulated immune response. This disease is mainly T cell-driven with Th1 cells suggested to be the main player for a long time. However, by now Th17 cells and to a lesser extend Th22 with the secreted cytokines IL-23, IL-17 and IL-22 were identified to be most important in disease pathogenesis [Fitch et al., 2010, Boniface and Guignouard, 2007]. Further, chemokine receptor CCR6, expressed on Th17 and $\gamma\delta$ T cells, is important for chemotaxis to

the site of inflammation (Fig 3) [Mabuchi et al., 2013].

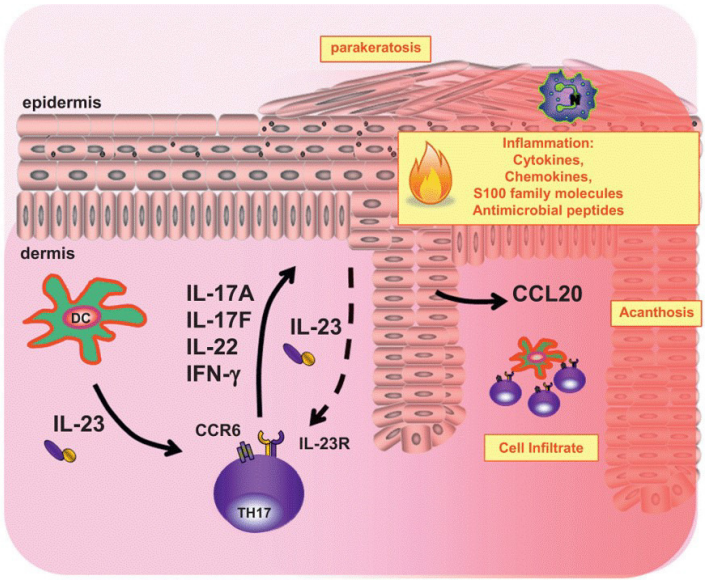


Figure 3 The pathogenesis of psoriasis is characterized by Th17 cells infiltrating the skin. Infiltrating and pro-inflammatory cytokine-producing Th17 cells are attracted by CCR6 ligands to the site of inflammation. IL-23 is important for maintenance of these cells. Th17 cells secrete effector cytokines IL-17A, IL-17F, IL-22 and IFN-γ causing typical features like parakeratosis and acanthosis as well as inducing inflammation, resulting in further immune cell infiltration and production of pro-inflammatory cytokines, chemokines, antimicrobial peptides and inflammatory S100 proteins [Di Meglio and Nestle, 2010].

The mechanism of the pathogenesis of psoriasis is still not fully understood. Whereas in murine psoriasis models $\gamma\delta$ T cells are the main producers of IL-17, the contribution of $\gamma\delta$ T cells to the human disease pathogenesis is widely unknown [Atenhan, 2014, Mabuchi et al., 2012, Mabuchi et al., 2013].

Recently, the anti-microbial peptide LL-37 was identified as an auto-antigen, underlining the assumption of psoriasis being an autoimmune disease [Lande et al., 2014]. LL-37 specific, Th17 cytokine-producing T cells were identified in blood and lesional skin of psoriasis patients. In addition, their presence correlated with disease severity. This assumption is further supported by the Koebner phenomenon that describes the appearance of sudden skin lesions at sites of mechanical injury of the skin [Mabuchi et al., 2012, Sagi and Trau, 2011].

Besides UV-therapy and topical application of corticosteroids, classical treatment strategies involve systemic therapies with the immunosuppressive drugs methotrexate or cyclosporine [Heydendael et al., 2003]. New treatment approaches using biologics directed against Th17 cytokines, e.g., IL-17 and IL-23, show outstanding results in psoriasis patients, once again highlighting the importance of Th17 cells in psoriasis pathogenesis [Wasilewska et al., 2016].

Atopic eczema is a Th2-driven disease with Th22 participation in the chronic state
Atopic eczema (AE) is a chronic inflammatory skin disease. The triggers are thought to be a combination of environmental factors and genetic preposition, and skin-barrier defects are observed in patients [Brandt and Sivaprasad, 2011]. AE manifests in red, dry and itching skin, often in combination with asthma or allergic rhinitis [Novak and Leung, 2011, Peng and Novak, 2015]. The acute phase is characterized by accumulation of Th2 cells and their corresponding cytokines like IL-4 and IL-13. However, in the chronic phase a switch to Th1 cells and associated cytokines is observed [Novak and Leung, 2011]. In addition, IL-22 produced by Th22 cells becomes more important in this chronic phase [Nograles et al., 2009, Fujita, 2013]. IL-22 expression in chronic AE skin lesions is even higher than in the Th17-driven disease psoriasis, and IL-22 levels are also systemically elevated in the serum [Hayashida et al., 2011]. The high IL-22 amounts in chronic lesions cause acanthosis, a typical characteristic of psoriasis that makes the differentiation of these two diseases in the chronic state of AE difficult [Fujita, 2013, Quaranta et al., 2014]. Further, atopic eczema in Europe and Asia differs both on molecular and histological level. Asian AE shows increased IL-17 levels and Th17 cell numbers in lesional skin as well as histological features typical for psoriasis (e.g., parakeratosis and hyperplasia), again highlighting the role of Th17 and Th22 cytokines in this disease [Noda et al., 2015].

The treatment of inflammatory skin diseases is difficult. Either the antigen cannot be removed (in case of autoimmunity) or the trigger is often even not known. For skin diseases, the first line of therapy is topical treatment, often with glucocorticosteroids due to their efficient anti-inflammatory effect. However, they act unspecific and have strong side effects, including strong and permanent immunosuppression when taken systemically as a later step of treatment [Rosenblum et al., 2014, Fauci et al., 1983]. Therefore, new approaches are needed to suppress autoimmune and dysregulated immune responses more specifically by addressing responsible molecules like cytokines or by intervening into involved pathways (e.g., blocking receptors on specific cell types, mainly T or B lymphocytes) [Rider et al., 2016, Rosman et al., 2013]. These new classes of drugs are called biologics and are proteins acting as agonists or antagonists of receptors or neutralizing antibodies that target specific proteins, mainly cytokines [Rider et al., 2016]. So far, the most common used biologics are antibodies directed against TNF-α, IL-1 and IL-6. Th17 cells are further targeted by antibodies directed against IL-12 and IL-23, used for treatment in RA, psoriasis and other diseases with mostly good results [Rider et al., 2016, Rosman et al., 2013].

For successful treatment and new therapeutic approaches it is essential to understand in detail the involved cells and their mechanisms. Therefore, the differentiation processes for Th17 and Th22 cells that are involved in many autoimmune and inflammatory diseases have to be further investigated to gain full and detailed knowledge.

1.3 Newly identified potential factors for regulation of T helper cell phenotypes

1.3.1 The LINGO protein family

The leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor interacting protein (LINGO) family consists of 4 proteins, LINGO1 to LINGO4. Among these, LINGO1 is the only protein properly characterized, though [Carim-Todd et al., 2003]. LINGO proteins are transmembrane proteins with a single transmembrane domain and are localized in the plasma membrane [Carim-Todd et al., 2003, Mi et al., 2004, Clark et al., 2003, Haines and Rigby, 2008]. With 44 - 61 % sequence identity they are highly homologous and highly conserved in vertebrates like humans, mice, monkeys and chicken [Carim-Todd et al., 2003]. So far, their expression was mainly described in context of the central nervous system and the brain and partly associated with neurogenerative disorders [Carim-Todd et al., 2003, Mi et al., 2004, Llorens et al., 2008, Vilarino-Guell et al., 2010]. LINGO1 forms homotetramers in the plasma membrane and is highly expressed in adult human brain tissues like cerebal cortex, hippocampus or thalamus. It is part of the nogo receptor complex. This complex responds to myelin associated inhibitory proteins (MAIs) and prevents myelination and therefore axon regeneration through intracellular signaling [Mosyak et al., 2006, Llorens et al., 2008]. Due to its function in prevention of axon regeneration, LINGO1 is connected to different neurodegenerative diseases. It was shown that LINGO1 antagonists have neuroprotective effects in Glaucoma, an eye disease caused by degeneration of retinal ganglion cells and axons [Fu et al., 2009]. Further, LINGO1 is associated with diseases like essential tremor and multiple sclerosis, a Th17 mediated disease, and a possible candidate as drug target for therapy [Mi et al., 2013, Rudick et al., 2008]. Contrary to LINGO1, the other members of this family are far less characterized. For instance, *LINGO2* mRNA expression was found exclusively in the adult mouse brain [Haines and Rigby, 2008, Homma et al., 2009]. Regarding functional aspects, an association with essential tremor and Parkinson's disease was identified, as single nucleotide polymorphisms (SNPs) were found to represent a risk factor for these diseases [Vilarino-Guell et al., 2010, Wu et al., 2011]. *LINGO3* mRNA expression was found to be ubiquitous at an early time point in mouse embryogenesis, while later expression concentrated in mesodermal tissues with higher levels in the branchial arches and head mesoderm [Haines and Rigby, 2008].

LINGO4

LINGO4 was first discovered in 2003 by Clark *et al.* within the "secreted protein discovery initiative" (SPDI). Based on bioinformatic calculations it is predicted as a transmembrane protein with 593 amino acids and a molecular weight of around 64 kDa [Clark et al., 2003]. The function of this protein is still unknown and only two studies were conducted in the context of mouse embryogenesis. These identified a weak expression of *Lingo4* on mRNA level in the central nervous system of 10 days old mouse embryos [Haines and Rigby, 2008, Homma

et al., 2009]. Contrary to the family members LINGO1 and LINGO2, LINGO4 does not play a role in the neurodegenerative disease essential tremor [Liang et al., 2012].

1.3.2 The FOXO protein family

The forkhead box protein O (FOXO) protein family consists of four members (FOXO1, FOXO3, FOXO4 and FOXO6) representing an important group of transcription factors that all share the conserved winged-helix DNA-binding domain forkhead box [Kaestner et al., 2000]. The specific DNA sequence for binding and target gene regulation was described as TTGTTTAC [Kumar et al., 2015, Eijkelenboom and Burgering, 2013, Li et al., 2016, Martins et al., 2016, Furuyama et al., 2000]. In invertebrates, FOXO proteins were shown to extend longevity and also in mammalians these proteins are discussed to increase the lifespan as they are regulated by the insulin pathway, a pathway that has been implicated in aging [Lin et al., 1997, Ogg et al., 1997, Blueher et al., 2003, Holzenberger et al., 2003, Furuyama et al., 2003]. FOXOs are further associated with metabolic processes, tumor suppression, DNA repair, cell cycle arrest and apoptosis, development and the regulation of stress resistance [Martins et al., 2016, Calnan and Brunet, 2008]. This wide range and even antagonistic functions are induced and regulated by several different factors, e.g., insulin, growth factors, oxidative stress and nutrients [Calnan and Brunet, 2008]. Further, the function of FOXO proteins is regulated by post-translational modifications (e.g., phosphorylation, acetylation, methylation and ubiquitinaion) that subsequently determine the subcellular translocation of the transcription factors [Martins et al., 2016, Calnan and Brunet, 2008].

FOXO1 is mainly expressed in B and T cells as well as ovaries, but has important functions in different tissues [Hedrick et al., 2012, Kerdiles et al., 2009, Tothova et al., 2007]. FOXO3 shows expression in a wide range of tissues as well as both in lymphocytes and myeloid cells while FOXO4 is also expressed in several tissues, but at low levels [Hedrick et al., 2012, Hosaka et al., 2004, Kerdiles et al., 2009]. The generation of knock-out mice revealed different functions of FOXO proteins. While FOXO1 deficient mice already died in the embryogenic phase due to impaired development of vascular vessels, FOXO3 deficient mice were viable besides female mice getting infertile with age. FOXO4 deficiency did not result in consistent abnormalities [Hosaka et al., 2004]. However, FOXO4 was identified as regulator of inflammation by, e.g., inhibiting NFκB, and protecting against colon inflammation [Zhou et al., 2009b].

FOXO transcription factors, in particular FOXO1 and FOXO3, influence development and cell survival of T cells. FOXO1 seems to be more important for survival of naive T cells as it regulates IL-7Rα expression, a cytokine important for survival, maintenance and expansion of T cells after antigen-stimulation, although the mechanism is still unknown [Rathmell et al., 2001, Ouyang et al., 2009]. FOXO3 regulates apoptosis/cell survival of effector cells, e.g., by controlling pro-apoptotic factors like BIM and Fas-ligand. Upon stimulation by TCR signaling, FOXO3 is inhibited and thereby its effect on apoptosis. However, these findings origin

primarily from non-lymphocytic cells and were not confirmed for T cells yet [Hedrick et al., 2012].

FOXO4

FOXO4 functions as transcription factor and is located on the X chromosome [Parry et al., 1994, Weigel and Jäckle, 1990]. FOXO4 is involved in different cellular processes like stress response induced by reactive oxygen species (ROS), metabolic processes and tumorigenesis, but also cell survival as a common feature of the FOXO protein family. In the insulin signaling pathway, it is negatively regulated by protein kinase B (PKB). PKB phosphorylates FOXO4, keeping it as an inactive form in the cytosol. Only dephosphorylated FOXO4 can translocate to the nucleus and initiate transcription of the target genes. This regulatory mechanism is also described for another isoform of FOXO4 that misses the first 16 amino acids of the protein [Yang et al., 2002, Matsuzaki et al., 2005]. However, an alternative activation pathway via Ras/Ral exists. Ras signaling can lead to activation of FOXO4 independently from PKB [Kops et al., 1999]. This pathway is controlled by c-Jun N-terminal kinase (JNK) signaling and activated by ROS-induced stress. Therefore, FOXO4 can keep cells in homeostasis by both regulating metabolism and stress responses via two independent mechanism [Essers et al., 2004]. In addition, nemo-like kinases (NLK) can regulate ROS-induced stress responses as a negative regulator leading to the phosphorylation of FOXO4. This kinase further prevents monoubiquitinylation, a post-translational modification required for FOXO4 activation and initiation of the anti-stress response [Szypowska et al., 2011, van der Horst et al., 2006]. FOXO4 is further related to cancer and tumor suppression. It can suppress tumor and metastasis growth in gastric cancer by inducing G1 arrest and downregulation of S phase entering cells [Su et al., 2014]. Additionally, overexpression of FOXO4 can downregulate a mitogenic and pro-transforming factor, the oncogene human epidermal growth factor receptor 2 (HER2) in cancer and, therefore, is dealt as possible anti-cancer agent [Yang et al., 2005]. However, not much is known about the role of FOXO4 in T cells. One study showed a FOXO4 dependent regulation of the Treg response by IL-10 production upon stimulation with progranulin (PGRN), mediating an anti-inflammatory effect. This process is JNK-dependent and in addition to FOXO4, STAT3, a known transcription factor of Treg regulation as well as Th17 cells, was described to function as transcription factor in this case [Fu et al., 2016]. Of note, increased FOXO4 levels were observed in transcriptome analysis of Th22 cells compared to other subsets, suggesting a transcriptional role in this relatively recently described T cell subset that has to be investigated in detail [Eyerich et al., 2009].

1.4 Objective

T cells play a major role in the regulation and successful performance of an immune response against both intracellular and extracellular pathogens. In particular, $CD4^+$ T helper cells direct adaptive immune responses by activating and recruiting other cell types to the site of inflammation in order to clear an infection. However, an imbalance in the T cell compartment leading to dysfunctional responses is highly associated with inflammatory disorders like the skin affecting diseases atopic eczema and psoriasis, but also autoimmunity. The number of people affected by T cell-mediated diseases is increasing, particularly in the Western, developed countries. Therefore, profound knowledge about the pathogenic mechanisms is important, particularly regarding identification and development of new therapeutic approaches. In this context, a complete understanding of the regulation and differentiation within the involved T helper cell subsets is crucial. While Th1 and Th2 subsets are well described, detailed knowledge of the differentiation process of Th17 and Th22 cells is still lacking, particularly in humans. This work addresses the role of two novel factors, LINGO4 and FOXO4, in the differentiation and regulation of Th17 and IL-22 producing/Th22 cells, respectively. Elevated LINGO4 mRNA expression were detected in whole genome expression arrays of Th17 clones compared to other T helper subsets derived from psoriasis or atopic eczema patient samples, indicating a possible role of this protein in Th17 cells. On the other hand, Th22 cells showed elevated FOXO4 mRNA expression in whole genome expression arrays. Based on these preliminary studies, potential regulatory functions of these proteins will be investigated. First, the transcription pattern will be investigated to validate previous made observations. Lentiviral overexpression and knockdown will address functional features. These results will give deeper insight into the regulation and differentiation of these T helper subsets. This knowledge might be important for the general understanding of Th17 and Th22-driven diseases and therefore contribute to the development of new therapeutic approaches.

2. Materials

2.1 Devices

Table 1 Devices

Device	Type	Company, Headquarter
Agarose gel electrophoresis module	Compact Line	Biometra, Göttingen, Germany
Balance	ALJ	Kern& Sohn, Balingen-Frommern, Germany
Bioanalyzer	2100	Agilent, Santa Clara, USA
Blot imaging system	ECL ChemoCam Imager	Intas Science Imaging, Göttingen, Germany
Blot module	Mini Blot Module	Life Technologies, Carlsbad, USA
Centrifuges	Megafuge 1.0R	Heraeus, Hanau, Germany
	Megafuge 40R	Thermo Fisher Scientific, Waltham, USA
	Perfect Spin 24 R	Peqlab, Erlangen, Germany
	Universal 32R	Hettich, Tuttlingen, Germany
ELISA reader	Epoch	BioTek, Winooski, USA
ELISA washer	hydrospeed	Tecan, Münnedorf, Switzerland
Flow cytometer	BD LSRFortessa	Becton Dickinson, Franklin Lakes, USA
Freezer (-80 °C)	Hera freeze	Thermo Fisher Scientific, Waltham, USA
Gel imaging system	Gel iX Imager	Intas Science Imaging, Göttingen, Germany
Gel system	Mini Gel Tank	Life Technologies, Carlsbad, USA
Heating block	Thermomixer 5437	Eppendorf, Hamburg, Germany
Hemocytometer	Neubauer	Superior Marienfeld, Lauda Königshofen, Germany
Hood	Cell Safe	Heraeus, Hanau, Germany
Incubator	Hera Cell	Heraeus, Hanau, Germany
MACS	AutoMACS Pro	Miltenyi Biotec, Bergisch Gladbach, Germany
Mass spectrometer	Q Exactive HF	Thermo Fisher Scientific, Waltham, USA
Microarray Scanner System	iScan	Agilent Technologies, Santa Clara, USA

Table 1 Devices

Device	Type	Company, Headquarter
Microscope	Axiovert 25	Zeiss, Oberkochen, Germany
pH meter	inoLab pH7110	WTW, Weilheim, Germany
pH meter electrode	SenTix 81	WTW, Weilheim, Germany
Pipet boy	Strippetor Ultra	Corning, Corning, USA
Pipets	Reference	Eppendorf, Hamburg, Germany
Plate shaker	MTS 2/4	IKA, Staufen, Germany
Power supply	Standard Power Pack P25T	Biometra, Göttingen, Germany
Real-time PCR machine	ViiA7	Applied Biosystems, Foster City, USA
RSLC system	Ultimate 3000	Dionex, Sunnyvale, USA
Shaker	Unitwist RT	UniEquip, Martinsried, Germany
Spectrophotometer	NanoDrop-1000	Peqlab, Erlangen, Germany
Thermal cycler	TC-412	Techne, Stone, UK
TissueLyser	-	Qiagen, Hilden, Germany
Transfer pipets	Transferpipette-8	Brand, Wertheim, Germany
Vacuum pump	BVC control	Vacuubrand, Wertheim, Germany
Vortex	Genie2	Bender + Hobein AG, Bruchsal, Germany
Water bath	SW22	Julabo, Seelbach, Germany

2.2 Chemicals

Table 2 Chemicals

Chemicals	Company, Headquarter
Acetic acid (100%) (CH_3COOH)	Merck, Darmstadt, Germany
Acetonitrile (ACN)	Sigma-Aldrich, St. Louis, USA
Agar-Agar	Roth, Karlsruhe, Germany
Agarose “peqGold Universal“	Peqlab, Erlangen, Germany
Bicine	ChemCruz, Dallas, USA
Bis-Tris	Amresco, Solon, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, USA
Citric acid anhydrous ($C_6H_8O_7$)	Sigma-Aldrich, St. Louis, USA
Dithiothreitol (DTT)	AppliChem, Darmstadt, Germany
Dimethylsulfoxid (DMSO)	AppliChem, Darmstadt, Germany

Table 2 Chemicals

Chemicals	Company, Headquarter
Ethanol (C_2H_5OH)	Merck, Darmstadt, Germany
Glycerol ($C_3H_8O_3$)	Alfa Aesar, Karlsruhe, Germany
Hydrogen peroxide 30% (H_2O_2)	Sigma-Aldrich, St.Louis, USA
Iodoacetamide (IAA)	Sigma-Aldrich, St. Louis, USA
Isopropyl alcohol (C_3H_7OH)	Roth, Karlsruhe, Germany
LB Broth (Miller)	Sigma-Aldrich, St. Louis, USA
Lys-C	Thermo Fisher Scientific, Waltham, USA
Methanol (CH_3OH)	Merck, Darmstadt, Germany
MOPS	AppliChem, Darmstadt, Germany
Non-fat dried milk powder	AppliChem, Darmstadt, Germany
Paraformaldehyde	Sigma-Aldrich, St. Louis, USA
Potassium chloride (KCl)	Merck, Darmstadt, Germany
MG-132	EMD Millipore Corp., Billerica, USA
Monopotassium phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Sodium azide (NaN_3)	Merck, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
10 % Sodium dodecylsulfate (SDS)	Gibco Life Technologies, Carlsbad, USA
Disodium phospate (Na_2PO_4)	Merck, Darmstadt, Germany
Sulfuric acid (H_2SO_4)	Merck, Darmstadt, Germany
Tetramethylbenzidine (TMB)	Sigma-Aldrich, St. Louis, USA
Trifluoroacetic acid (TFA)	Sigma-Aldrich, St.Louis, USA
Triton-X100	Sigma-Aldrich, St. Louis, USA
Trizma base	Sigma-Alrdich, St. Louis, USA
Trizma hydrochloride	Sigma-Aldrich, St. Louis, USA
Tween-20 Detergent	EMD Millipore Corp., Billerica, USA

2.3 Buffers

Table 3 Buffers

Buffer	Recipe
20x PBS buffer	110 mM KCl, 58 mM KH_2PO_4, 33 mM Na_2PO_4, 5.5 M NaCl
50x TAE buffer	2 M Tris, 5 % acetic acid, 50 mM EDTA

Table 3 Buffers

Buffer	Recipe
10x TBS buffer	152 mM Tris-HCl, 46 mM Tris-base, 1.5 M NaCl, pH 7.6
ABC buffer (Mass spectrometry)	50 mM ammonium bicarbonate
Antibody dilution buffer (Immunofluorescence)	1 % BSA in PBS^{def}, 0.03 % Triton-X100
Blocking buffer (ELISA)	1 % BSA in PBS^{def}, pH 7.2 - 7.4 (for R&D ELISA)
	10 % FCS in PBS^{def}, pH 7.0 (for BD ELISA)
Blocking buffer (Immunofluorescence)	5 % BSA in PBS^{def}, 0.3 % Triton-X100
Blocking buffer (Western Blot)	5 % milk powder in 1xTBS, 0.1 % Tween-20
Citrate buffer (ELISA)	190 mM citric acid monohydrate, pH 3.9
Coating buffer (ELISA, BD)	0.1 M Na_2CO_3, pH 9.5
FACS buffer	0.02 % NaN_3, 5 % FCS in PBS^{def}
Guanidine buffer (50 μl)	6 M guanidinium chloride, 100 mM Tris pH 8.5, 1x complete protease inhibitor
Running buffer (SDS-PAGE)	50 mM MOPS, 50 mM Tris-base, 0.1 % SDS, 1 mM EDTA, pH 7.7
Stop solution (ELISA)	2 N H_2SO_4
Substrate solution (ELISA)	1 mM TMB, 0.05 % H_2O_2 in citrate buffer
1x TAE	40 mM Tris, 0.1 % acetic acid, 1 mM EDTA
Transfer buffer (Western Blot)	25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.7
Urea buffer (Mass spectrometry)	8 M urea, 100 mM Tris pH 8.5
Washing buffer (ELISA)	1x PBS, 0.05 % Tween-20, pH 7.2 - 7.4
Washing buffer 1x TBST (Western Blot)	1x TBS, 0.1 % Tween-20

2.4 Enzymes and Reagents

Table 4 Enzymes/reagents

Enzymes/Reagents	Company, Headquarter
4x sample buffer (Laemmli, for SDS PAGE)	Thermo Fisher Scientific, Waltham, USA
6x loading dye (for agarose gels)	Thermo Fisher Scientific, Waltham, USA
7.5 % BSA in DPBS	Sigma-Alrdich, St. Louis, USA
DAPI	Thermo Fisher Scientific, Waltham, USA
DEPC-treated water	Invitrogen, Carlsbad, USA
DNA/RNA dye, PeqGreen	Peqlab, Erlangen, Germany

Table 4 Enzymes/reagents

Enzymes/Reagents	Company, Headquarter
DPBS w/o $Ca^{2+}Mg^{2+}$	Gibco Life Technologies, Carlsbad, USA
EcoRV	Thermo Fisher Scientific, Waltham, USA
Fast Digest Green Buffer	Thermo Fisher Scientific, Waltham, USA
Gene ruler DNA ladder	Thermo Fisher Scientific, Waltham, USA
GolgiPlug (Brefeldin A)	BD Bioscience, San Jose, USA
GolgiStop (Monensin)	BD Bioscience, San Jose, USA
Heparin sodium	Merck, Darmstadt, Germany
IL-1β, human, recombinant	PromoKine, Heidelberg, Germany
IL-2, human, recombinant	Novartis Pharma, Nürnberg, Germany
IL-4, human, recombinant	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-6, human, recombinant	PromoKine, Heidelberg, Germany
IL-12, human, recombinant	PromoKine, Heidelberg, Germany
IL-17A, human, recombinant	PromoKine, Heidelberg, Germany
IL-23, human, recombinant	PromoKine, Heidelberg, Germany
Ionomycin	Sigma-Aldrich, St. Louis, USA
Lipopolysaccharide (LPS)	Sigma-Aldrich, St.Louis, USA
Lymphoprep	Progen Biotechnik, Heidelberg, Germany
Phorbol-12-myristat-13-acetat (PMA)	Sigma-Aldrich, St. Louis, USA
Phusion Hot Star II	Thermo Fisher Scientific, Waltham, USA
Phytohaemagglutinin (PHA)	Sigma-Aldrich, St. Louis, USA
Protein ladder	Thermo Fisher Scientific, Waltham, USA
Polybrene	Santa Cruz Biotechnologies, Santa Cruz, USA
RNaseZap	Sigma-Aldrich, St. Louis, USA
Streptavidin-Peroxidase	R&D, Minneapolis, USA
Stripping buffer (for Western Blot)	Thermo Fisher Scientific, Waltham, Germany
TGF-β, human, recombinant	PromoKine, Heidelberg, Germany
TNF-α, human, recombinant	R&D, Minneapolis, USA
Trypsin	Thermo Fisher Scientific, Waltham, USA
Turbofect	Thermo Fisher Scientific, Waltham, USA
0.4 % Trypane blue	Gibco, Life Technologies, Carlsbad, USA
Vectashield Mounting Medium	Vector Laboratories, Burlingame, USA

2.5 Kits

Table 5 Kits

Kit	Company, Headquarter
Agilent Low Input Quick Amp Labeling Kit	Agilent Technologies, Santa Clara, USA
BCA Protein Assay	Pierce Biotechnology, Rockford, USA
CD14 MicroBeads, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
$CD4^+$ T Cell Isolation Kit, human	Miltenyi Biotec, Germany
CD45RO MicroBeads, human	Miltenyi Biotec, Bergisch-Gladbach, Germany
ECL Western Blotting Substrate	Thermo Fisher Scientific, Waltham, USA
EndoFree Plasmid Maxi Kit	Qiagen, Hilden, Germany
FastDigest Eco32I	Thermo Fisher Scientific, Waltham, USA
Fast Start Universal SYBRGreen Master (Rox)	Roche, Basel, Switzerland
Fixation/Permeabilization Solution Kit	BD Bioscience, San Jose, USA
High capacity cDNA Reverse Transcription Kit	Applied Biosystems, Foster City, USA
HRM Calibration Kit	Biognosys, Schlieren, Switzerland
Human IL-4 ELISA Set	BD Bioscience, San Jose, USA
Human IL-17 DuoSet ELISA	R&D, Minneapolis, USA
Human IL-22 DuoSet ELISA	R&D, Minneapolis, USA
Human IFN-γ DuoSet ELISA	R&D, Minneapolis, USA
Human TNF-α DuoSet ELISA	R&D, Minneapolis, USA
In-Fusion HD Cloning Kit	Clontech Takara, Mountain View, CA, USA
InviTrapSpin Universal RNA Mini Kit	Stratec Biomedical, Birkenfeld, Germany
Live/Dead Fixable Aqua Dead Cell Stain Kit	Thermo Fischer Scientific, Waltham, USA
miRNase Mini Kit	Qiagen, Hilden, Germany
PAXgene Tissue RNA Kit	Qiagen, Hilden, Germany
QIAprep spin Miniprep Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
RIPA Lysis Buffer System	Santa Cruz Biotechnology, Dallas, USA
SuperSignal West Femto Chemiluminescent Substrate	Thermo Fisher Scientific, Waltham, USA

2.6 Media and supplements

2.6.1 Medium components

Table 6 Medium components

Medium/Supplement	Company, Headquarter
AIM-V, AlbuMAX Supplement	Gibco Life Technologies, Carlsbad, USA
DermaLife K Keratinocyte Medium Complete Kit	LifeLine Cell Technology, Frederick, USA
DMEM	Gibco Life Technologies, Carlsbad, USA
0.5 % EDTA pH 8.0	Invitrogen Life Technologies, Grand Island, USA
Fetal Calf Serum (FCS)	GE Healthcare Life Science, Chicago, USA
Human Serum	Sigma-Aldrich, St. Louis, USA
L-Glutamine 200 mM (100x)	Gibco Life Technologies, Carlsbad, USA
Minimal essential medium Non-essential amino acids (MEM NEAA) (100x)	Gibco Life Technologies, Carlsbad, USA
Na-Pyruvate (100x)	Gibco Life Technologies, Carlsbad, USA
Penicillin/Streptomycin (100x)	Gibco Life Technologies, Carlsbad, USA
RPMI 1644	Gibco Life Technologies, Carlsbad, USA
0.05 % Trypsin (1x)	Gibco Life Technologies, Carlsbad, USA

2.6.2 Supplemented medium

Table 7 Supplemented media

Supplemented medium	Recipe
1 % T Cell Medium	1x L-Glutamine, 1x Na-Pyruvate, 1x NEAA, 1x P/S, 1 % human serum in RPMI
5 % T Cell Medium	1x L-Glutamine, 1x Na-Pyruvate, 1x NEAA, 1x P/S, 5 % human serum in RPMI
HEK medium	10 % FCS, 1x NEAA, 1x P/S in DMEM
HEK medium for Transfection	10 % FCS, 1x NEAA in DMEM
HEK medium for Virus production	2 % FCS, 1x NEAA in DMEM
Jurkat medium	10 % FCS, 1x L-Glutamine, 1x Na-Pyruvate, 1x NEAA, 1x P/S in RPMI
Primary Fibroblast Medium	1x P/S, 1x L-Glutamine, 20 % FCS in DMEM

2.7 Antibodies

Table 8 Antibodies

Specificity	Fluorochrom/Enzyme	Host	Company, Headquarter
Cell Culture			
CD3	-	mouse	BD Bioscience, San Jose, USA
CD28	-	mouse	BD Bioscience, San Jose, USA
IFN-γ, human	-	mouse	eBioscience, San Diego, USA
IL-4, human	-	rat	eBioscience, San Diego, USA
IL-12, human	-	rat	eBioscience, San Diego, USA
Flow cytometry			
CD4	APC-Cy7	mouse	BD Bioscience, San Jose, USA
CD14	AF700	mouse	BD Bioscience, San Jose, USA
CD45RA	V450	mouse	BD Bioscience, San Jose, USA
IFN-γ	FITC	mouse	BD Bioscience, San Jose, USA
IL-17A	PE	mouse	BD Bioscience, San Jose, USA
IL-22	eFluor660	mouse	eBioscience, San Diego, USA
TNF-α	AF700	mouse	BD Bioscience, San Jose, USA
Immunofluorescence and Western Blot (primary antibodies)			
FOXO4	-	rabbit	Cell Signaling Technologies, Boston, USA
HA-tag	HRP	mouse	Cell Signaling Technologies, Boston, USA
HA-tag	-	mouse	Cell Signaling Technologies, Boston, USA
LINGO4	-	rabbit	Abcam, Cambridge, UK
LINGO4	-	rabbit	LifeSpan BioScience, Seattle, USA
LINGO4	-	rabbit	Novus Biologicals, Littleton, USA
Ubiquitin	-	rabbit	Cell Signaling Technologies, Boston, USA
Immunofluorescence and Western Blot (secondary antibodies)			
Mouse IgG	HRP	goat	Jackson ImmunoResearch, West Grove, USA
Mouse IgG	NL557	donkey	R&D, Minneapolis, USA
Rabbit IgG	AF488	goat	Life Technologies, Carlsbad, USA
Rabbit IgG	AF647	goat	Life Technologies, Carlsbad, USA
Rabbit IgG	HRP	goat	Santa Cruz Biotechnologies, Dallas, USA
Immunoprecipitation (couled to Beads)			
HA-tag	-	mouse	Santa Cruz Biotechnolgies, Dallas, USA

2.8 Primer

Table 9 Human primers

Target (human)	Direction	Sequence (5 ’ - 3 ’)	Company, Headquarter
AHR	fw	AGTTATCCTGGCCTCCGTTT	Metabion, Martinsried, Germany
	rev	TCAGTTCTTAGGCTCAGC	Metabion, Martinsried, Germany
EF1α	fw	CTGAACCATCCAGGCCAAAT	Metabion, Martinsried, Germany
	rev	GCCGTGTGGCAATCCAAT	Metabion, Martinsried, Germany
FOXO4	fw	CCTCGTTGTGAACCTTGATG	Metabion, Martinsried, Germany
	rev	AAGGGTGACAGCAACAGCTC	Metabion, Martinsried, Germany
GAPDH	fw	GAAGGTGAAGGTCGGAGT	Metabion, Martinsried, Germany
	rev	GAAGATGGTGATGGGATT	Metabion, Martinsried, Germany
GATA-3	fw	GCGGGCTCTATCACAAAATGA	Metabion, Martinsried, Germany
	rev	GCTCTCCTGGCTGCAGACAGC	Metabion, Martinsried, Germany
GFP	fw	CTGCTGCCCGACAACCAC	Metabion, Martinsried, Germany
	rev	CATGCCGAGAGTGATCCCG	Metabion, Martinsried, Germany
IFN-γ	fw	TCAGCCATCACTTGGATGAG	Metabion, Martinsried, Germany
	rev	CGAGATGACTTCGAAAAGCTG	Metabion, Martinsried, Germany
IL-4	fw	GTGTCCTTCTCATGGTGGCT	Metabion, Martinsried, Germany
	rev	CAGACATCTTTGCTGCCTCC	Metabion, Martinsried, Germany
IL-10	fw	CTCATGGCTTTGTAGATGCCT	Metabion, Martinsried, Germany
	rev	GCTGTCATCGATTTCTTCCC	Metabion, Martinsried, Germany

Table 9 Human primers

Target (human)	Direction	Sequence (5 ’ - 3 ’)	Company, Headquarter
IL-17A	fw	CCATCCCCAGTTGATTGGAA	Metabion, Martinsried, Germany
	rev	CTCAGCAGCAGTAGCAGTGACA	Metabion, Martinsried, Germany
IL-17F	fw	CAGCGCAACATGACAGTGAA	Metabion, Martinsried, Germany
	rev	CCAATATCGACAGCAGCAAGTACT	Metabion, Martinsried, Germany
IL-17RA	fw	CTGCCCAGAAATGCCAGACAC	Metabion, Martinsried, Germany
	rev	AGATGCCCGTGATGAACCAGTA	Metabion, Martinsried, Germany
IL-22	fw	ACAGCAAATCCAGTTCTCCAA	Metabion, Martinsried, Germany
	rev	TCCAGAGGAATGTGCAAAAG	Metabion, Martinsried, Germany
LINGO4 (endogenous)	fw	GGCAAACTTGATGCCACCTT	Metabion, Martinsried, Germany
	rev	CTCAGTGTCCAGTGGGAGTC	Metabion, Martinsried, Germany
LINGO4 (cloning)	fw	TAGAGAGCTAGCGATATCGCCAC-CATGGATGCAGCCACAGCTC	Metabion, Martinsried, Germany
	rev	TGGGTACTCGAGGATATCGA-AGAGCTTGGCAGTGACC	Metabion, Martinsried, Germany
LINGO4 (overexpression)	fw	TGGCAGATAACGCCCTTCAG	Metabion, Martinsried, Germany
	rev	CAGCCTCAAGGTGACCAGTT	Metabion, Martinsried, Germany
LINGO4-HA	fw	CTCGGCCCTCTGGGGATAAA	Metabion, Martinsried, Germany
	rev	GTCTGGGACGTCGTATGGGT	Metabion, Martinsried, Germany
LOXL3	fw	CGCAAGTGTGCGACAAAGG	Metabion, Martinsried, Germany
	rev	GGACACGGGCCTGTAGAAG	Metabion, Martinsried, Germany

Table 9 Human primers

Target (human)	Direction	Sequence (5 ' - 3 ')	Company, Headquarter
NLRP3	fw	GATGAGCCGAAGTGGGGTTC	Metabion, Martinsried, Germany
	rev	TCAATGCTGTCTTCCTGGCA	Metabion, Martinsried, Germany
REL	fw	GGCCTCCTGACTGACTGACT	Metabion, Martinsried, Germany
	rev	GGTTATACGCACCGGAGGC	Metabion, Martinsried, Germany
RORα	fw	TCGCAGCGATGAAAGCTCAAAT	Metabion, Martinsried, Germany
	rev	GCCTTCACATGTAATGACACCATA	Metabion, Martinsried, Germany
RORC2	fw	CAGTACTGAGAACACAAATTGAAGTG	Metabion, Martinsried, Germany
	rev	CAGGTGATAACCCCGTAGTGGAT	Metabion, Martinsried, Germany
18S	fw	GTAACCCGTTGAACCCCATT	Metabion, Martinsried, Germany
	rev	CCATCCAATCGGTAGTAGCG	Metabion, Martinsried, Germany
TBET	fw	GATGCGCCAGGAAGTTTCAT	Metabion, Martinsried, Germany
	rev	GCACAATCATCTGGGTCACATT	Metabion, Martinsried, Germany
TNF-α	fw	GCCAGAGGGCTGATTAGAGA	Metabion, Martinsried, Germany
	rev	TCAGCCTCTTCTCCTTCCTG	Metabion, Martinsried, Germany

Table 10 Mouse primers

Target (mouse)	Direction	Sequence (5 ' - 3 ')	Company, Headquarter
Lingo4	fw	AGGCGACTGGACACTATTCC	Metabion, Martinsried, Germany
	rev	TCAGGGTGAGTAGACTTTGT	Metabion, Martinsried, Germany
RORγt	fw	GATCTAAGGGCTGAGGCACC	Metabion, Martinsried, Germany

Table 10 Mouse primers

Target (mouse)	Direction	Sequence (5 ’ - 3 ’)	Company, Headquarter
	rev	AGGGATCACTTCAATTTGTG	Metabion, Martinsried, Germany

2.9 Vectors

Table 11 Vectors

Vector	Company, Headquarter
pLenti-GIII-CMV-C-term-HA	ABM, Richmond, Canada
pLenti-GIII-CMV-FOXO4-C-term-HA	ABM, Richmond, Canada
pLenti-GIII-CMV-GFP-2A-Puro	ABM, Richmond, Canada
shRNA Lentiviral vectors	ATCGbio, Burnaby, Canada
ViraSafe Lentiviral Packaging System	Cell Biolabs, San Diego, USA

2.10 small hairpin RNA

Table 12 small hairpin (sh) RNA

shRNA	Sequence (from 5 ’ - 3 ’)	Company, Headquarter
sh RNA FOXO4	ACATATCGGCTTCTTCACGGTTT	ATCGbio, Burnaby, Canada
shRNA LINGO4	GGCTTAGAGAACTGGATAT	ATCGbio, Burnaby, Canada
shRNA ctrl	-	ATCGbio, Burnaby, Canada

2.11 Consumables

Table 13 Consumables

Consumables	Company, Headquarter
6-well TC plate	Falcon (BD), Franklin Lakes, USA
24-well Non-tissue Culture Treated Plate (for T cells)	Falcon (BD), Franklin Lakes, USA
96-well Non-tissue Culture Treated Plate flat (for T cells)	Falcon (BD), Franklin Lakes, USA
96-well TC plate, flat	Sarstedt, Nümbrecht, Germany
96-well TC plate, round	Sarstedt, Nümbrecht, Germany

Table 13 Consumables

Consumables	Company, Headquarter
348-well Microplate Frame Star (for qRT-PCR)	4titude, Dorking, UK
Acquity UPLC M-Class HSS T3 Column (1.8 μm, 75 μm x 250 mm)	Waters, Milford, USA
Amicon Ultra-15 Centrifuge Filter Devices	EMD Millipore Corp., Billerica, USA
Chamber slides 8-well	Thermo Fisher Scientific, Waltham, USA
Cluster tubes	Thermo Fisher Scientific, Waltham, USA
CoStar Assay plate, 96-well, flat (for ELISA)	Corning, Corning, USA
Cover slips	Menzel, Braunschweig, Germany
CryoPure tubes	Sarstedt, Nümbrecht, Germany
Culture flasks (T75, T175)	Greiner Bio-one, Frickenhausen, Germany
Cytoclips	Thermo Fisher Scientific, Waltham, USA
Cytospin Filter Cards	Thermo Fisher Scientific, Waltham, USA
Cytospin Funnel Chamber	Thermo Fisher Scientific, Waltham, USA
Falcon tubes (15 ml, 50 ml)	Greiner Bio-one, Frickenhausen, Germany
Filter 30 kDa cut-off	Sartorius, Göttingen, Germany
Filter flasks Filtropur (250 ml, 500 ml)	Sarstedt, Nümbrecht, Germany
Gels 4-12 % Bolt Bis-TrisPlus	Invitrogen, Carlsbad, USA
Innoculation loop	Sarstedt, Nümbrecht, Germany
Microscope Slides Superfrost	Menzel, Braunschweig, Germany
MicroTubes SafeSeal (1.5 ml, 2 ml)	Sarstedt, Nümbrecht, Germany
Nano Trap Column (300 μm inner diameter x 5 mm)	LC Packings, Sunnyvale, USA
PAXgene Tissue Container	Qiagen, Hilden, Germany
PCR 8-tube strips and caps	Greiner Bio-one, Frickenhausen, Germany
PetriDish (94x16)	Greiner Bio-one, Frickenhausen, Germany
Pipet tips epT.I.P.S Standard (20 μl, 200 μl, 1000 μl)	Eppendorf, Hamburg, Germany
Pipet tips SurPhob Safe Seal (10 μl, 100 μl, 200 μl, 1250 μl)	Biozym Scientific, Hessisch Oldendorf, Germany
Plastic pipets (1 ml, 5 ml, 10 ml, 25 ml)	Greiner Bio-one, Frickenhausen, Germany
PVDF Immobilon-P Membrane	EMD Millipore Corp., Billerica, USA
qPCR Seal	4titude, Dorking, UK
SurePrint G3 Human GE 8X60K BeadChip	Agilent Technologies, Santa Clara, USA
Syringes Omnifix (5 ml, 10 ml, 50 ml)	Braun, Melsungen, Germany
Syringe filters (0.22 μm, 0.45 μm)	EMD Millipore Corp., Billerica, USA

Table 13 Consumables

Consumables	Company, Headquarter
Tissue Culture Dish (10 cm)	Sigma-Aldrich, St. Louis, USA
Whatman paper (Western Blotting Filter Paper)	Thermo Fisher Scientific, Waltham, Germany

2.12 Cell lines

Table 14 Cell lines

Cell line	Company, Headquarter
HEK 293LTV	Cell Biolabs, San Diego, USA
Jurkat Cells	Helmholtz Center Munich, Munich, Germany
Stellar Competent Cells	Clontech Takara, Mountain View, CA, USA

2.13 Sofware

Table 15 Software

Software	Company, Headquarter
Adobe Illustrator	Adobe Systems Incorporated, San Jose, USA
Agilent Feature Extraction software	Agilent Technologies, Santa Clara, USA
BD FACSDiva Software	Becton Dickinson, Franklin Lakes, USA
Byonic search engine 2.0	Proteinmetrics, San Carlos, USA
FlowJo V10	Tree Star, Ashland, USA
GraphPad Prism 6	GraphPad Software, San Diego, USA
ImageJ	Wayne Rasband
Intas ChemoStar	Intas Science Imaging, Göttingen, Germany
Intas GDS	Intas Science Imaging, Göttingen, Germany
Microsoft Office	Microsoft, Redmond, USA
Primer blast	NCBI, Bethesda, USA
Proteome Discoverer 2.1	Thermo Fisher Scientific, Waltham, USA
Spectronaut 10	Biognosys, Schlieren, Switzerland
ViiA v1.0	Applied Biosystems, Foster City, USA

3. Methods

3.1 Ethical statement and study participants

For transcriptome analysis of skin lesions, data from 24 patients with psoriasis (mean age: 42±14.7 years; 67.6 % male, Psoriasis Area and Severity Index [PASI] score: 6.2 to 43.2, mean 14.9), 15 patients with AE (37±20.7 years; 46 % male, SCORAD score: range 13.3 to 55.5, mean 38) and 26 healthy volunteers (mean age: 48±14.2 years, 65 % male) were used that have been pre-published by Quaranta *et al.* and were re-analyzed for this study [Quaranta et al., 2014]. Severity scores were obtained using the PASI and SCORAD system, respectively [Quaranta et al., 2014]. For blood analysis, 12 psoriasis patients and 10 healthy donors were enrolled. All patients and healthy volunteers gave their written consent to participate in the study. The study was approved by the local ethical committee (project number 5060/11).

3.2 Primary cells and cell lines

Working with primary cells was performed under a sterile bench of biosafety level 1 or 2, respectively. All materials used for cell culture were sterile, either cleaned, disinfected, sterilized or autoclaved. All media were sterile filtrated before use. If not indicated otherwise, cells were cultured at 37 °C, 5 % CO_2 and 100 % air humidity.

3.2.1 Isolation of PBMCs from human blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole venous heparinized blood taken from patients/healthy donors. PBMCs were isolated by Ficoll-Hypaque (lymphoprep) making use of the density differences between the different blood cell types for separation. By this method, monocytes, T and B lymphocytes as well as NK cells can be separated from other blood cells like erythrocytes or neutrophils.

- A 50 ml syringe was filled with 100 μl Heparin
- Blood was drawn from a forearm vein after local skin disinfection using an adaptor for the 50 ml syringe
- 15 ml lymphoprep were filled into a sterile 50 ml tube
- Blood was diluted 1:2 with PBS^{def}
- 25 ml of diluted blood were carefully layered onto the lymphoprep without mixing both solutions and centrifuged at RT, 1000x g, 15 min with brake switched off
- PBMC band was collected with a 5 ml pipette. Two bands were combined in a new sterile

50 ml tube

- The tube was filled up to 50 ml with PBS^{def} + 5mM EDTA and centrifuged at RT, 530x g, 10 min
- The liquid was discarded without disturbing the PBMC pellet
- Two PBMC pellets were combined, the tube was filled up to 50 ml with PBS^{def} + 5mM EDTA and centrifuge at RT, 300x g, 10 min
- Again, two PBMC pellets were combined, filled-up to 50 ml with PBS^{def} + 5mM EDTA and centrifuged at RT, 300x g, 10 min
- Cell number was determined

3.2.2 Isolation of cell populations via Magnetic-Activated Cell Sorting (MACS)

For isolation of different cell types from PBMCs via magnetic-activated cell sorting (MACS), kits from Miltenyi Biotec were used. Cell isolation was performed following the manufacturer's instructions. Isolation steps were performed by using the AutoMACS Pro device (Miltenyi Biotec).

Table 16 MACS kits used for cell isolations

Kit	Isolated cell type	Fraction with desired cells
$CD4^+$ T Cell Isolation Kit, human	$CD4^+$ T cells	neg fraction
$CD14^+$ MicroBeads, human	monocytes	pos fraction
$CD45RO^+$ MicroBeads	$CD45RO^+$ T cells	pos fraction
$CD45RO^+$ MicroBeads	$CD45RO^-$ T cells	neg fraction

Naive T cell isolation

For naive T cell isolation, PBMCs were isolated as described in 3.2.1. Untouched naive T cells were then purified in a two-step MACS separation using first "$CD4^+$ T Cell Isolation Kit" (Miltenyi Biotec) and then "$CD45RO^+$ MicroBeads" (Miltenyi Biotec) for depletion of effector T cells. For both kits, separation program "depletes" was used whereby the negative fraction contained the untouched cells of interest.

Isolation of monocytes

For monocyte isolation, PBMCs were isolated as described in 3.2.1. Subsequently, monocytes were isolated via their surface marker CD14 using the "CD14 MicroBeads" (Miltenyi Biotec) following the manufacturer's instructions. For separation, program "possel" was used. The positive fraction contained the bound monocytes.

3.2.3 T cell stimulation

T cells can be stimulated in different ways, depending on the desired read-out. TCR-dependent stimulation by anti-CD3 and anti-CD28 antibodies was used to measure cytokines in the T cell cultures. For a more general and unspecific stimulation, PHA was used to stimulate PBMCs and $CD4^+$ T cells for *LINGO4* mRNA expression, and for cytokine analysis by intracellular flow cytometry staining PMA and Ionomycin were used. These stimuli can also be used for T cell stimulation in PBMCs.

Stimulation with anti-CD3/anti-CD28 antibodies

T cells can be stimulated *in vitro* with anti-CD3 (plate-bound) and anti-CD28 (soluble) monoclonal antibodies (mAbs) to activate the T cell receptor. This method is useful for the generation of supernatant to measure secreted cytokines in ELISA, for investigating gene expression on mRNA level; or expansion of T cells, e.g., derived from skin. Depending on the read-out, stimulation times varied. For analysis of mRNA expression, cells were stimulated for 6 hours as transcription is quickly upregulated. For generation of ELISA supernatants, a defined cell number was restimulated for 48-72 hours as protein translation takes longer than mRNA transcription and therefore potential effects on protein level can only be observed after longer stimulation.

- Anti-CD3 mAb was diluted to a final concentration of 0.75 μg/ml in PBS^{def}
- 100 μl were added to a 96-well or 1 ml to a 24-well
- Plate was incubated at 37 °C for 2 h
- T cells were resuspended and adjusted to $1x10^6$ cells/ml
- Anti-CD3 mAb solution was discarded and 100 μl with $1x10^5$ cells or 1 ml with $1x10^6$ cells/ml, respectively, were added
- 100 μl or 1 ml of fresh T cell culture medium containing 1.5 μg/ml anti-CD28 mAb were added (final concentration 0.75 μg/ml)
- For ELISA supernatant generation, T cells were restimulated for 48 - 72 hours at 37 °C. Cell-free supernatant was transferred into a new plate/tube and stored at -80 °C.
- For mRNA analysis, T cells were restimulated for 6 h at 37 °C. T cells were resuspended and transferred into a new tube. Well was washed with 1 ml of PBS^{def} and cell suspension was centrifuged at 300x g for 10 min. Up to $3x10^6$ cells were resuspended in 350 ml lysis buffer and stored at -80 °C.

Stimulation with PHA

- 1 - 2x10^6 T cells/ $CD4^+$ cells or 3x10^6 PBMCs, respectively, were seeded per 24-well in 5 % T cell medium
- Cells were stimulated with 10 μg/ml PHA for 4 hours at 37 °C
- Cells were harvested, well was washed once with cold PBS and cells were centrifuged
- For mRNA analysis, cells were resuspended in RNA lysis buffer and stored at -80 °C

3.2.4 T cell differentiation

Naive T cells were differentiated into T helper cell subsets using different combinations of cytokines and neutralizing antibodies in addition to the T cell receptor stimulation via anti-CD3/CD28 mAbs.

For differentiation, either untouched naive $CD4^+CD45RA^+$ T cells, isolated by a two-step MACS procedure described in 3.2.2, or untouched $CD4^+$ T cells, isolated using the "$CD4^+$ T Cell Isolation Kit" (Miltenyi Biotec), were used. Differentiation was performed in 24-well plates with 1x10^6 cells per well for 6 - 7 days. The distinct cytokine combinations used to differentiate T helper subsets are listed in Tables 17 - 20. Th17 differentiation was performed in AIM-V medium, supplemented with 1 % HS without IL-2 from day 2/3 on, in a total volume of 1.5 ml per well. Medium was changed every 2 - 3 days.

Th1 and Th2 differentiation were performed in 2 ml of 1 % T cell medium. Medium was changed every 2 - 3 days, supplemented with 20 U/ml of IL-2.

Th22 differentiation was performed with 1 % T cell medium in a total volume of 2 ml. Medium was changed on day 5 and cells were withdrawn from anti-CD3 coated well. For medium change, medium was supplemented with 20 U/ml IL-2.

For cytokine analysis by ELISA, cell culture supernatant was taken directly from differentiation culture at the last day/indicated time point or a defined cell number (150000/96-well) was restimulated for 72h with anti-CD3/CD28 mAbs. For mRNA analysis, 1x10^6 were optionally restimulated with anti-CD3/CD28 mAbs for 6 hours.

Table 17 Cytokine/antibody cocktail for Th17 differentiation

Cytokine/Antibody	Stock concentration	Final concentration
IL-1β	10 μg/ml	20 ng/ml
IL-6	20 μg/ml	30 ng/ml
IL-23	20 μg/ml	30 ng/ml
TGF-β	10 μg/ml	3 ng/ml
anti-CD28	1 mg/ml	1 μg/ml
anti-IFN-γ	1 mg/ml	1 μg/ml
anti-IL-4	1 mg/ml	5 μg/ml

Table 18 Cytokine/antibody cocktail for Th1 differentiation

Cytokine/Antibody	Stock concentration	Final concentration
IL-12	10 μg/ml	25 ng/ml
anti-CD28	1 mg/ml	1 μg/ml
anti-IL-4	1 mg/ml	5 μg/ml

Table 19 Cytokine/antibody cocktail for Th2 differentiation

Cytokine/Antibody	Stock concentration	Final concentration
IL-4	10 μg/ml	40 ng/ml
anti-CD28	1 mg/ml	1 μg/ml
anti-IL-12	1 mg/ml	5 μg/ml

Table 20 Cytokine/antibody cocktail for Th22 differentiation

Cytokine/Antibody	Stock concentration	Final concentration
IL-6	20 μg/ml	50 ng/ml
TNF-α	100 μg/ml	20 ng/ml
anti-CD28	1 mg/ml	1 μg/ml
anti-IFN-γ	1 mg/ml	1 μg/ml
anti-IL-4	1 mg/ml	5 μg/ml
anti-IL-12	1 mg/ml	5 μg/ml

3.2.5 Isolation and cultivation of human primary fibroblasts

Fibroblasts were isolated from lesional skin biopsies of psoriasis or atopic eczema patients. Biopsies were kept in T25 flasks with primary fibroblast medium until confluency was reached. Medium was changed once a week. Cells of one flask were frozen in freezing medium in one vial and kept at -80 °C. Thawed fibroblasts were cultured in 6-well plates with primary fibroblast medium until reaching appropriate confluence for experiments. Fibroblasts were cultured at 37 °C, 6.5 % CO_2 and 100 % air humidity.

3.2.6 Cultivation of primary keratinocytes

Primary human keratinocytes were isolated from healthy donors by suction blister. This method was already described in the 1960s for mechanical separation of epidermis from dermis ([Kiistala and Mustakallio, 1964, Kiistala, 1968, Albanesi et al., 2000]). Isolation was performed with some modifications. “Briefly, blisters were induced by generating a vacuum on normal skin of the forearms. Epidermal sheets were obtained from blister roofs, treated with 0.05 % trypsin to obtain single cell suspension, and seeded on a feeder layer of [Mitomycin-treated] 3T3/J2 fibroblasts in modified Green‘s medium. At 70 – 80 % confluence, keratinocytes were detached with 0.05 % trypsin, aliquoted, and cryopreserved in liquid nitrogen” (passage P1) [Eyerich et al., 2009]. For experiments, passage P1 keratinocytes of healthy donors were thawed and grown in 6-well plates for 5 - 7 days until reaching 70 - 80 % confluency. For culture, LifeLine medium supplemented with the delivered supplements and hydrocortison was used. Medium was changed the day after thawing.
For stimulation of keratinocytes with IFN-γ, cells were starved with basal LifeLine medium for 6 hours previous to 12 hour stimulation with 50 ng/ml IFN-γ in LifeLine medium with supplements but without hydrocortison. Cells were harvested for RNA isolation by using trypsin.
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3.2.7 Cultivation of HEK cells

HEK cells were cultured in HEK medium containing 10 % FCS and 1x P/S. Confluent cells were splitted in a T75 flask every second day in a ratio of 1:5. For transfection, HEK cells were cultured in HEK medium containing 10 % FCS w/o P/S. For virus supernatant production, they were cultured in HEK medium containing 2 % FCS w/o P/S.

3.2.8 Cultivation of Jurkat T cells

Jurkat T cells were cultured at numbers around 15x10^6/50 ml Jurkat medium in T75 flasks. Confluent cells were splitted every second day in a 1:4 or 1:5 ratio (approx. 0.3x10^6/flask).

3.2.9 Freezing and thawing of cells

Cells were preserved in liquid nitrogen in the corresponding freezing medium (Tab 21). Freezing procedure:

- PBMCs, T cells, monocytes, fibroblasts or keratinocytes were centrifuged at RT, 300x g, 10 min, HEK cells at 350x g, 5 min, 4 °C
- Cells were resuspended in 1 ml of cold freezing medium per defined cell number and immediately cooled
- Cells were transferred to a freezing container and put at -80 °C

- Cells were transferred to liquid nitrogen the next day

Table 21 Freezing media for different cell types and cell numbers

Cell type	freezing medium	cell number/ml
HEK cells	DMEM 40 % FCS + 10 % DMSO	$5x10^6$
Fibroblasts	DMEM 40 % FCS + 10 % DMSO	$0.5x10^6$
Keratinocytes	DMEM 40 % FCS + 10 % DMSO	$3x10^6$
Monocytes	90 % FCS + 10 % DMSO	$3x10^6$
PBMCS	RPMI + 40 % FCS + 10 % DMSO	$25x10^6$
T cells	RPMI + 40 % FCS + 10 % DMSO	$10x10^6$

Thawing procedure:

- Cells were removed from liquid nitrogen and put into hot water
- As soon as suspension was thawed cells were transferred into 15 ml of medium to dilute DMSO contained in freezing medium
- Cells were centrifuged at 4 °C, 300x g, 10 min or 350x g, 5 min (HEK cells)
- Cells were resuspended in appropriate amount of medium and cell number was determined

3.2.10 HEK cell transfection and lentiviral transduction of T cells

Lentiviral transduction is a system for permanent gene transfer into the genome of cells. Contrary to retroviruses, lentiviruses can also infect non-dividing cells, making them a precious tool for overexpression or knockdown of genes of interest for different kinds of cells. Lentiviral vectors are restricted to the genes necessary for stable integration of the target gene into the genome of the host cell. Therefore, virus particles cannot replicate in transduced cells anymore. For lentivirus production, one transfer and three packaging vectors of the third generation were used. HEK 293 LTV was used as packaging cell line.

Transfection of HEK cells and virus production

Day 0: Transfection of HEK 293 LTV cells for RNA isolation, protein lysates, immunofluorescence staining or virus production

- In the morning, $3.2x10^6$ HEK cells/ 10 cm dish/15 ml were plated in HEK medium containing 10 % FCS w/o P/S for virus production to reach semi-confluence in the evening. For the other approaches, adequate cell numbers (to reach 70 - 80 % confluence at transfection time point) were plated in HEK medium containing 10 % FCS w/o P/S (Tab 23)
- In the evening, cells were transfected with Turbofect and vector ratios of 4:1:1:2 (transfer vector : pCMV-VSV-G : pRSV-REV : pCgpV) for virus production (Tab 22). For other

approaches, cells were transfected with Turbofect and transfer vector (Tab 23)

- Vectors were diluted in Polypropylene tubes with DMEM (without supplements) and gently mixed
- Transfection reagent Turbofect was briefly vortexed, 30 μl added to the diluted vectors and mixed immediately by pipetting or vortexing
- Mixture was incubated for 15-20 min at RT
- 1.5 ml of the DNA/Turbofect complex were added drop-wise to the 10 cm dish. Plates were gently rocked back and forth for mixing
- Incubation at 37 °C in 5 % CO_2, overnight

Table 22 Pipet scheme for transfection of HEK cells for virus production with 15 μg total DNA

Vector	Amount/10 cm dish
DMEM	ad 1500 μl
transfer	7.5 μg
pCMV-VSV-G	1.875 μg
pRSV-REV	1.875 μg
pCpgV	3.75 μg
Turbofect	30 μl

Table 23 Scheme for HEK transfection

Format	Cell number/volume	DNA for transfection	Turbofect
10 cm dish	3.2x10^6/15 ml	15 μg transfer vector	30 μl ad 1500 μl DMEM
6-well plate	0.51x10^6/4 ml	4 μg transfer vector	6 μl ad 400μl DMEM
8-well chamber slide	0.05x10^6/250 μl	0.25 μg transfer vector	0.4 μl ad 25 μl DMEM

Day 1: Medium was changed in the morning with 6 ml of pre-warmed HEK medium containing 2 % FCS w/o P/S for virus production. For the other approaches, medium volume was adjusted to plate size.

Day 3: Harvesting of cells (for RNA isolation, protein lysates and immunofluorescence staining)

- Cell culture medium was removed completely and ice-cold PBS^{def} was added to cells
- Cells were resuspended and collected. Well was washed once with ice-cold PBS^{def} to collect remaining cells.
- Centrifuged at 350x g, 5 min, 4 °C
- For RNA isolation, cells were resuspended in RNA lysis buffer and stored at -80 °C until use. For protein lysates, cells were resuspended in RIPA buffer. For immunofluorescence stainings, cells were fixed in PFA.

T cell transduction

Day 2: 1st Infection

T Cell Isolation

- PBMCs were isolated as described in 3.2.1
- Naive T cells were purified by MACS separation by first $CD4^+$ via "CD4 T cell Isolation Kit" (Miltenyi Biotec), followed by depletion of CD45RO cells using "CD45RO MicroBeads" (Miltenyi Biotec) as described in 3.2.2
- $1.5x10^6$ T cells /24-well were used

Harvesting of virus supernatant, 6x concentration, 1st T cell infection

- Virus supernatants were harvested 24 h after medium change and were replaced by 6 ml pre-warmed HEK medium containing 2 % FCS w/o P/S.
- Supernatants were centrifuged at 350x g for 5 min to remove cell debris and were filtered on 0.45 μm filter
- Virus supernatant was 6x concentrated using Amicon Ultra-15 Centrifugal Devices, 100 kDa (pre-rinsed with PBS^{def}): centrifuged at 1500x g for 5 - 10 min until desired volume was reached
- Virus supernatant was filtered on 0.45 μm filters and filled up to required volume with HEK medium containing 2 % FCS w/o P/S (1 ml/24-well for infection) and supplemented with 10 U/ml IL-2 and 6 μg/ml polybrene
- T cells were resuspended in virus supernatant and spin-infected at 800x g, 90 min, 32 °C
- Virus supernatant was removed and 2 ml of pre-warmed 1 % T cell medium or AIM-V medium supplemented with 10 U/ml IL-2 were added

Day 3: 2nd Infection

as Day 2. HEK cells were analyzed by flow cytometry for GFP expression (empty vector, shRNAs)

Day 4: Puromycin selection/ Cell harvesting/ Th17 differentiation

Transduced cells were selected by supplementing puromycin (0.2 μg/ml) and, depending on the experimental setup, either harvested for RNA isolation, protein lysates or immunofluorescence staining. Further, cells were stimulated with anti-CD3/CD28 mAbs for 6 h or *in vitro* differentiated into Th17/Th22 cells for 7 d (3.2.4).

3.3 Molecular biological methods

3.3.1 RNA isolation from (primary) cell culture cells

RNA was isolated from (primary) cell culture cells by using the column-based "InviTrapSpin Universal RNA Mini Kit" (Stratec Biotechnology) following the manufacturer's instructions. Workplace and pipets were cleaned with RNase Zap prior to isolation to prevent degradation of RNA. Briefly, cells were lysed in provided Lysis Buffer supplemented with 100 mM DTT and stored at -80 °C until isolation. Samples were thawed on ice and applied to a DNA-binding column to remove DNA. RNA was precipitated by adding 70 % ethanol and applied to a RNA-binding column. Bound RNA was washed twice with provided washing buffers and remaining ethanol was removed by centrifugation. RNA was eluted in 30 μl of elution buffer and concentration as well as purity was determined by spectrophotometric measurement via NanoDrop.

3.3.2 RNA isolation from mouse tissue

B6.129P2-Rorctm1Litt/J mice (The Jackson Laboratory, Bar Harbor, USA) with a complete knock-out of the *Ror*γ gene, depleting both isoforms *Ror*γ and *Ror*γt (*Ror*γt (-/-)), and heterozygous mice were used, bred and kindly provided by Dr. Caspar Ohnmacht and Maria Fedoseeva, Helmholtz Center Munich. RNA from thymus and small intestine was isolated using the "miRNeasy Mini Kit" (Qiagen), following the manufacturer's instructions for "Purification of Total RNA from Animal Tissue". Tissues were first chopped using TissueLyser. To remove DNA traces, samples were incubated with DNase.

3.3.3 RNA isolation from skin biopsies

Total RNA isolation from skin and microarrays were performed by Maria Quaranta and microarray data was published by our working group in 2014 [Quaranta et al., 2014].

RNA of skin biopsies was isolated using the "PAXgene Tissue RNA Kit" (Qiagen) following the manufacturer's instructions. Briefly, biopsies were stored in Paxgene Tissue Containers until use. Tissue was cut into small pieces and further chopped using TissueLyser, followed by the column-based RNA isolation. RNA concentration and integrity was measured by NanoDrop and Bioanalyzer, respectively.

3.3.4 cDNA synthesis

RNA was reversely transcribed into cDNA for subsequent quantitative real-time PCR (qRT-PCR) (3.3.5) with the "High capacity cDNA Reverse Transcription Kit" (Applied Biosystems). Up to 1 μg RNA in a total volume of 20 μl was transcribed, depending on the sample con-

centration. RNA was diluted with DEPC-treated water up to a volume of 14.2 μl and 5.8 μl of reaction mix were added (Tab 24 and 25).

Table 24 Reaction mixture for reverse transcription

Substance	Stock concentration	End concentration
RT Buffer	10x	1x
RT Random Primers	10x	1x
dNTP Mix	25x (100 mM)	1x (4 mM)
Reverse Transcriptase	50 U/μl	2.5 U/μl

Table 25 cDNA synthesis reaction conditions

Step	Temperature	Time
1	25 °C	10 min
2	37 °C	120 min
3	85 °C	5 min
4	4 °C	∞

3.3.5 Quantitative real-time PCR (qRT-PCR)

Gene expression was analyzed by qRT-PCR. Therefore, cDNA was mixed with “Fast Start Universal SYBRGreen Master (Rox) Mix” (Roche), containing FastStart Taq DNA Polymerase, reaction buffer and nucleotides as well as SYBR Green I. Target specific forward and reverse primers were added. As reference, housekeeping genes EF1α or GAPDH were used. 10 ng cDNA was used in each reaction performed in 384-well plates. cDNA was diluted with DEPC-treated water to a final volume of 3.4 μl per reaction and added to 6.6 μl reaction mix, consisting of SYBR Green Mix and primers (Tab 26 and 27). Primers are listed in 2.8.
Data was analyzed by the Δ CT or $\Delta\Delta$ CT method and is thereby presented as Δ CT values normalized to the housekeeping gene or as log2 expression when normalized to the housekeeping gene and a control condition ($\Delta\Delta$ CT method).

Table 26 Reaction mixture for qRT-PCR

Substance	Stock concentration	End concentration
Fast Start Universal SYBR-Green Master (Rox)	2x	1x
Primer fw	100 μM	0.64 μM
Primer rev	100 μM	0.64 μM
DEPC-treated H_2O		ad 6.6 μl

3.3.6 Nested PCR

For nested PCR, two primer pairs were designed using NCBI primer blast software. In a first step, PCR reaction was performed with 100 - 200 ng of cDNA and an outer primer

Table 27 qRT-PCR reaction conditions

Step	Temperature	Time	Cycles
Hold stage	50 °C	2 min	1x
	95 °C	10 min	
PCR stage	95 °C	15 s	40x
	60 °C	1 min	

pair. Therefore, “Phusion Hot Start II Kit” (Thermo Fisher Scientific) was used following the manufacturer’s instructions and using buffer for GC-rich regions. Annealing temperatures were adjusted to primer pairs (outer primer pair: 59.4 °C; inner primer pair: 64.3 °C). 1 μl of the reaction product was used for the second reaction using the same kit (Tab 28). Products of both reactions were analyzed by agarose gel electrophoresis (3.3.7).

Table 28 Nested PCR reaction conditions

Step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1x
Denaturation	98 °C	10 s	35x
Annealing	59.4/64.3 °C	30 s	
Extension	72 °C	30 s/kb	
Final Extension	72 °C	10 min	1x
Hold stage	4 °C	∞	

3.3.7 Gel electrophoresis of DNA

Agarose gel electrophoresis was used to verify successful PCR reactions, purify DNA or qualitative analysis.

- 1 % Agarose was diluted in 1x TAE buffer and boiled
- Liquid was briefly cooled down, 1x DNA dye peqGreen (stock concentration 20000x) was added and gel was casted
- Electrophoresis chamber was filled with 1x TAE and gel was placed in buffer
- DNA samples were diluted with 6x loading buffer and placed on the gel
- As reference, gene ruler 1 kb was loaded (0.5 μg)
- Gel was run for 5 min at 80 V, then for 30 - 45 min at 120 V
- DNA bands were detected by UV exposure and documented by Gel Imaging System
- When required, relevant bands were cut from the gel and purified via “QIAquick Gel Extraction Kit” (Qiagen) following the manufacturer’s instructions
- DNA concentration was determined using spectrophotometric measurement via NanoDrop

3.3.8 Cloning of pLenti-GIII-CMV-LINGO4-HA vector

For analysis of LINGO4 function, a lentiviral-mediated overexpression system was used. The LINGO4 transfer vector was generated by including the LINGO4-specific sequence into the commercially available pLenti-GIII-CMV-C-term-HA vector. For cloning, “In-Fusion HD Cloning Kit” (Clontech) was used following the manufacturer’s protocol. Successful linearization of the vector prior to cloning as well as successful insertion of the target sequences after cloning was confirmed by agarose gel electrophoresis and renewed vector digestion with FastDigest Eco321 (Fig 4).

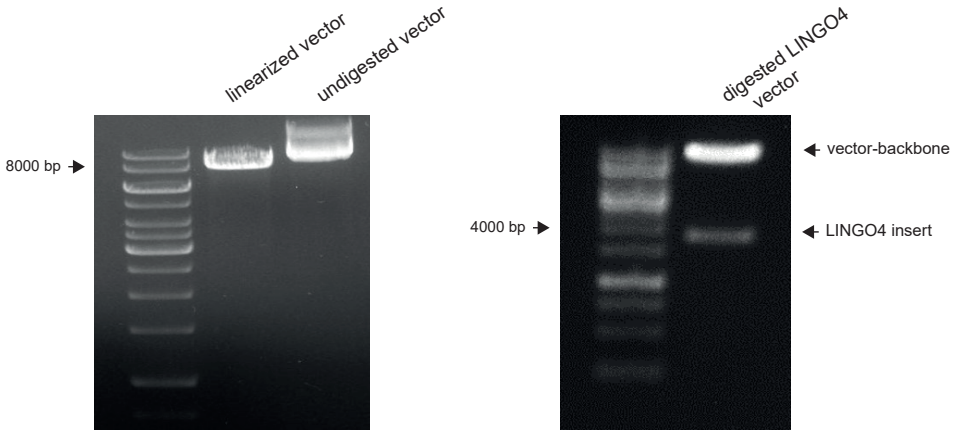

Figure 4 Validation of vector cloning
Linearized vector was applied to a gel after purification. As control, undigested vector was loaded (left). Cloned LINGO4 vector was digested with FastDigest Eco32l and loaded on a 1 % agarose gel to verify right fragment formation and successful target gene insertion (right).

Transformation

For Transformation, Stellar Competent Cells were used according to manufacturer’s instruction. Successfully transformed cells were selected by antibiotics appropriate for the corresponding selection cassette in the transformed vector (Tab 29).

Table 29 Antibiotic resistances of vectors

Vector	Antibiotic resistance
pLenti-GIII-CMV-FOXO4-C-term-HA	kanamycin
pLenti-GIII-CMV-GFP-2A-Puro	kanamycin
pLenti-GIII-CMV-LINGO4-C-term-HA	kanamycin
shRNA Lentiviral vector	ampicillin
ViraSafe Lentiviral Packaging System	ampicillin

MiniPrep

For further clone amplification, mini cultures of the picked clones were started.

- Per clone, 14 ml round bottom tube were prepared with 3.5 ml of 2.5 % LB medium supplemented with antibiotics (Tab 29)
- Clones were picked and cultures were incubated for 12-16 h at 37 °C, 200 rpm
- Mini cultures were stored at 4 °C until use
- Vector was purified using "QIAprep spin Miniprep Kit" (Qiagen) following the manufacturer's instructions
- Vector purification was analyzed by agarose gel electrophoresis

MaxiPrep

- 200 - 300 ml of 2.5 % LB medium supplemented with antibiotics were inoculated with 100 μl of the Mini culture
- Incubation for 12-16 h at 37 °C, 200 rpm
- Culture was centrifuged at 4000x g for 15 min
- Vector was isolated using the "EndoFree Plasmid Maxi Kit" (Qiagen) following the manufacturer's instructions
- DNA was resuspended in 100 μl of deionized H_2O and DNA concentration was determined via NanoDrop

3.3.9 Measurement of DNA and RNA concentration

Concentration of DNA or RNA was determined in 1 μl of sample via spectrophotometric measurement using the NanoDrop system (Thermo Fisher Scientific). Absorption of nucleic acid was measured at 260 nm. To determine purity, the ratio of 260 nm/280 nm was used as it gives information about protein contamination. A ratio of 1.8 for DNA and 2.0 for RNA indicates high purity.

3.4 Protein biochemical methods

3.4.1 Production of whole cell lysates

Whole cell lysates were generated using the "RIPA Lysis buffer system" (Santa Cruz Biotechnology) for downstream analysis of proteins via western blot (WB). All steps were performed on ice.

- 1 - 3x10^6 cells were centrifuged and resuspended in 80 - 100 μl RIPA buffer supplemented with 1:75 proteinase inhibitor, 1:100 Na_3VO_4 and 1:100 PMSF (always prepared freshly)
- Lysates were shaken for 30 min at 4 °C

- Lysates were centrifuged for 10 min at 10000x g, 4 °C, and supernatant was taken
- Protein lysates were stored at -20 °C or -80 °C

3.4.2 Production of cytoplasmic extracts

Cytoplasmic protein extracts were generated using the "NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit" (Thermo Fisher Scientific) following the manufacturer's instructions. Cells were harvested in cold PBS^{def}, centrifuged and treated according to the protocol for "Cytoplasmic and Nuclear Protein Extraction". Protein concentration was determined by BCA assay.

3.4.3 BCA Assay

The protein concentration of cell lysates was determined using the "bicinchoninic acid assay (BCA) kit" (Pierce) according to the manufacturer's instructions.

3.4.4 SDS-Polyacrylamide gel electrophoresis

Proteins were separated under denaturing conditions according to their molecular weight by SDS-Polyacrylamide gelelectrophoresis (SDS-PAGE) for downstream analysis in western blot (WB) 3.4.5. For SDS-PAGE, Novex Bolt 4-12 % Bis-Tris Plus Gels (Invitrogen) were used according to the manufacturer's instructions in Mini Gel Tanks.

- 20 μg of protein were loaded per well
- Prior to gel loading, protein lysates were supplied with 4x loading buffer and filled up with RIPA buffer to a final volume of 30 μl, boiled at 96 °C for 5 min and quickly centrifuged.
- Tank with inserted gel cassette was filled with 1x MOPS running buffer and gel wells were washed before loading samples
- 5 μl of PageRuler were loaded on the gel
- Gel was run for 5 min at 80 V and for additional 45 - 60 min at 160 V.

3.4.5 Western blot

For specific detection of proteins by antibodies, proteins separated by SDS-PAGE were transferred from the SDS gel onto a PVDF membrane. For transfer, the "Mini Tank - Blot system" (Life Technologies) was used following the manufacturer's instructions. The membrane was either stained with specific antibodies directly coupled to horseradish peroxidase (HRP) for detection or with a primary target-specific antibody that was detected by a secondary antibody coupled to HRP. As substates for HRP, ECL or SuperSignal^{TM} West Femto Maximum Sensi-

tivity Substrate (Thermo Scientific) were used. The chemoluminescence produced by HRP-mediated conversion of luminol (substrate) was detected by the Intas Imaging System.

- 1x Transfer buffer supplemented with 10 % of methanol was prepared freshly
- PVDF membrane was activated for 15 s in methanol
- Whatman paper, sponges and membrane were equilibrated in transfer buffer
- Blot module was put together following the manufacturer's instructions and filled up with transfer buffer
- Proteins were transferred to the PVDF membrane for 75 min at 30 V
- After blotting, membrane was incubated in methanol for 15 s and air-dried for 15 min to reduce background
- For immune detection, membrane was activated for 15 s in methanol and incubated with blocking buffer (5 % milk powder, 1x TBS, 0.01 % Tween) for 1.5-2 h at RT, shaking
- Primary antibodies were diluted in blocking buffer (Tab 30) and membrane was incubated overnight at 4 °C, shaking. If using a target-specific HRP-coupled antibody, membrane was only incubated for 1 h at RT, shaking, followed by one washing step and direct detection.
- Membrane was washed 3x 10 min with TBST
- Secondary HRP-coupled antibody diluted in blocking buffer (Tab 31) was applied for 1 h at RT, shaking
- Membrane was washed 3x 10 min with TBST and once in TBS for 10 min
- For detection, either ECL or SuperSignal™ West Femto Maximum Sensitivity substrate was diluted according to the manufacturer's instructions and membrane was incubated in the dark for 1 - 5 min
- The ECL ChemoCam Imager system (Intas) was used for detection. Sequential integrate function was used over 1 - 15 min of detection

3.4.6 Immunoprecipitation

Specific proteins can be isolated by immunoprecipitation (IP) from whole cell extracts to identify and verify these proteins by, e.g., mass spectrometry. Therefore, specific antibodies against the target protein or an attached tag are binding to Protein A/G-coupled agarose beads. After binding of the bead-coupled antibody to its target, the complex is separated by a centrifugation step. This method also enables identification of potential binding partners if purification is not performed under denaturing conditions. For identification of LINGO4 protein, LINGO4-HA was overexpressed in HEK cells (3.2.10) and purified by bead-coupled anti-HA

Table 30 Human specific primary antibodies and dilutions for western blot

Primary antibody (clonality)	Dilution
mouse anti-β-Actin (mAb)	1:10000 in blocking buffer
rabbit anti-AHR (pAb)	1:1000 in blocking buffer
rabbit anti-FOXO4 (mAb)	1:1000 in blocking buffer
rabbit anti-GAPDH (mAb)	1:1000 in blocking buffer
mouse anti-HA-HRP (mAb)	1:1000 in blocking buffer
rabbit anti-LINGO4(mAb) Abcam (a)	1:1000 in blocking buffer
rabbit anti-LINGO4 (mAb) LSbio (b)	1:1000 in blocking buffer
rabbit anti-LINOG4 (mAb) Novus (c)	1:1000 in blocking buffer
rabbit anti-Ubiquitin (mAb)	1:1000 in blocking buffer

Table 31 Secondary HRP-coupled antibodies and dilutions for western blot

Secondary antibody	Dilution
goat anti-mouse	1:10000 in blocking buffer
goat anti-rabbit	1:10000 in blocking buffer

antibody.

- HEK cells were harvested in cold PBS^{def} (3 ml/10 cm dish) and washed with additional 2 ml PBS^{def}
- Cells were centrifuged at 350x g, 5 min, 4 °C
- Pellet was resuspended in 900 μl of RIPA buffer supplemented with inhibitors (3.4.1)
- Suspension was shaken for 25 min at 4 °C
- Suspension was centrifuged at 10000x g, 20 min, 4 °C
- In the meantime, beads were prepared: 20 μl/reaction of protein A beads for pre-clearance and 40 μl/reaction (corresponds to 20 μl packed beads) of anti-HA beads were used, washed 2x with 500 μl of RIPA buffer (centrifuged at 350x g, 1 min, 4 °C) and resuspended in 20 μl or 40 μl of RIPA buffer supplemented with inhibitors, respectively.
- Supernatant was transferred into a new tube. 100 μl were taken for WB analysis
- Protein A beads were added to pre-clear sample and shaken for 1 h, 4 °C
- After incubation, sample was centrifuged at 350x g, 1 min, 4 °C
- Supernatant was transferred into a new tube
- anti-HA beads (40 μl/reaction) were added and sample was incubated overnight at 4 °C, shaking
- Beads were washed with 750 μl of RIPA buffer supplemented with inhibitors (350x g, 1 min, 4 °C)
- Beads were washed 3x with 1x TBS (350x g, 1 min, 4 °C)

- Beads were resuspended in 30 μl of 1x Laemmli buffer and boiled for 15 min at 75 °C
- Sample was centrifuged and supernatant collected (bead free)
- Supernatant was stored at -80 °C until use

3.4.7 Enzyme-linked Immunoabsorbent Assay

Cytokine concentrations in cell-free culture supernatants were detected via Enzyme-linked Immunoabsorbent Assay (ELISA). Following the principle of a "Sandwich-ELISA", two different antibodies specific for the same cytokine but different epitopes are used. Therefore, a specific antibody (capture antibody) was immobilized to a 96-well Microplate (Corning). The target cytokine in the added cell culture supernatant was then captured by this antibody. For detection, a second biotinylated antibody specific for the same cytokine was used. HRP-coupled streptavidin was added in the next step to bind the biotinylated secondary antibodies. In a photometric reaction, the substrate TMB was converted by HRP, causing a change in absorption wavelength. Concentration of measured samples was then determined by comparison with standard curves done with known protein concentrations. ELISA kits for the cytokines IFN-γ, IL-17, IL-22, TNF-α (R&D) and IL-4 (BD) were used to determine cytokine concentrations in cell culture supernatants. ELISA was performed following the manufacturer's instructions.

3.5 Immunofluorescence

For immunofluorescence staining, cells were either directly grown in chamber slides or applied to slides by cytospin.

3.5.1 Chamber Slides

Chamber slides can be used to culture adherent cells and directly stain them in the same chamber slides afterwards. HEK cells were cultured (and transfected) in chamber slides, followed by immunofluorescence staining for overexpressed LINGO4-HA protein. 0.5x10^5 HEK cells/chamber slide/250 μl were seeded and transfected with the transfer vector (3.2.10).

3.5.2 Cytospin

Suspension cells like T cells can be applied to slides for immunofluorescence staining via cytospins.

- Cytotunnels were put together following the manufacturer's instructions by putting a filter paper on the slide and fixing the cytotunnel.
- 50.000 - 100.000 cells were resuspended in 7.5 % BSA in PBS^{def} and loaded on the

cytotunnels.

- Cytotunnels were centrifuged for 10 min at 460x g to apply cells to the slides.
- Slides were immediately used for immunofluorescence staining (3.5.3)

3.5.3 Immunofluorescence staining

All steps were performed with 100 μl liquid per spot. When many slides were stained at the same time containers were used for fixation, permeabilization and blocking. Excess liquid was carefully and thoroughly removed after blocking without disturbing the cell spot.

- For fixation, cell spots were covered with 4 % PFA and incubated for 15 min at RT
- Fixative was aspirated and slides were washed 3x with PBS^{def} for 5 min each
- For permeabilization, cell spots were covered with 100 % ice-cold methanol for 10 min at -20 °C
- Slides were washed 1x with PBS^{def} for 5 min
- For blocking, slides were incubated in blocking buffer (PBS^{def}, 5 % BSA, 0.3 % Triton X-100) for 60 min at RT
- Blocking buffer was aspirated and primary antibody, diluted in antibody dilution buffer (PBS^{def}, 1 % BSA, 0.3 % Triton X-100) was applied overnight at 4 ° (Tab 32).
- Slides were rinsed 3x in PBS^{def} for 5 min each
- Secondary fluorescently-labeled antibody diluted in antibody dilution buffer and combined with DAPI (1 μg/ml) was applied for 1-2 h at RT, protected from light (Tab 32)
- Slides were rinsed 3x with PBS^{def} for 5 min each
- Excess liquid was removed carefully around cell spots and coverslips were applied with sufficient amount of mounting medium
- To avoid drying out of the cell spots, coverslips were sealed with nail polish
- For long-term storage, slides were stored at 4 °C in the dark.

Table 32 Antibody concentrations for immunofluorescence staining

Antibody (clonality)	Label	Dilution
mouse anti-human HA (mAb)	-	1:100 in antibody diluent buffer
donkey anti-mouse (pAb)	NL557	1:500 in antibody diluent buffer

3.6 Flow cytometry

Flow cytometry, also known as FACS (fluorescent-activated cell sorting) analysis, allows the analysis of cells based on cell size and granularity. Each cell runs through a flow cell where it

passes laser beams of defined wavelengths and intensities. Fluorescent and scattered light are detected and used for quantification. The forward scatter (FSC) gives information about the size of cells, while the sideward scatter (SSC) determines the granularity. Further, surface proteins or intracellular components can be stained by fluorescently-labeled antibodies and quantified by flow cytometry. This method allows frequency determination of cells positive for different markers, but also can give information about, e.g., surface marker density by determining the mean fluorescence intensity. FACS analysis was performed using the LSR Fortessa flowcytometer (BD Bioscience) and analyzed with the FlowJo software.

3.6.1 Surface marker staining

- 100.000 - 500.000 cells were centrifuged at 800x g, 1 min in a 96-well round bottom plate
- Cells were washed 1x with FACS buffer
- Antibodies were diluted to working concentrations in FACS buffer, total staining volume 10 μl (Tab 33)
- Cells were resuspended in staining solution and incubated for 30 min at 4 °C, in the dark.
- Cells were washed 2x with FACS buffer, resupsended in 200 μl of FACS buffer, transferred to FACS cluster tubes and analyzed

Table 33 Antibody concentrations for flow cytometry

Antibody	Fluorochrom	Dilution
mouse anti-human CD4	APC-Cy7	1:20 in FACS buffer
mouse anti-human CD14	AF700	1:100 in FACS buffer
mouse anti-human CD45RA	V450	1:50 in FACS buffer

3.6.2 Intracellular cytokine staining

Intracellular staining of cytokines requires stimulation of the cells with a combination of PMA and ionomycin in presence of the protein transport inhibitors Monensin and Brefeldin A that prevent secretion of cytokines. This artificial stimulation gives an overview about the potential ability of a cell to produce cytokines, but does not reflect the cytokine secretion under physiological conditions. Further, cells have to be fixed and permeabilized prior to cytokine staining so that staining antibodies can penetrate the cell (Tab 34).

- Cells were resuspended in 1 ml RPMI/24-well
- Stimulation mix containing PMA, ionomycin and GolgiStop is prepared in 3x concentration in 500 μl of RPMI to final concentrations of 50 ng/ml PMA, 1 μg/ml ionomycin and 1:1430 GolgiStop
- 500 μl of stimulation mix were added to the plated cells, incubation for 2-3 hours at 37 °C

- GolgiPlug was diluted 1:100 in 166 μl RPMI and added to cells to a final dilution of 1:1000
- Incubation for another 2-3 hours at 37 °C
- Cells were harvested and washed 2x with cold PBS^{def} in 96-well round bottom plate
- 1:100 fixable Aqua solution was prepared in cold PBS^{def} and added to the cells
- Incubation for 30 min at 4 °C, in the dark
- Cells were washed 1x and fixed using Fix-solution (BD Bioscience) for 20 min, 4 °C in the dark
- Cells were washed 2x with FACS buffer and permeabilized in 1x Perm-solution (BD Bioscience) for one centrifugation step (800x g, 1 min)
- Cells were stained with antibody mix diluted in 1x Perm-solution for 30 min, RT, in the dark, slightly shaking
- Cells were washed 1x with FACS buffer, resuspended in 200 μl of FACS buffer and transferred to cluster tubes for measurement

Table 34 Antibody concentrations for intracellular cytokine staining

Antibody	Fluorochrom	Dilution
mouse anti-human IFN-γ	FITC	1:1000 in 1x Perm-solution
mouse anti-human IL-17A	PE	1:50 in 1x Perm-solution
mouse ant-human IL-22	eFluor660	1:20 in 1x Perm-solution
mouse anti-human TNF-α	AF700	1:1000 in 1x Perm-solution

3.7 Mass spectrometry analysis

Mass spectrometry analysis was performed in cooperation with the Research Unit Protein Science of the Helmholtz Center Munich (Dr. Stefanie Hauck and Marlen F. Lepper). The following steps were carried out by the cooperation partners. For analysis, HEK cells overexpressing LINGO4 (3.2.10) were generated and LINGO4-HA protein was isolated by immunoprecipitation (3.4.6). Further, *in vitro* differentiated Th1, Th2, Th17, Th22 as well as the corresponding control cells (3.2.4) were analyzed for LINGO4 protein expression.

3.7.1 Filter-aided sample preparation (FASP)

Approximately 300.000 *in vitro* differentiated Th17, Th1, Th2, Th22 and control cells corresponding to $\sim$10 μg of protein were lysed in 50 μl guanidine buffer (6 M guanidinium chloride, 100 mM Tris pH 8.5, 1x complete protease inhibitor). Cell lysates were boiled for 5 min at 95 °C and subjected to 5 min waterbath sonication. The entire protein lysate was subsequently digested with trypsin using a modified filter-aided sample preparation (FASP) protocol [Wisniewski et al., 2009]. Briefly, protein lysates were filled up to a volume of 200 μl with ABC buffer (50 mM ammonium bicarbonate) and reduced for 30 min at 60 °C with 1 μl of 1 M

dithiothreitol (DTT). Samples were diluted in urea buffer (8 M urea, 100 mM Tris pH 8.5) to a final concentration of 4 M urea. For alkylation of cysteine residues, 10 μl of freshly prepared 300 mM iodoacetamide (IAA) solution were added, and the samples were incubated in the dark for 30 min at RT. Unreacted IAA was quenched with 2 μl of 1 M DTT, followed by centrifugation of samples through a 30 kDa cut-off filter. The filter was washed 3x with urea buffer and 2x with ABC buffer by centrifugation for 15 min at 14000x g. On-filter digestion of proteins was performed with 1 μg Lys-C in 40 μl ABC buffer for 2 hours at RT, followed by 1 μg trypsin in 10 μl ABC buffer for 16 hours at 37 °C. The peptides were collected by centrifugation for 10 min at 14000x g, and the filters were washed with 20 μl ABC buffer containing 5 % ACN. Prior to mass spectrometric analysis, the peptides were acidified with 2 μl 100 % TFA.

3.7.2 Mass spectrometry

Proteomic analysis of *in vitro* differentiated Th17, Th1, Th2, Th22 and control cells was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in the data-independent acquisition (DIA) mode with a Q Exactive HF mass spectrometer coupled to a rapid separation liquid chromatography (RSLC) system. All samples were spiked with 1 injection unit of the HRM Calibration Kit for retention time indexing. Approximately 1 μg of sample was automatically loaded on the HPLC system, which was equipped with a nano trap column (packed with Acclaim PepMap100 C18, 5 μm, 100 Å). After 5 min, the peptides were eluted from the trap column and separated using reversed-phase chromatography using a gradient of 7 – 27 % acetonitrile (ACN) at a flow rate of 250 nl/min over a period of 90 min, followed by two short gradients of 27 – 41 % ACN (15 min) and 41 – 85 % ACN (5 min). After 5 min at 85 % ACN, the gradient was set back to 3 % ACN over a period of two minutes and allowed to equilibrate for 8 min. All ACN solutions contained 0.1 % TFA. The DIA method consisted of a full MS scan at 120000 resolution ranging from 300 to 1650 m/z with automatic gain control target set to $3x10^6$ and a maximum injection time of 120 ms. Subsequently, 37 DIA windows with a variable width spanning from 300 to 1650 m/z were acquired at a resolution of 30000. Normalized collision energy was set to 28, and the spectra were recorded in profile type.

3.7.3 Generation of a T cell spectral library

Prior to analysis of DIA LC-MS/MS raw data obtained for *in vitro* differentiated Th17, Th1, Th2, Th22 and control cells, a comprehensive human T cell spectral library was generated, specifically focusing on good proteomic coverage of LINGO4 protein. To this end, data-dependent acquisition (DDA) LC-MS/MS data comprising 18 raw files of different *in vitro* differentiated T cell subsets (control cells, Th1, Th2, Th17, Th22 (3.2.4)), LINGO4-HA-IP samples from HEK cells and naive T cells lentivirally transduced with LINGO4 overexpression vector which were all spiked with the HRM Calibration Kit, were analyzed using Proteome Discoverer. The latter two sample types were included in the library generation to guarantee good proteomic coverage of LINGO4 protein. Proteome Discoverer was embedded with Byonic search en-

gine. Database search identifications were filtered to satisfy the 1 % peptide and protein level false-discovery rate (FDR) and combined in a multi-consensus result file maintaining the 1 % FDR threshold. The peptide spectral library was generated in Spectronaut with default settings using the Proteome Discoverer combined result file. Spectronaut was equipped with the Swissprot human database (Release 2016.02, www.uniprot.org) with a few spiked proteins (Biognosys iRT peptide sequences). The resulting spectral library generated in Spectronaut contained 5880 protein groups and 104621 peptide precursors.

3.7.4 Label-free Quantification

DIA LC-MS/MS raw files were analyzed using Spectronaut with slightly modified default settings for the spectral library search. Spectronaut HTRMS converter was used to convert the raw files. In brief, the data and extracted Ion chromatogram (XIC) extraction settings were set to dynamic with a correction factor of 1. Automatic calibration mode was chosen with precision indexed retention time (iRT) enabled for applying the non-linear iRT calibration strategy. Peptide identification was filtered to satisfy a FDR of 1 %. Only proteotypic peptides were considered for protein quantification applying averaged precursor quantities based on MS2 area quantity. Data filtering was set to “q value complete” indicating that only peptide precursor signals passing the 1 % FDR threshold in all analyzed samples were considered for quantification. Lastly, cross-run normalization was enabled.

3.8 Statistics

Statistical analysis was performed using GraphPad Prism. Statistical significance was determined using Mann-Whitney test for unpaired and Wilcoxon test for paired samples and defined as *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Mean $\pm$ SD are presented additionally to single data points.
Data of whole genome expression arrays from both skin lesions of patients and T cell clone subsets were analyzed in R and statistical significance was determined by Welch 2-sample t-test [R Core Team, 2016]. This analysis was performed by Linda Krause, PhD student at the Institute of Computational Biology and Center of Allergy and Environment.

4. Results

The results are divided into two parts dealing with LINGO4 and FOXO4 as possible novel regulators of Th17 and Th22 cells, respectively. The first part focuses on a possible impact of LINGO4 on Th17 cells by analyzing mRNA expression profiles during *in vitro* Th17 differentiation and the correlation with RORC2, the key transcription factor of Th17 cells, followed by overexpression and knockdown of LINGO4 to obtain functional insight. This part closes with the investigation of *LINGO4* expression in lesional skin of patients affected by the inflammatory skin diseases psoriasis or atopic eczema.

The second part focuses on the transcription factor FOXO4 that was identified to be upregulated in Th22 cells by whole genome expression arrays. FOXO4 is investigated for its role in the regulation of IL-22 expression in T cells, especially in Th22 cells.

4.1 LINGO4 and its association with the Th17 subset

In preliminary experiments, T cell clones from different subsets were isolated from lesional skin or blood of patients suffering from the inflammatory skin diseases psoriasis or atopic eczema (AE). Clones were clonally expanded and their transcriptome analyzed by whole genome expression arrays. It was found that mRNA expression of LINGO4 - a protein not much is known about so far - was upregulated significantly in IL-17 expressing T cells representing either pure Th17 cells or IL-17$^+$IFN-γ^+ double positive T cells (Th1/Th17) as well as Th22 cells that share expression of AHR with Th17 cells (Fig 5).

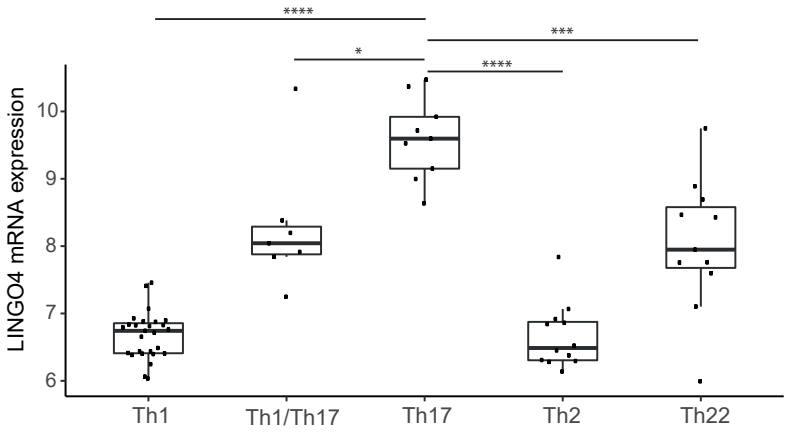

Figure 5 *LINGO4* mRNA expression is upregulated in Th17 or Th17-associated T cell clones. Whole genome expression arrays of different T helper cell clones. T cell clones were obtained from skin biopsies or blood of psoriasis and AE patients. Th1 n=26, Th17 n=9, Th2 n=12, Th22 n=11. Statistical significance was calculated by Welch 2-sample t-test and defined as *P<0.05, ***P<0.001, ****P<0.0001

To confirm the association of *LINGO4* mRNA expression within the Th17 subset, expression was investigated in other immune and non-immune cells as well as in *in vitro* generated T cell subsets. Furthermore, *LINGO4* mRNA expression was investigated in the kinetics of *in vitro* Th17 differentiation. Following validation of *LINGO4* expression on mRNA level, protein LINGO4 levels were investigated by western blot. This finding led to the hypothesis that *LINGO4* expression is involved in IL-17 regulation and differentiation of Th17 cells.

4.1.1 *LINGO4* is expressed in Th17 cells

LINGO4 expression in different immune and non-immune cells revealed up-regulation in Th17 cells

To validate *LINGO4* up-regulation in Th17 cells, different cell types were examined for *LINGO4* expression levels. Primary fibroblasts, keratinocytes and monocytes as well as PBMCs, $CD4^+$ T cells and *in vitro* differentiated Th17 cells and corresponding control cells were analyzed for *LINGO4* expression. Primary fibroblasts and keratinocytes were cultured as described in 3.2.5 and 3.2.6. PBMCs were isolated from blood of healthy donors (3.2.1). Subsequently, monocytes, $CD4^+$ T cells and naive T cells were isolated by MACS, the latter consecutively from $CD4^+$ cells in a two-step-protocol (3.2.2 and 3.2.2). Purity of isolated populations was analyzed by flow cytometry. Monocytes were identified by expression of the CD14 surface marker, $CD4^+$ T cells by CD4 and naive $CD4^+$ T cells by the combination of CD4 and CD45RA surface marker expression as shown representatively for one donor (Fig 7). Purity was above 94.1 % for all cell populations. Naive T cells were differentiated into Th17 cells for 7 days (3.2.4) or stimulated with anti-CD3/CD28 only for the time of differentiation (Th control cells).

Different stimulation conditions were tested. Fibroblasts, monocytes and keratinocytes were either used unstimulated or stimulated with a combination of IFN-γ and LPS for 6h or in case of keratinocytes IFN-γ alone for 12 hours. PBMCs and $CD4^+$ T cells were stimulated for 4 hours with phytohaemagglutinin (PHA), an unspecific stimulus for T cells. *In vitro* differentiated Th17 and the corresponding control cells (Th control cells) were used directly after 7 days of differentiation. RNA was isolated from all cell types and analyzed for *LINGO4* expression by quantitative real-time PCR. As stimulation of fibroblasts, monocytes and keratinocytes with IFN-γ (and LPS) did not change *LINGO4* expression significantly compared to unstimulated cells, only results of unstimulated conditions are shown.

Fibroblasts, keratinocytes and monocytes did not or at least at very low levels express *LINGO4*, indicated by high Δ CT values ($\geq$20) (Fig 6). However, elevated *LINGO4* levels could be detected in PBMCs and T cells. Highest *LINGO4* levels were found in Th17 differentiated T cells with Δ CT values of 13.08 ± 0.82, while PBMCs showed mean Δ CT values of 13.52 ± 0.25. Th control cells showed slightly lower *LINGO4* mRNA expression while $CD4^+$ T cells expressed it at significantly lower levels than Th17 differentiated cells, assigning *LINGO4* up-regulation to Th17 cells.

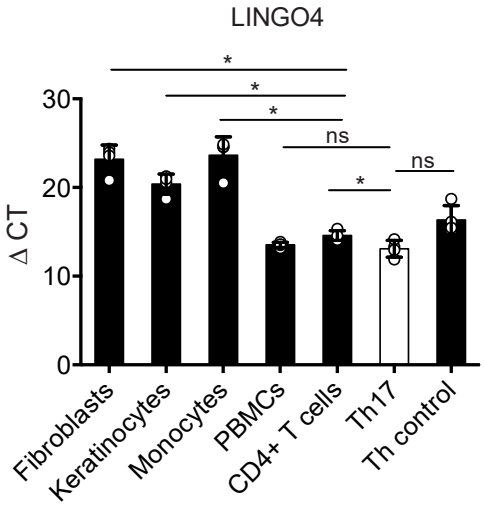

Figure 6 *LINGO4* mRNA expression is upregulated in PBMCs and *in vitro* differentiated Th17 cells.
qRT-PCR analysis of *LINGO4* expression of *in vitro* differentiated Th17 cells compared to Th control cells, $CD4^+$ T cells and PBMCs, both stimulated with PHA for 4 hours, monocytes isolated from peripheral blood of healthy donors as well as non-immune cells represented by unstimulated primary fibroblasts and keratinocytes. Shown are Δ CT (threshold CT) values normalized to the housekeeping gene EF1α with the lower value representing the higher expression. n=4 individual donors each. Statistical significance was calculated by Wilcoxon (Th17 and Th control cells) or Mann-Whitney test, respectively, and was defined as *P<0.05.

Establishment of *in vitro* Th17 differentiation from naive T cells

To examine *LINGO4* expression in Th17 cells *in vitro* differentiation of this T cell subset was established. Naive $CD4^+CD45RA^+$ T cells were isolated by magnetic-activated cell sorting (MACS) in a two-step protocol (3.2.2) from PBMCs of healthy donors.

For T cell receptor stimulation, isolated naive T cells were further activated with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. In addition, a cytokine cocktail consisting of IL-6, IL-1β, IL-23, TGF-β as well as neutralizing antibodies for IL-4 and IFN-γ were added to the culture to induce the differentiation of Th17 cells and to prevent differentiation into Th1 or Th2 cells (17). Serum-free AIM-V medium was used for differentiation cultures as RPMI-based medium is known to inhibit IL-17A secretion [Hakemi et al., 2011]. After 2 days, medium was changed supplemented with 1 % human serum followed by medium changes every 2-3 days until a total culture time of 7 days. For verification of successful Th17 differentiation, cells were restimulated for 6 hours with anti-CD3/CD28 mAbs to induce cytokine expression for analysis on mRNA level. As control, naive $CD4^+$ T cells were stimulated under the same conditions with anti-CD3/CD28 mAbs only, resulting in unspecific differentiation of T cells.

Stimulation of naive T cells with the Th17-polarizing cytokine cocktail led to significant upregulation of *IL-17A* and *RORC2* compared to control cells, while *IL-22* was significantly lower expressed in these cells (Fig 8A). Restimulation with anti-CD3/CD28 mAbs for 6 hours

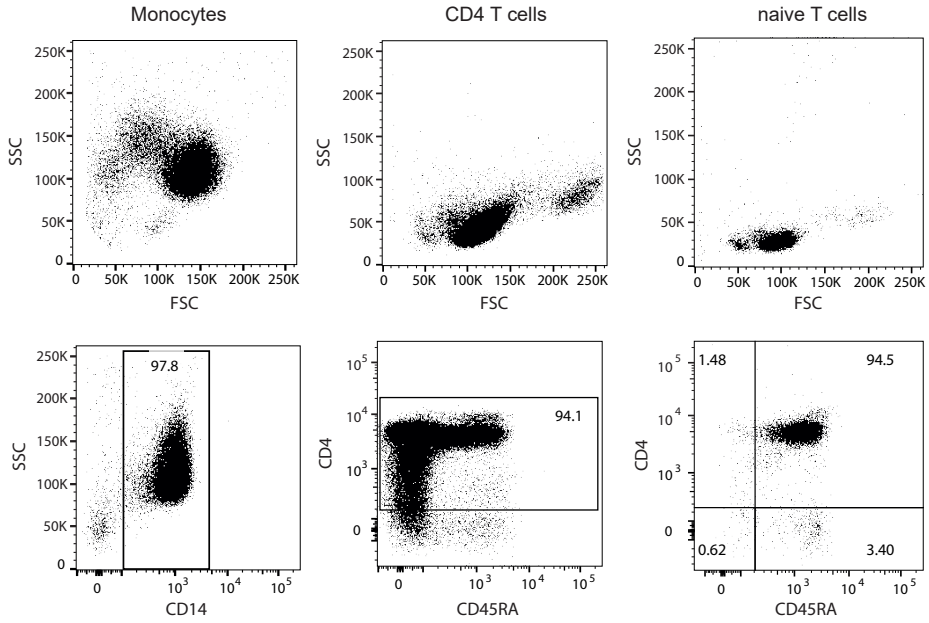

Figure 7 Purity of isolated cell populations.
Flow cytometry analysis of purified monocytes, $CD4^+$ T cells and naive T cells after MACS. Cell populations are identified in the FSC-SSC and further specified by staining with anti-CD14 mAb (monocytes) as well as anti-CD4 mAb and anti-CD45RA mAb as markers for $CD4^+$ T cells and naive T cells.

did not further induce *IL-17A* or *IL-22* levels, but reduced expression of *RORC2* in Th17 differentiated cells.

To confirm the stability of the induced Th17 phenotype, cells were kept in culture for up to 12 days and re-analyzed for mRNA expression of Th17 important genes like *RORC2*, *IL-17A* and *IL-22* after restimulation with anti-CD3/CD28 mAbs for 6 hours. No significant differences between 7 days and 12 days of culture could be identified, confirming a stable phenotype of the *in vitro* differentiated Th17 cells (Fig 8B).

Further, T cell supernatants were generated by restimulating cells for 48 - 72 hours with anti-CD3/CD28 mAbs to measure secreted cytokine concentrations by ELISA, especially IL-17A and IL-22. Increased IL-17A protein levels could be detected in T cell supernatants of Th17 cells, confirming qRT-PCR results (Fig 8C). However, a clear inter-individual difference between the different donors was observed. One donor showed no IL-17 secretion, one only low secretion of 53 pg/ml while the highest IL-17A concentration was at 433 pg/ml. IL-22 concentrations in T cell supernatants corresponded to qRT-PCR results as Th17 differentiated cells secreted significantly lower amounts than control cells.

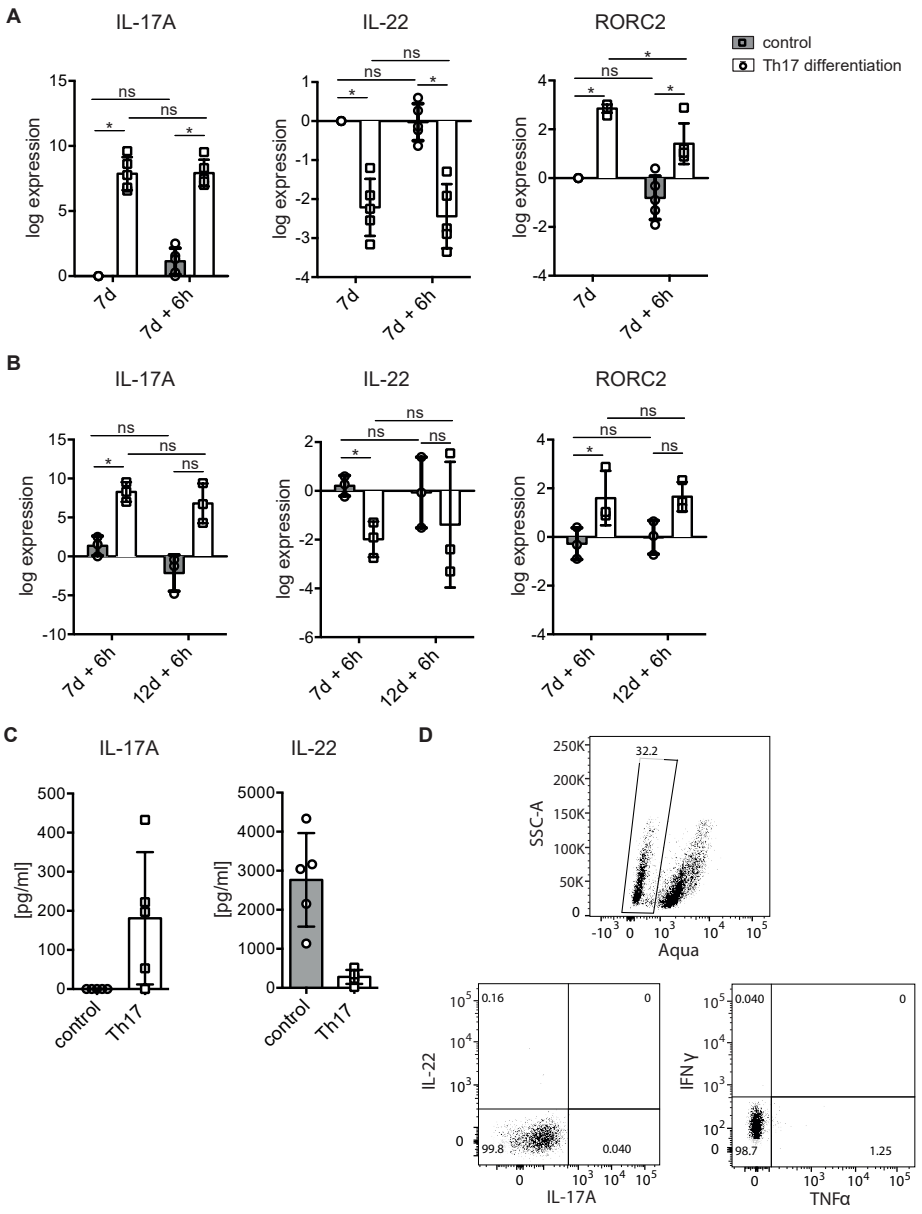

Figure 8 Naive T cells can be successfully differentiated *in vitro* into Th17 cells. $CD4^+CD45RA^+$ T cells were differentiated into Th17 cells *in vitro* by adding a cocktail of IL-6, IL-1β, IL-23 and TGF-β as well as neutralizing antibodies against IL-4 and IFN-γ in the presence of plate-bound anti-CD3 and soluble anti-CD28 mAbs (each 0.75 μg/ml) for 7 days with medium changes every 2-3 days. As control, naive T cells with TCR stimulus only were cultured for the same time. (**A**) qRT-PCR analysis of Th17 relevant gene expression of *IL-17A*, *IL-22* and *RORC2* after 7 days of culture or restimulation with anti-CD3/CD28 mAbs for 6 hours. (**B**) qRT-PCR analysis of the same Th17 relevant genes after 7 days and 12 days of culture and anti-CD3/CD28 mAb restimulation for 6 hours to analyze the stability of the Th17 phenotype. (**C**) IL-17A and IL-22 protein concentrations measured by ELISA in cell-free T cell supernatants generated by restimulation with anti-CD3/CD28 mAbs for 48 hours after 7 days of differentiation. n=5 individual donors. Statistical significance was calculated by Wilcoxon test and defined as $^*P<0.05$. (**D**) Flow cytometry analysis of intracellular cytokine staining of *in vitro* Th17 differentiated cells. Cells were stimulated for 5 hours with 50 ng/ml PMA, 1 μg/ml ionomycin and 1:1430 GolgiStop. After 3 hours, 1:1000 GolgiPlug was added. Aqua staining was used to exclude dead cells. Cells were fixed using Fix/Perm Kit of BD and stained for IL-17A, IL-22, IFN-γ and TNF-α.

Therefore, successful *in vitro* Th17 differentiation manifested in up-regulation of *IL-17A* and *RORC2* mRNA levels and IL-17A protein secretion in supernatants, though these cells did not express IL-22 as naturally occurring Th17 cells do. For quantification of differentiation efficiency, intracellular cytokine staining was analyzed by flow cytometry. Therefore, cells were stimulated for another 5 hours with PMA and ionomycin. To prevent secretion of newly synthesized proteins, GolgiStop (Monsesin) and GolgiPlug (Brefeldin A) were added. Intracellular cytokine staining of *in vitro* differentiated Th17 cells failed, as neither positive cells for IL-17A, IL-22, TNF-α or IFN-γ could be detected (Fig 8D). The frequency of viable cells, identified by aqua-staining, was already quite low with only 32 %.

Analysis of *LINGO4* expression in different T helper cell subsets shows a clear association with the Th17 phenotype

Analysis of different cell types revealed *LINGO4* expression in immune cells, particularly in T cells, with highest expression in *in vitro* differentiated Th17 cells. To confirm *LINGO4* up-regulation in Th17 cells observed in T cell clones (Fig 5) and to exclude an *in vitro* artefact in differentiated Th17 cells (Fig 6), Th1, Th2 and Th22 cells were differentiated *in vitro* and compared to *in vitro* differentiated Th17 cells for *LINGO4* expression. Th cell subsets were differentiated from $CD4^+CD45RA^+$ T cells, obtained from PBMCs of healthy donors as previously described (3.2.1 and 3.2.4). For induction of distinct Th cell phenotypes, different cytokine cocktails were used in addition to TCR stimulus via anti-CD3/CD28 mAbs. Th1 cells were induced by IL-12 and neutralization of IL-4 (Tab 18). For Th2 differentiation, IL-4 and neutralizing IFN-γ mAb was added (Tab 19), while Th22 differentiation was initiated by IL-6 and TNF-α in combination with neutralizing antibodies against IL-4, IL-12 and IFN-γ to prevent differentiation into Th1 or Th2 cells (Tab 20). Contrary to Th17 differentiation, RPMI-based medium was used. Expression of specific transcription factors and cytokine secretion verified successful differentiation.

Again, *LINGO4* mRNA expression was significantly elevated in Th17 cells compared to the other three Th cell subsets (Fig 9A). Further, significantly elevated *IL-17A* and *RORC2* levels in Th17 cells confirmed successful *in vitro* differentiation into Th17 cells. Successful Th1 differentiation was confirmed by elevated levels of the key transcription factor *TBET* as was Th2 differentiation by expression of the transcription factor *GATA3* (Fig 9B). For Th22 cells, AHR is known as key transcription factor. Slightly elevated expression levels could be shown for this factor compared to Th1 and Th2 subsets, while Th17 cells, also IL-22 producers, expressed similar levels (Fig 9B).

Further confirmation of successful Th cell subset differentiation was shown on protein level by measuring signature cytokines of these Th cell subsets, namely IL-17A, IL-22, IL-4, IFN-γ and also TNF-α, in the cell culture supernatants. For Th17 cells, cytokine concentration was directly determined from the cell culture supernatant after 7 days of differentiation. Significantly elevated levels in Th17 cells confirmed successful differentiation (Fig 9C).

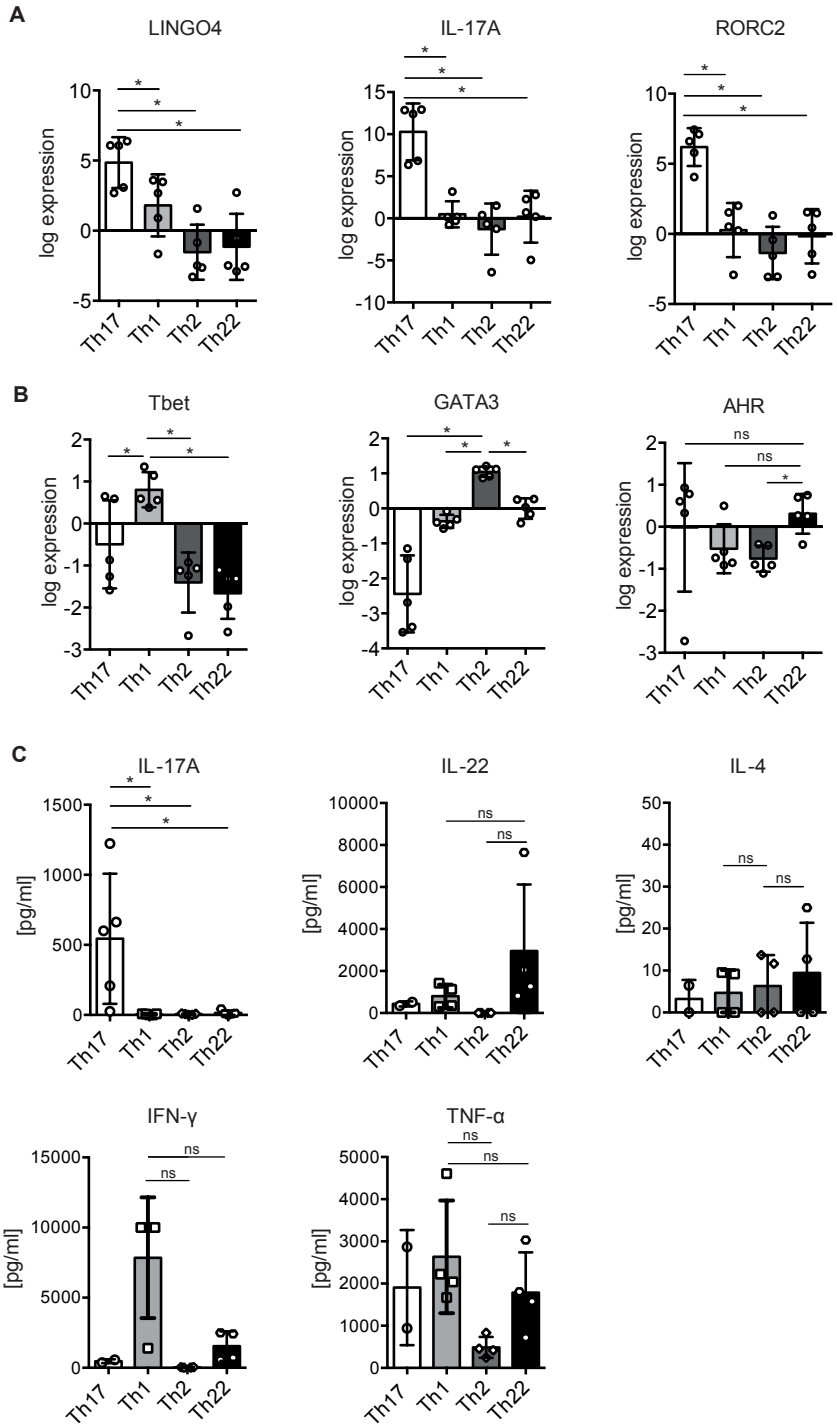

Figure 9 *LINGO4* expression in different Th cell subsets reveals up-regulation in Th17 cells.

qRT-PCR analysis of *in vitro* differentiated Th1, Th2 Th17 and Th22 cells for *LINGO4*, *IL-17A* and *RORC2* mRNA expression (**A**) as well as *TBET*, *GATA3* and *AHR* expression (**B**) in n=5 individuals, normalized to control cells stimulated with anti-CD3/CD28 mAbs only. (**C**) Protein concentrations measured by ELISA from cell culture supernatants after 7 days of differentiation (IL-17A, all Th cell subsets n=5 individuals) or from cells restimulated for 72 hours with anti-CD3/CD28 mAbs after 7 days of differentiation (IL-22, IL-4, IFN-γ, TNF-α, n=5 individuals, Th17 cells (n=2)). Statistical significance was calculated by Wilcoxon test and defined as *P<0.05 or P>0.05 as not significant (ns).

For the other *in vitro* differentiated Th cell subsets, a defined cell number was stimulated with anti-CD3/CD28 mAbs for 72 hours. Th22 cells secreted with 2951±2748 pg/ml highest amounts of IL-22 (801±482 pg/ml in Th1, 434±84 pg/ml in Th17 and 0±0 pg/ml in Th2), as Th1 cells did for IFN-γ with 7849±3726 pg/ml (468±98 pg/ml in Th17, 27±20 pg/ml in Th2 and 1547±908 pg/ml in Th22), confirming again successful differentiation. However, IL-4 was only secreted in very low amounts with mean concentrations of 3±3 pg/ml (Th17), 4±4 pg/ml (Th1), 6±6 pg/ml (Th2) and 9±10 pg/ml (Th2) and by most donors not at all (Fig 9C). TNF-α was secreted in similar amounts by Th1, Th17 and Th22 cells with concentrations of 1904±964 pg/ml (Th17), 2635±1157 pg/ml (Th1) and 1784±827 pg/ml (Th22), while Th2 cells secreted less TNF-α (487±214 pg/ml). However, high standard deviations indicate again high inter-individual differences between the donors regarding secretion of cytokine amounts.

LINGO4 protein cannot be detected due to missing specific antibodies

On mRNA level, LINGO4 could be assigned to Th17 cells with elevated expression levels compared to other Th cell subsets. To verify this finding on protein level, lysates of *in vitro* differentiated Th17 and control cells were generated and analyzed via western blot. Three different anti-LINGO4 antibodies (**a**, **b** and **c**, Tab 30) were tested for specific LINGO4-staining. Representatively, results of Th17 differentiation cultures of two donors are shown.

Western blot analysis revealed that none of the commercially available antibodies worked specifically for endogenous LINGO4 detection (Fig 10A). LINGO4 protein is predicted to have a molecular weight of around 64 kDa. Antibody **a** only showed bands at a molecular weight above 170 kDa. Although staining with antibody **b** resulted in a band of the expected weight, many unspecific bands were present. Antibody **c** only showed 2 bands, one at the expected height of 64 kDa and the other at around 35 kDa. However, the unspecific band of around 35 kDa was more concise. In addition, no difference between Th17 and control cells was observed with antibody **c**.

Nevertheless, to verify the presence of LINGO4 protein in *in vitro* differentiated Th cell subsets (Th17, Th1, Th2, Th22 and control cells), mass spectrometry analysis (LC-MS/MS) was performed (Fig 10B). LINGO4 was detected in all samples with two unique peptides that could be specifically assigned to LINGO4 protein. Peptides could only be identified by alignment with a previously generated library of overexpressed LINGO4 protein in HEK cells, isolated by immunoprecipitation prior to measurement.

LINGO4 protein in LC-MS/MS could only be detected using the sensitive data-independent acquisition (DIA) method. Further, Th17 cells did not show a differential LINGO4 protein expression to other Th cell subsets. Again, a high discrepancy between the donors was observed in Th1, Th22 and especially control cells (2 to 4-fold different levels of protein abundance discrepancy) as already observed on cytokine level in ELISA.

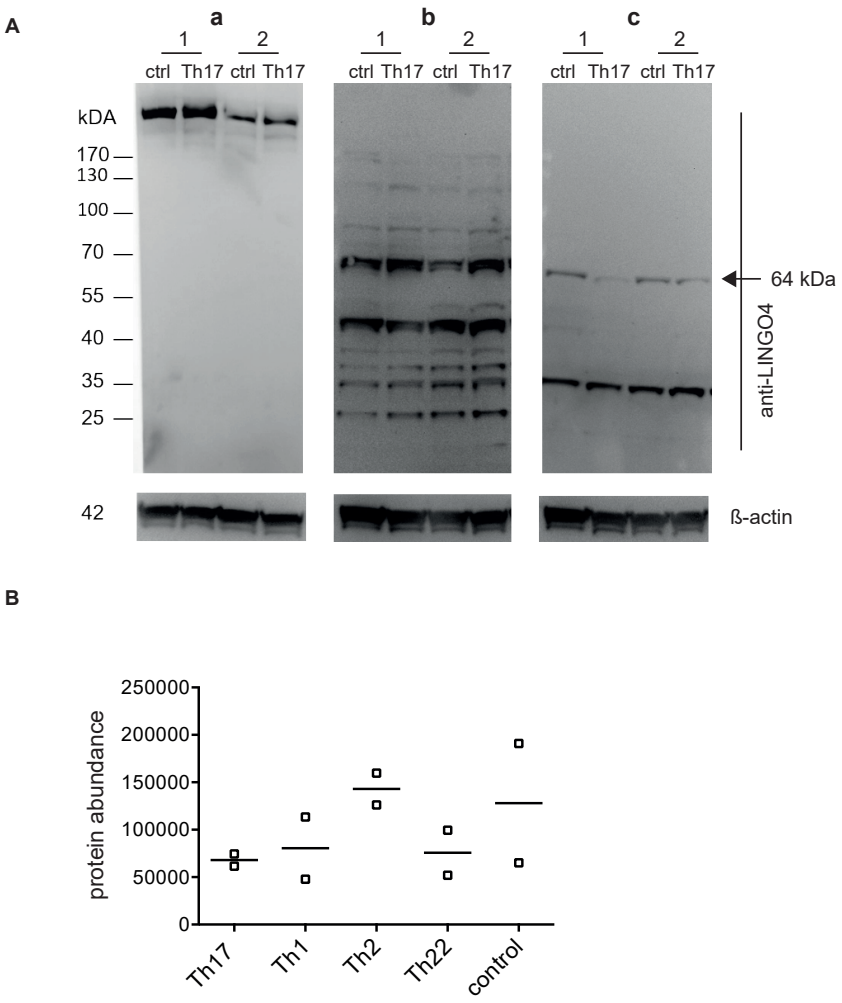

Figure 10 Endogenous LINGO4 protein cannot be detected by commercially available antibodies, but by LC-MS/MS analysis.
(**A**) Western blot analysis of *in vitro* differentiated Th17 and corresponding control cells after 6 days of culture. Shown are n=2 donors (1 and 2), representatively. Protein lysates were generated in RIPA buffer and 20 μg of protein - concentration determined by BCA assay - were applied on the gel. Proteins were transferred to PVDF membrane and stained with three different commercially available anti-LINGO4 antibodies (**a**, **b** and **c**). Anti-rabbit-HRP labeled secondary antibody was used and signals were detected using the SuperSignal West Femto Chemiluminescent Substrate. As housekeeping protein, β-actin was stained. (**B**) Mass spectrometry analysis of *in vitro* differentiated Th cell subsets (Th17, Th1, Th2, Th22 and control cells) of n=2 individuals. Signals were identified using a library, also generated with overexpressed LINGO4 protein in HEK cells, isolated by IP. DIA method was used for analysis. For each sample, LINGO4 could be identifed with two unique peptides specific to LINGO4. Values are presented as protein abundance.

LINGO4 expression is induced during Th17 differentiation

Previous results related increased *LINGO4* expression to Th17 cells, both generated *in vitro* as well as “natural”, in comparison with other Th cell subsets and different immune and non-immune cells. Therefore, it was of interest how *LINGO4* expression behaves during the differentiation process. To address this question, kinetics of Th17 differentiation were performed

(Fig 11). Naive CD4⁺ T cells were differentiated into Th17 cells for 7 days. Control cells were stimulated with anti-CD3/CD28 mAbs over the same time. Every day, cells were harvested for RNA isolation and cell culture supernatant was taken to determine cytokine secretion by ELISA.

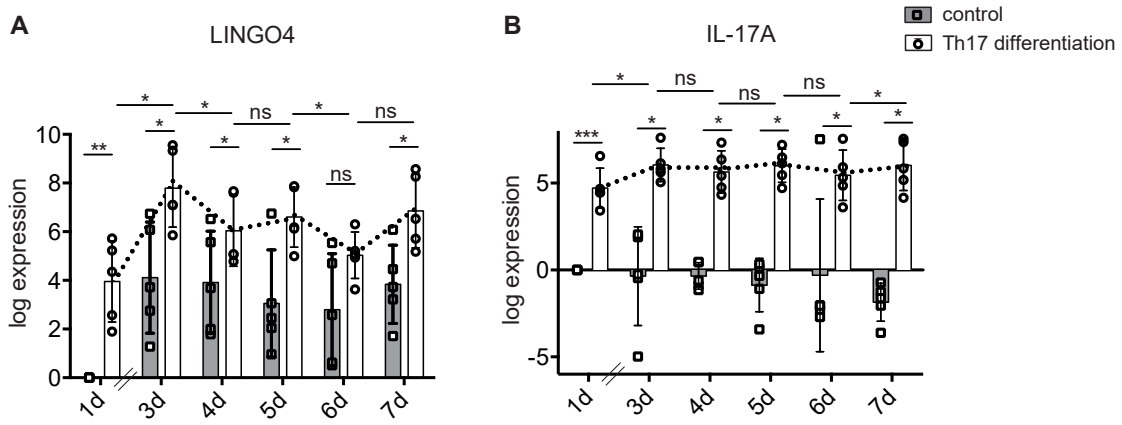

Figure 11 Time kinetics of Th17 differentiation reveals oscillatory *LINGO4* expression.
qRT-PCR analysis of *in vitro* differentiated Th17 cells for *LINGO4* (**A**) and *IL-17A* (**B**) mRNA expression levels. Naive CD4⁺ T cells were differentiated with Th17-polarizing cytokine cocktail and anti-CD3/CD28 mAbs for 7 days and harvested at indicated time points. Control cells were stimulated with anti-CD3/CD28 mAbs only. n=5 individual donors. Values were normalized to control cells at day 1. Statistical significance was calculated by Wilcoxon test and defined as **P*<0.05, ***P*<0.01.

Th17 differentiating cells expressed increased *LINGO4* mRNA levels already at day 1 compared to control cells and expression remained higher over the whole differentiation process (Fig 11A). Further, an oscillatory course was observed. *LINGO4* expression increased significantly between day 1 and 3, decreased significantly at day 4 and between day 5 and 6, while an increasing trend was again observed at day 7. Contrary to this, *IL-17A* mRNA levels were induced in Th17 differentiating cells at day 1 and stayed constantly increased over the time of differentiation (Fig 11B).

LINGO4 expression levels strongly correlate with *RORC2* expression during Th17 differentiation kinetics

RORC2 is known as the master transcription factor of Th17 cells and its expression as well as IL-17A protein concentration in cell culture supernatants were monitored during the differentiation course (Fig 11) to validate successful Th17 differentiation (Fig 12A). One donor is shown representatively.

Surprisingly, *RORC2* and *LINGO4* strongly correlated with an almost identical relative expression course during Th17 differentiation over the whole 7 days (Fig 12A). Therefore, the same oscillatory effects could be observed corresponding to the indicated medium changes every

two days. Medium change also had an impact on IL-17A protein concentration in the supernatants. After medium change at day 4, mRNA expression did not alter, but IL-17A protein levels increased, while at day 5 expression was decreased and protein level further increased. This slight anti-cyclical course was stronger observed at day 6, at which mRNA expression of both genes was increased while IL-17A protein level was decreased (Fig 12A).

The correlation of *LINOG4* and *RORC2* expression was further confirmed in whole genome expression arrays of different T cell clones (Fig 12B and Fig 5). A bimodal contribution was observed between Th1/Th2 cells and Th17 or IL-17A related cells like Th1/Th17 and Th22 cells. Strikingly, *RORC2* expression levels seemed to be capped at values around 14 as all *RORC* expressing cells (Th17, Th1/Th17 and Th22) showed similar expression levels, while *LINGO4* expression was clearly highest in Th17 cells. In addition, for all Th cell subsets a higher *RORC* and *LINGO4* expression could be observed when cells were stimulated with anti-CD3/CD28 mAbs prior to analysis.

The previously observed anti-cyclical course of *LINGO4/RORC2* mRNA expression and IL-17A protein (Fig 12A) led to the question whether regulatory effects of secreted IL-17A on the differentiating cells themselves occur. Therefore, expression of *IL-17RA*, the receptor for IL-17A, was examined in Th17 differentiated cells and control cells. Further, other cell types like monocytes, fibroblasts and keratinocytes, the last two cells known to be responsive to IL-17A, were tested. Indeed, all tested cell types expressed *IL-17RA*, but with lowest expression in *in vitro* differentiated Th17/control cells (Δ CT $\sim$10) (Fig 12C). However, though expressing *IL-17RA* and therefore being potentially able to respond to IL-17A, stimulation of Th17 and control cells after 7 days of *in vitro* differentiation with two different concentrations of recombinant IL-17A did not show any impact, neither on *LINGO4* nor on *IL-17A*, *RORC2* or *IL-17RA* expression (Fig 12D).

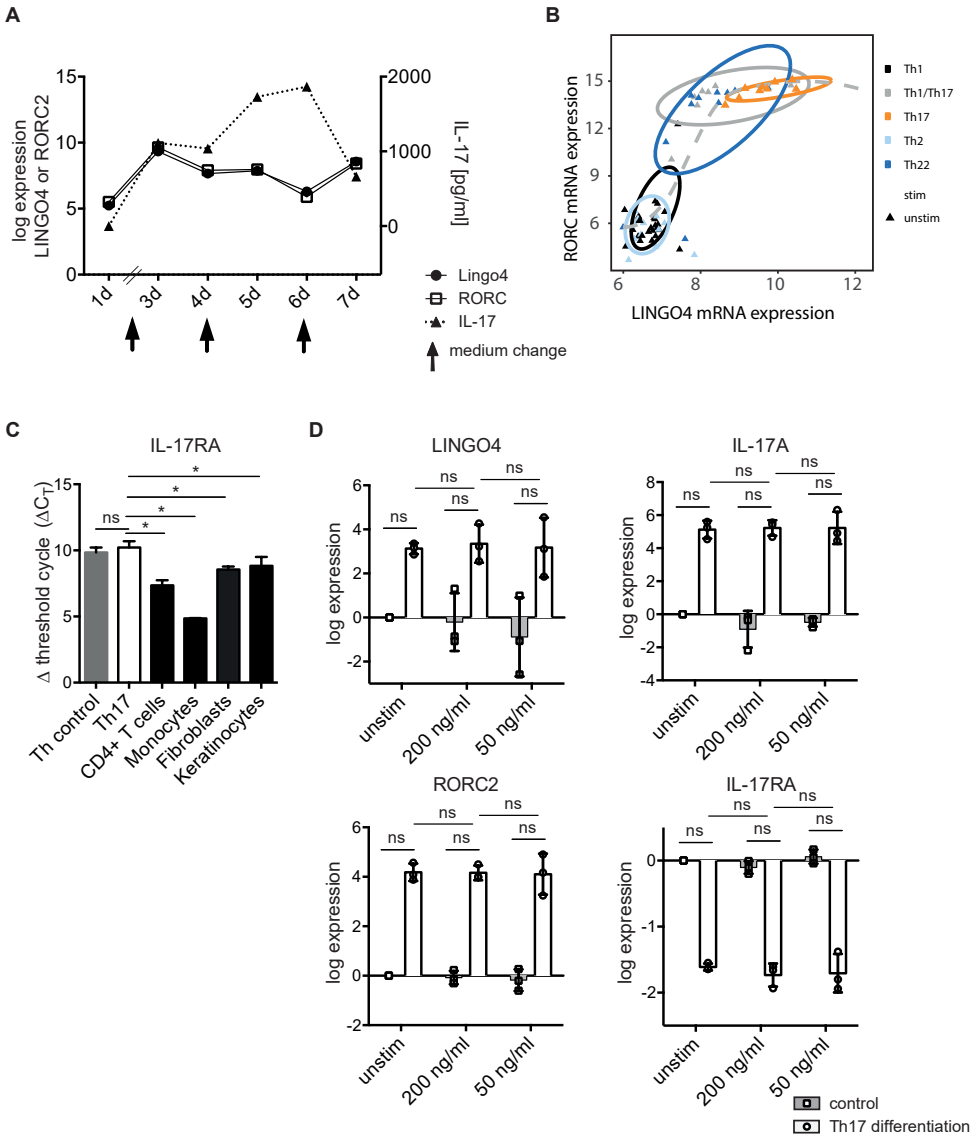

Figure 12 *LINGO4* expression strongly correlates with *RORC2* mRNA levels in Th17 differentiated cells.

(**A**) Time kinetics of Th17 differentiation was analyzed for *LINGO4* and *RORC2* mRNA as well as IL-17A protein expression by qRT-PCR or ELISA, respectively, at indicated time points. Cell culture medium was changed every second day indicated by arrows. n=2 individual donors, one shown representatively. Values were normalized to control cells at day 1. Statistical significance was calculated by Wilcoxon test and defined as $^{*}P<0.05$, $^{**}P<0.01$. (**B**) Correlation of *LINGO4* and *RORC* mRNA expression in different T cell clone subsets analyzed by whole genome expression arrays. Th1 n=26, Th17 n=9, Th2 n=12, Th22 n=11, each shown as unstimulated and stimulated sample with anti-CD3/CD28 mAbs for 6 hours. Level of confidence interval was 0.8. (**C**) qRT-PCR analysis of *IL-17RA* expression in different immune and non-immune cells represented by $CD4^{+}$ T cells, *in vitro* differentiated Th17 cells and corresponding control cells (Th control) as well as monocytes, fibroblasts and keratinocytes, all n=4 individual donors. Statistical significance was calculated by Wilcoxon (Th17 and control T cells) or Mann-Whitney test, respectively, and defined as $^{*}P<0.05$. Shown are Δ CT (threshold) values with the lower value representing the higher expression. (**D**) qRT-PCR analysis of *LINGO4*, *IL-17A*, *RORC2* and *IL-17RA* mRNA expression levels in *in vitro* differentiated Th17 cells after stimulation with recombinant IL-17 in indicated concentrations for 6 hours. Values were normalized to control cells. n=3 individual donors. Statistical significance was calculated by Wilcoxon test and defined as $^{*}P<0.05$.

LINGO4 and *RORC* genes are located in close proximity, but do not share a common transcript

Since *LINGO4* and *RORC2* showed an almost identical relative transcription course in kinetics of Th17 differentiation, the genomic organization of these genes was analyzed. It revealed that both genes lie on the same chromosome (chromosome 1) on the minus strand in direct proximity, even overlapping with 83 bp (Fig 13A).

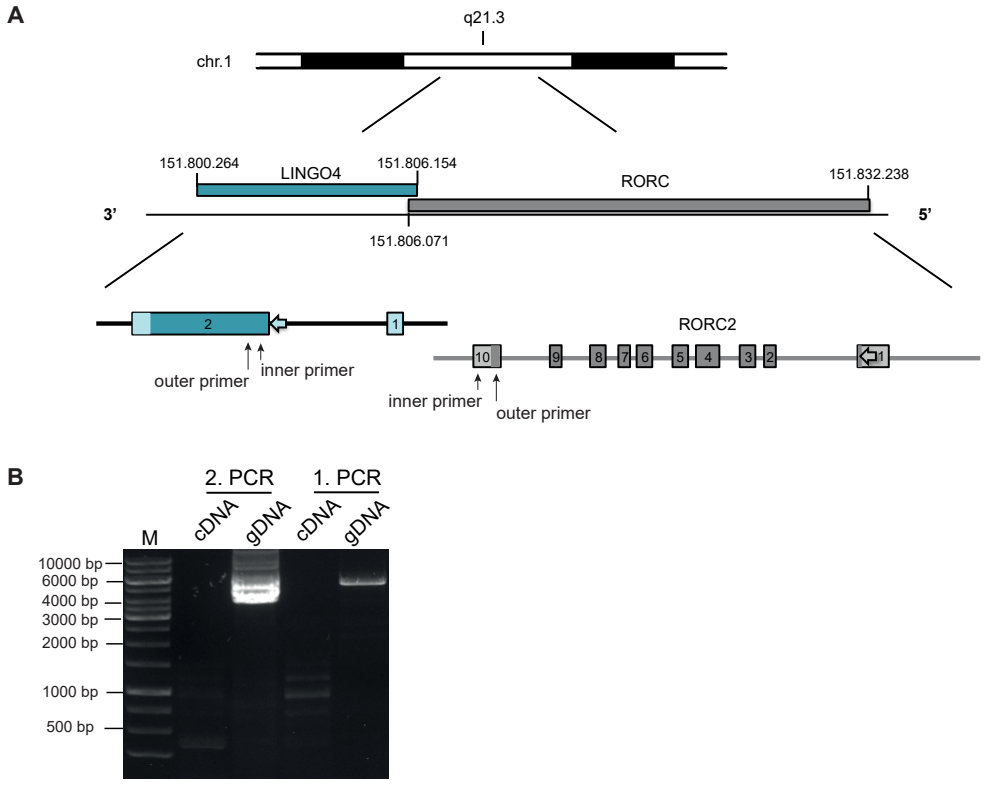

Figure 13 *LINGO4* and *RORC2* gene loci are in close proximity but not transcribed in one common mRNA.
(**A**) Analysis of genomic organization of *LINGO4* and *RORC* genes on chromosome 1. Exon regions of 3' and 5' UTR are displayed in light colours, coding regions in dark. Arrows indicate positions of primers for nested PCR. (**B**) Nested PCR of PHA stimulated PBMCs (n=2, one shown representatively) was performed with genomic DNA (gDNA) and mRNA, which was reversely transcribed to cDNA. Outer primers are localized in the CDS regions of *RORC* (fw) and *LINGO4* (rev), inner primers in the 3'UTR of *RORC* and CDS of *LINGO4*, respectively.

To rule out a common transcript explaining the identical expression levels and revealing *LINGO4* as a possible bystander product of *RORC2*, a nested PCR was performed (Fig 13B). Genomic DNA (gDNA) as well as mRNA (cDNA), both isolated from PHA stimulated PBMCs, were used. Outer primers span both coding regions of *RORC2* and *LINGO4*, while the inner primer pair span the 3'UTR of *RORC2* and the coding region of *LINGO4* leading to product sizes of $\sim$5140 bp in the first PCR reaction and $\sim$3800 bp in the second PCR reaction in

gDNA. In cDNA samples, the presence of a common transcript would result in products of ∼2000 bp and ∼650 bp in the first and second PCR reaction, respectively. However, only genomic DNA led to products in both PCR reactions, while only faint, unspecific bands were detected in cDNA samples in both reactions, excluding a common mRNA for *LINGO4* and *RORC2* (Fig 13B).

4.1.2 LINGO4 overexpression does not result in LINGO4 protein expression in T cells

Expression analysis of different cell types and Th cell subsets as well as Th17 differentiation kinetic analysis assigned *LINGO4* expression clearly to Th17 cells and revealed a strong correlation to expression of the Th17 transcription factor RORC2. However, functional data on LINGO4 are missing. To address a possible functional role of *LINGO4* in Th17 cells, an overexpression approach was taken.

Establishment of lentiviral *LINGO4* overexpression in naive T cells

As naive T cells are difficult to transfect, lentiviral transduction was used to achieve LINGO4 overexpression, a system enabling genetic modification even in resting cells. To produce virus containing either HA-tagged LINGO4 or empty GFP vector as control, the virus-packaging cell line HEK 293 LTV was transfected with transfer vector. Naive T cells were spin-infected with concentrated virus supernatant. Transduced naive T cells as well as transfected and virus-producing HEK cells were analyzed the next day for *LINGO4* mRNA expression by qRT-PCR (Fig 14A) and GFP expression by flow cytometry (Fig 14B).

LINGO4 mRNA expression was strongly elevated (14 - 15-fold) in both HEK cells and naive T cells transfected/transduced with LINGO4 overexpression vector, showing successful gene transfer (Fig 14A). Further, flow cytometric analysis showed GFP expression in HEK and T cells (Fig 14B), confirming successful transfection/transduction with empty vector. However, transfection efficiency of HEK cells was higher than transduction efficiency of naive T cells.

LINGO4 mRNA, but not protein can be overexpressed in T cells

Since transduction of naive T cells with LINGO4 overexpression vector resulted in high amounts of *LINGO4* mRNA (Fig 14), cells were further examined for protein overexpression. As commercially available antibodies specific for LINGO4 did not detect the protein reliably (Fig 10), exogenously overexpressed LINGO4 was detected by the HA-tag. Cells were harvested the day after the second infection and protein lysates were produced for western blot analysis. In addition, fluorescence staining was performed to further detect LINGO4 protein in the cells.

Western blot analysis of HEK cells showed clear overexpression of HA-tagged LINGO4 pro-

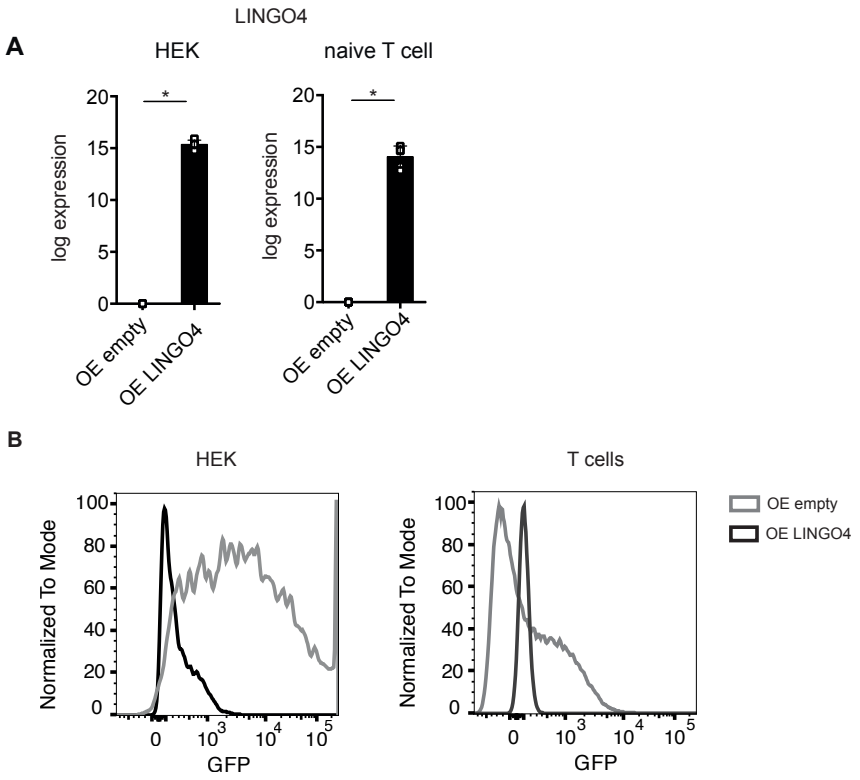

Figure 14 Validation of *LINGO4* mRNA overexpression in naive T cells and HEK cells.
(**A**) *LINGO4* was overexpressed in HEK cells by transfection as well as in naive T cells by lentiviral transduction. *LINGO4* mRNA was measured by qRT-PCR and normalized to empty vector transfected/transduced cells. Statistical significance was calculated by Wilcoxon test and defined as $*P<0.05$, $**P<0.01$. HEK: n=5 experiments, T cells: n=5 individual donors. (**B**) Flow cytometric analysis of the transfected HEK and transduced naive T cells, respectively. GFP was measured in cells transfected/transduced with empty vector. Data of one experiment/donor is shown representatively. OE: overexpression.

tein compared to empty transfected cells (Fig 15A). However, protein was not detected in transduced naive T cells (Fig 15A). Immunofluorescence staining confirmed this finding (Fig 15B). While in HEK cells LINGO4-HA protein appeared as red circles around the nuclei, fluorescent LINGO4 staining was not observed in naive, LINGO4 overexpressing T cells, although overexpression on mRNA level was at similar levels in both cell types (Fig 14A). Even later after transduction (up to 96 hours) immunofluorescence staining and western blot analysis did not reveal LINGO4-HA protein in naive T cells (data not shown).

Missing LINGO4 protein overexpression is not caused by increased proteasomal degradation

To examine if the phenomenon of missing LINGO4 protein overexpression in primary naive T cells was specific to this cell type, the same lentiviral overexpression setup was repeated in Jurkat T cells, an immortalized human T cell line. qRT-PCR analysis of *LINGO4* expression revealed a 8-fold up-regulation of *LINGO4* in overexpressing Jurkat T cells compared to

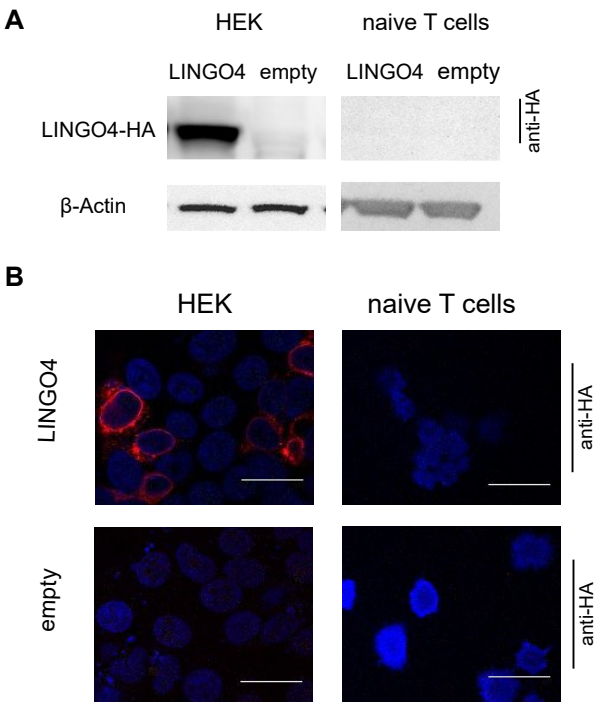

Figure 15 Validation of LINGO4 protein overexpression in naive T cells and HEK cells. (**A**) Representative western blot analysis of HA-tagged LINGO4 protein in *LINGO4* mRNA overexpressing HEK and naive T cells (n=5) detected by anti-HA-HRP mAb. (**B**) Immunofluorescence staining of HA-tagged LINGO4 protein in transfected HEK as well as transduced naive T cells. HA was stained using anti-HA mAb and anti-mouse-NL557 pAb (red) as secondary antibody. Nuclei were stained with DAPI (blue). Scale bar = 25 μm.

empty vector control cells indicating that lentiviral transduction is efficient in this cell line as well (Fig 16A). However, also in Jurkat T cells LINGO4-HA protein could not be detected by immunofluorescence staining (Fig 16B). One reason for lack of LINGO4 protein overexpression might be direct degradation by the proteasome. Proteasomal degradation is involved in gene expression control by degrading misfolded proteins. Ubiquitinylation of proteins is the signal for their transfer into the proteasome, where they are degraded into small peptides that can later be used for new protein synthesis.

To rule out proteasomal degradation as cause of missing LINGO4 protein overexpression despite the presence of high amounts of *LINGO4* mRNA, Jurkat T cells were treated with proteasome inhibitor MG-132. MG-132 not only inhibits the proteasome, but also influences other cellular processes (e.g., induction of apoptosis and inhibition of NFκB pathway). Due to easier handling and the ability to test more conditions at the same time, Jurkat T cells were used for proteasome inhibition experiments instead of primary naive T cells. In total, 3 different concentrations of MG-132 were tested for 2 incubation times to rule out that side-effects of MG-132 (e.g., induction of apoptosis) diminish possible impacts on LINGO4 protein expression.

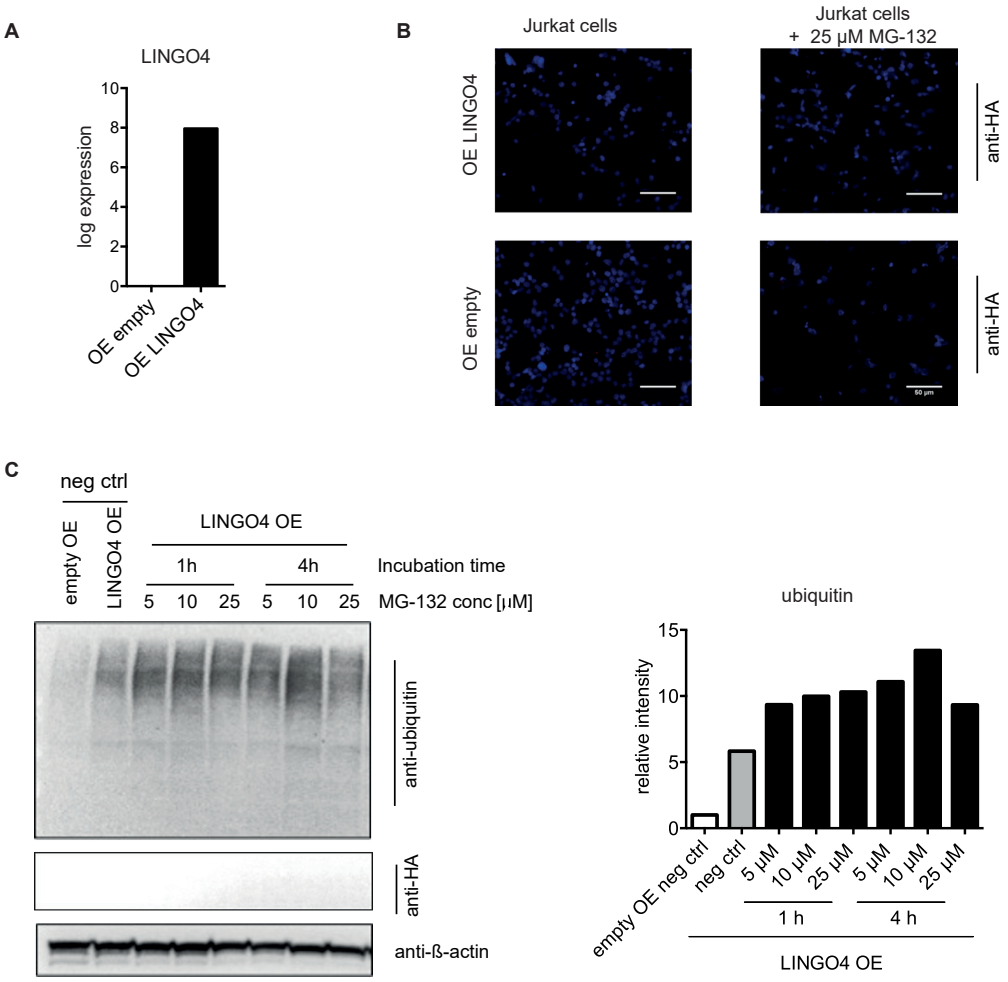

Figure 16 Increased proteasomal degradation is not the reason for missing LINGO4 protein overexpression in T cells.
(**A**) qRT-PCR analysis of *LINGO4* mRNA expression in Jurkat T cells transduced with LINGO4 overexpression vector or empty vector as control, respectively. Values were normalized to empty control. n=1 experiment. (**B**) Immunofluorescence staining of HA-tagged LINGO4 protein in transduced Jurkat T cells (left) and transduced Jurkat T cells treated with 25 μM MG-132 for 4 hours (right), respectively. HA was stained with anti-HA mAb and anti-mouse-NL557 pAb (red) as secondary antibody. Nuclei were stained with DAPI (blue). Empty vector transduced Jurkat T cells treated in the same way were used as control. Scale bar = 25 μm. (**C**) Western blot analysis of Jurkat T cells transduced with LINGO4 overexpression vector and treated with proteasome inhibitor MG-132 96h after transduction (left). Different concentrations and incubation times were tested as indicated. Blot was stained with anti-ubiquitin mAb and anti-rabbit-HRP, anti-HA-HRP mAb and anti-β-actin mAb and anti-mouse-HRP. As control, Jurkat T cells were transduced with empty vector. n=1 experiment. For quantification, intensities were corrected for the background and normalized on β-actin. Shown are the relative intensities to highest β-actin intensity (right).

However, LINGO4 protein still could not be detected after proteasome inhibition in immunofluorescence staining, as shown representatively for one tested condition in Fig 16B (right). Western blot analysis showed that proteasome inhibition was efficient, as accumulation of ubiquitinylated protein was observed, while LINGO4-HA still could not be detected in any of

the samples (Fig 16C). Quantification of ubiquitinylation showed more ubiquitinylated proteins in overexpressing LINGO4-HA cells without MG-132 treatment (neg ctrl) compared to the empty transduced cells. Nevertheless, MG-132 treatment led to higher levels of ubiquitinylation without differences in concentration and incubation times, showing again successful proteasome inhibition without any impact on LINGO4 protein overexpression.

4.1.3 *LINGO4* mRNA overexpression does not impact on the phenotype of Th17 cells

Since overexpression of LINGO4 in T cells was efficient on mRNA level while protein could not be detected, it can be speculated that *LINGO4* RNA itself might have a regulatory function particularly in T cells. To investigate whether high *LINGO4* mRNA levels have an impact on Th17 cells and their differentiation, naive $CD4^+$ T cells were transduced with LINGO4 overexpression and empty vector, respectively, and *in vitro* differentiated into Th17 cells. For analysis of cytokine expression on mRNA and protein level, cells were restimulated with anti-CD3 and anti-CD28 mAbs for 6 hours and 48 hours, respectively.

qRT-PCR analysis revealed increased *LINGO4* expression in both Th17 differentiated and control cells when transduced with LINGO4 overexpression vector, although Th17 differentiation itself seemed to decrease *LINGO4* overexpression (Fig 17A). Elevated expression levels of *IL-17A* and *RORC2* in Th17 differentiated cells, both overexpressing *LINGO4* or empty vector transduced, confirmed successful Th17 differentiation. However, *LINGO4* mRNA overexpression did not lead to altered *IL-17A* or *RORC2* mRNA levels, neither in Th17 nor control cells compared to the empty vector transduced cells (Fig 17A). The same was observed for *IL-22*, *IFN-*γ, *IL-4* and *TNF-*α mRNA levels. *LINGO4* overexpression did neither have an impact on Th17 differentiation or phenotype nor control cells (Fig 17B).

Cytokine analysis by ELISA in cell culture supernatants of differentiation cultures gave similar results. IL-17A was measured in cell culture supernatants directly resulting from differentiation cultures at day 7 (Fig 17C) as well as in supernatants generated by restimulation of a defined cell number for 72 hours after differentiation (Fig 17D).

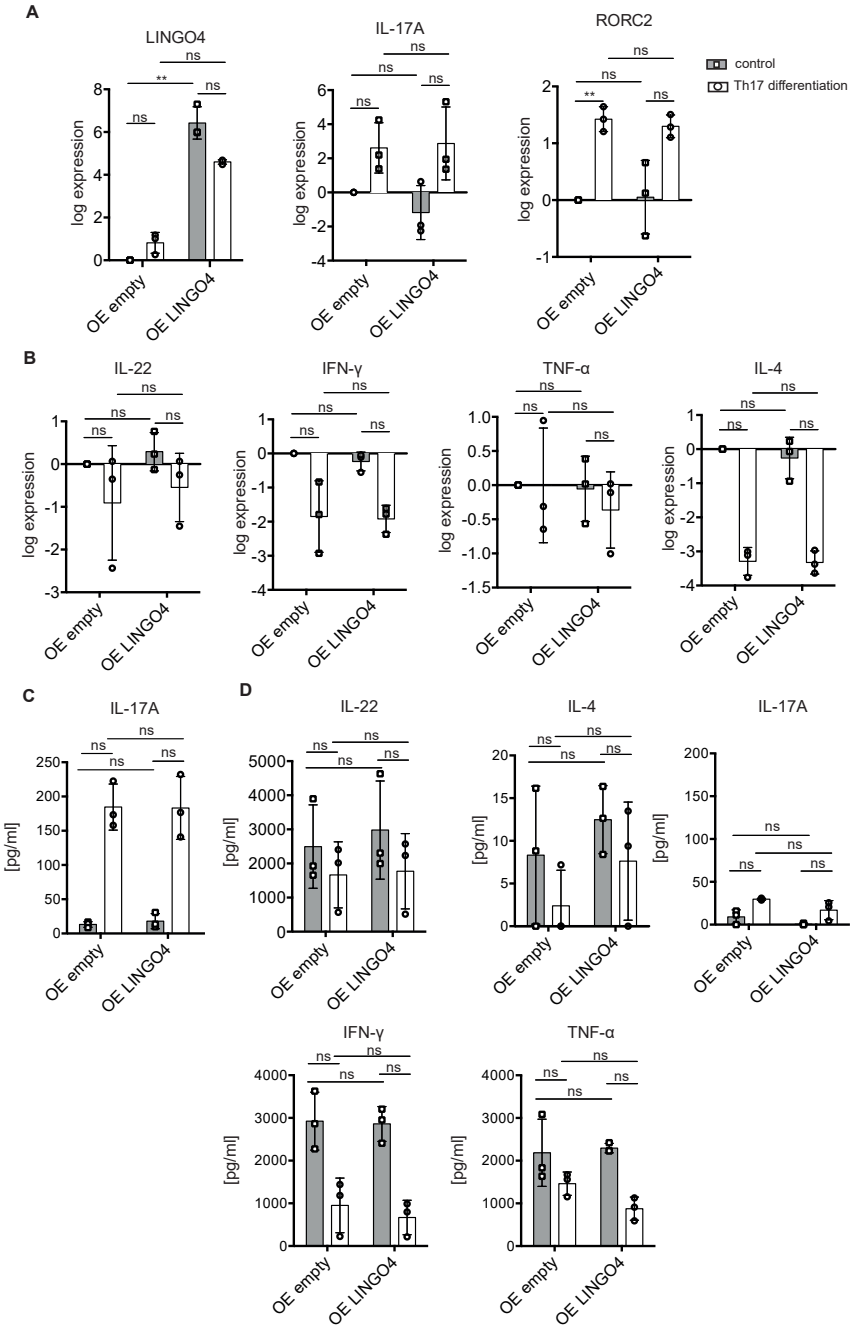

Figure 17 *LINGO4* mRNA overexpression does not affect the differentiation of Th17 cells. Naive CD4$^+$ T cells were lentivirally transduced with LINGO4 overexpression vector or empty vector and differentiated into Th17 and control cells, respectively, the next day for 7 days. Transduced cells were selected by addition of puromycin as resistance was committed by transfer vectors. After 7 days of differentiation, 1x10^6 cells were restimulated with anti-CD3/CD28 mAbs for 6 hours. Expression levels of *LINGO4*, *IL-17A* and *RORC2* (**A**) as well as *IL-22*, *IFN-*γ, *IL-4* and *TNF-*α (**B**) were analyzed by qRT-PCR. After 7 days of differentiation, cell culture supernatant was collected for measurement of IL-17A protein (**C**). In addition, a defined cell number was restimulated with anti-CD3/CD28 mAbs for 72 hours to generate cell culture supernatants for measurement of IL-17A, IL-22, IFN-γ, IL-4 and TNF-α protein (**D**). n=3 individual donors. Statistical significance was calculated by Wilcoxon test and defined as **P*<0.05, ***P*<0.01.

Control cells did not secrete IL-17A while no difference between *LINGO4* overexpressing and empty vector transduced Th17 differentiated cells was detectable. In supernatants generated by restimulation, however, IL-17A could not be detected in any of the samples (Fig 17D). Further, no influence of *LINGO4* overexpression could be detected on IL-22, IL-4, IFN-γ and TNF-α secretion.

4.1.4 *LINGO4* knockdown decreases *RORC2* and *IL-17A* expression levels

Due to missing protein of LINGO4 in (naive) T cells, lentiviral-mediated gene knockdown with small hairpin (sh) RNA was performed to get insight into the possible function of *LINGO4* in Th17 cells. Naive $CD4^+$ T cells were isolated from healthy donors and transduced with sh RNAs directed against a coding region sequence of *LINGO4* (sh LINGO4) or unspecific control sh RNA (sh ctrl). The day after second infection, a defined number of T cells was differentiated into Th17 cells or, as control, only stimulated with anti-CD3/CD28 mAbs, respectively. Transduced cells were enriched by supplemented puromycin as transfer vectors committed resistance upon successful gene transfer.

Successful transduction and LINGO4 knockdown was monitored on mRNA level after 7 days of differentiation. Further, due to a GFP site in both transfer vectors, virus-producing HEK cells and transduced differentiated T cells were analyzed for GFP expression by flow cytometry.

Knockdown by lentiviral-mediated transduction with sh LINGO4 resulted in significantly lower levels (-0.76$\pm$0.33) of *LINGO4* mRNA in Th17 differentiated cells (Fig 18A), confirming successful LINGO4 knockdown with an efficiency of 30 - 70 %. In control cells, *LINGO4* levels did not differ in sh LINGO4 and sh control transduced cells as for both expression levels were already quite low compared to Th17 cells. Flow cytometric analysis of virus particles-producing HEK cells showed successful transfection with a frequency of GFP-positive cells between 98.4 and 98.8 %, shown representatively for one experiment (Fig 18B). Further, transduced T cells showed a high frequency of GFP-expressing cells with at lowest 90.7 % in Th17 differentiated cells and 64 - 69 % in control cells. (Fig 18B).

After confirming successful gene knockdown, cells were further analyzed for additional transcription factor and cytokine expression to see if knockdown had an impact on the Th17 phenotype. *IL-17A* mRNA levels showed a decreasing tendency (mean -0.46$\pm$0.5) upon *LINGO4* knockdown. However, *RORC2* expression was significantly downregulated upon LINGO4 knockdown (-0.31$\pm$0.29), resulting in a decrease of 10 - 40 % compared to sh control transduced cells. Interestingly, *RORA* expression showed increasing, however not significant, expression upon *LINGO4* knockdown (Fig 19A). In general, high inter-individual differences between the donors were observed for all measured transcription factors.

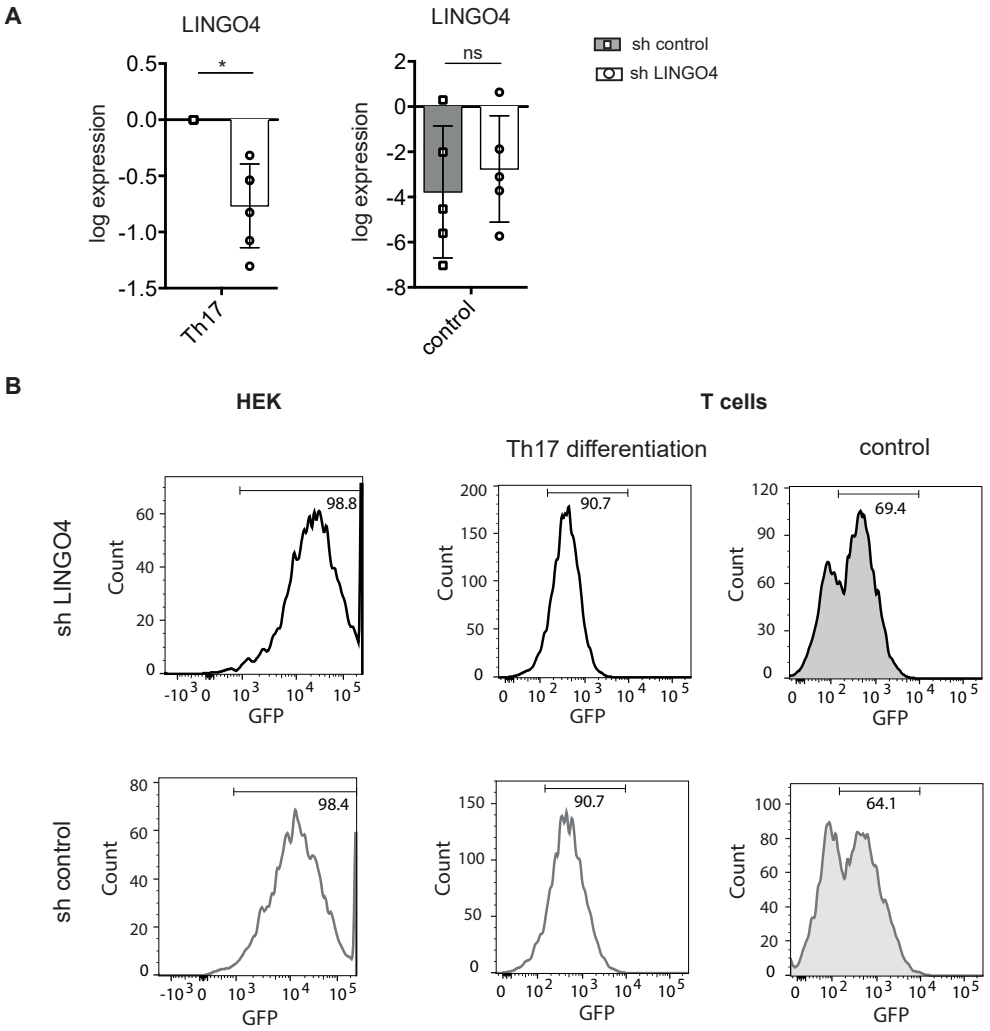

Figure 18 Validation of *LINGO4* knockdown in HEK and T cells.
(**A**) qRT-PCR analysis of lentiviral-mediated *LINGO4* knockdown in naive $CD4^+$ T cells subsequently differentiated into Th17 cells for 7 days or control cells (only anti-CD3/CD28 mAb stimulated) in n=6 individual donors. Values were normalized to Th17 differentiated cells transduced with sh control for both Th17 and control cells. Statistical significance was calculated by Wilcoxon test and defined as $^*P<0.05$. (**B**) Flow cytometric analysis for GFP expression in HEK and differentiated T cells transfected/transduced with sh LINGO4 and sh ctrl vector and Th17 differentiated or stimulated under control conditions with anti-CD3/CD28 mAbs (one representative experiment is shown for each cell type).

Control cells that were either transduced with sh control or sh LINGO4 RNA and only kept on TCR stimulation for the time of differentiation did not show altered expression levels of *IL-17A*, *RORC2*, *LINGO4* or *RORA* (Fig 19B). However, all expression levels were, independent of knockdown, below those of Th17 differentiated cells, confirming successful Th17 differentiation. In addition, *LINGO4* knockdown in Th17 differentiated cells resulted in significantly lower levels of *IL-22* (mean -0.54$\pm$0.38), and *TBET* (-0.33$\pm$0.20), while other cytokines and

transcription factors used for characterization of other Th cell subsets (i.e., *IFN-*γ, *TNF-*α, *IL-4* and *GATA3*) did not show altered expression levels upon LINGO4 knockdown (Fig 19C).

Altered expression levels of Th17-associated factors like *RORC2*, *IL-17A* and *RORA* resulting from *LINGO4* knockdown further supported the hypothesis of a functional role based on the previously observed Th17-associated *LINGO4* expression (4.1.1). Therefore, other factors annotated to the Gene Ontology (GO)-term "Th17 differentiation" were analyzed. The Gene Ontology Consortium summarizes connections of genes and proteins by cluster formation into functional classes or biological processes, based on their interaction. Th17 differentiation GO-terms were, e.g., Th17 transcription factors STAT3 and REL as well as LOXL3, an inhibitor of Th17 differentiation by interaction with STAT3 and NLRP3, a part of the inflammasome and shown to regulate Th1 and Th17 cells [Bateman et al., 2017, Gris et al., 2010]. Expression analysis of these factors showed only significantly decreased levels of *NLRP3* in Th17 cells upon *LINGO4* knockdown. However, an increasing tendency was observed for *REL* expression after *LINGO4* knockdown in Th17 cells (Fig 19D). *LOXL3* and *STAT3* expression revealed a high discrepancy between the different donors. Control cells showed no difference between *LINGO4* knockdown and control condition.

To examine the effect of LINGO4 knockdown on Th17 cells on protein level, cell culture supernatants of respective cells were analyzed for protein concentrations of IL-17A, IL-22, IFN-γ, TNF-α and IL-4 at the endpoint of differentiation (7 days). The decreasing tendency of *IL-17A* mRNA expression levels was not mirrored on protein level (Fig 20). Concentrations of IL-17A in sh LINGO4 compared to sh control transduced cells were not altered and successful differentiation was shown by higher IL-17A secretion of Th17 cells than control cells. The other measured cytokines were as well unaffected by the knockdown.

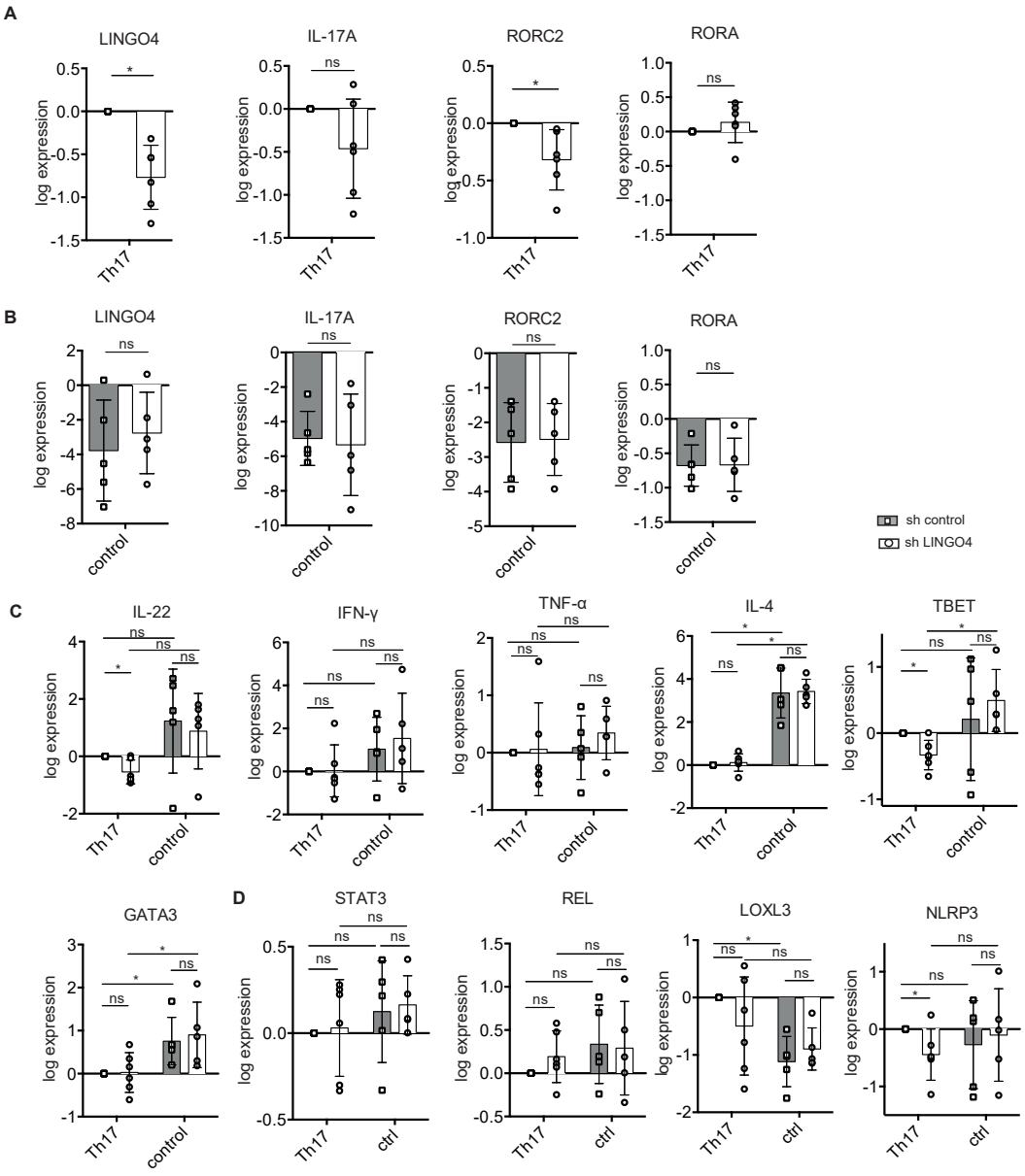

Figure 19 *LINGO4* knockdown decreases *RORC2* and *IL-17A* expression.
Naive CD4$^+$ T cells were subsequently differentiated into Th17 cells or control cells (only anti-CD3/CD28 mAbs stimulated) for 7 days after lentiviral-mediated knockdown. *IL-17A*, *RORC2* and *RORA* mRNA expression levels were analyzed by qRT-PCR in Th17 differentiated cells (**A**) and control cells (**B**) in n=6 individual donors. (**C**) *IL-22*, *IFN-*γ, *TNF-*α, *IL-4*, *TBET*, and *GATA3* mRNA expression in the same cells analyzed by qRT-PCR. (**D**) Gene expression of *STAT3*, *REL*, *LOXL3* and *NLRP3*, all "Th17 differentiation"-GO-term associated genes, was analyzed by qRT-PCR. Values were normalized to Th17 differentiated cells transduced with sh control vector. Statistical significance was calculated by Wilcoxon test and defined as $^*P<0.05$.

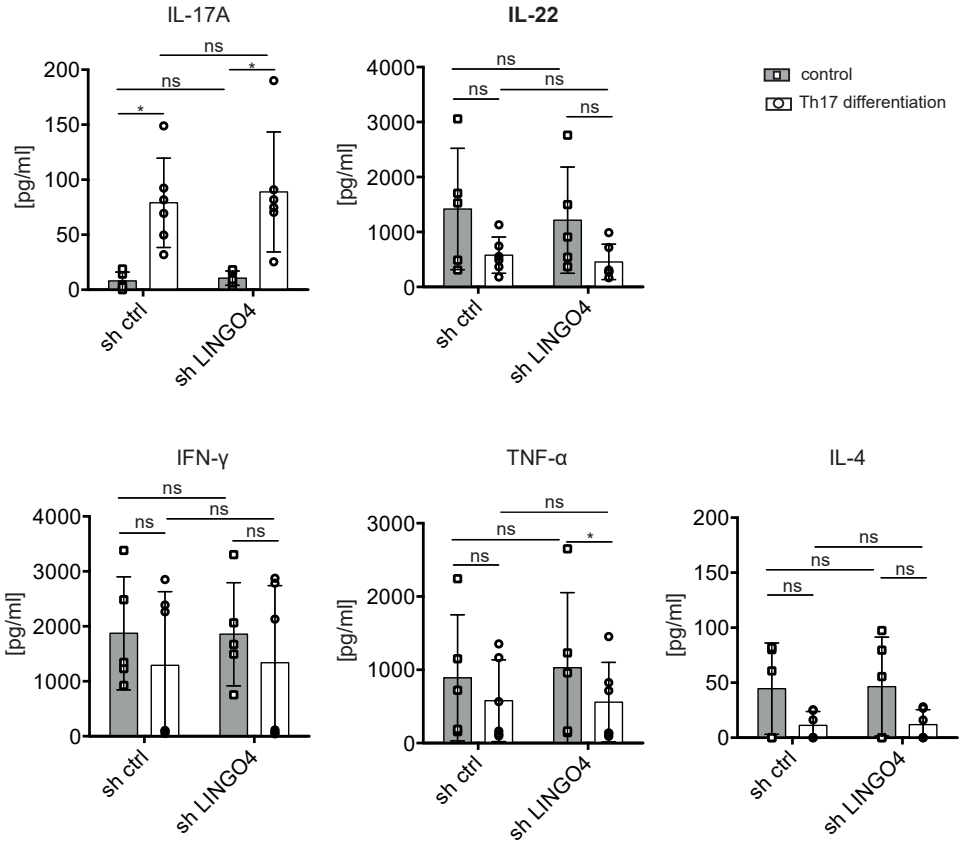

Figure 20 *LINGO4* knockdown has no impact on Th17 cytokine secretion.
Cytokine concentrations in cell culture supernatants of naive $CD4^+$ T cells undergone lentiviral-mediated *LINGO4* (or sh control RNA) knockdown and subsequently differentiated into Th17 cells for 7 days or control cells (stimulated only with anti-CD3/CD28 mAbs) were measured by ELISA. For determination of protein concentrations, supernatants were collected after 7 days of differentiation and analyzed for IL-17A, IL-22, IFN-γ, TNF-α and IL-4 secretion. Statistical significance was determined by Wilcoxon test in n=6 (Th17 cells) and n=5 (control cells) individuals and defined as $*P<0.05$.

4.1.5 Psoriasis patients reveal elevated *LINGO4* expression levels

LINGO4 expression could be assigned to Th17 cells and knockdown experiments suggested a potential regulatory role of LINGO4 on the Th17 phenotype. Th17 cells are known to play an important role in many (auto-)immune disorders, among them multiple sclerosis, rheumatoid arthritis, but also in the inflammatory skin disease psoriasis. To prove the hypothesis that elevated *LINGO4* levels are related to the Th17 phenotype based on "*ex vivo*" evidence, skin and blood cells of psoriasis patients were investigated for *LINGO4* expression levels. Lesional as well as non-involved skin biopsies were collected from patients suffering from psoriasis or as control atopic eczema and analyzed by whole genome expression arrays [Quaranta et al., 2014].

Indeed, psoriatic skin lesions showed the highest expression of *LINGO4* with significant dif-

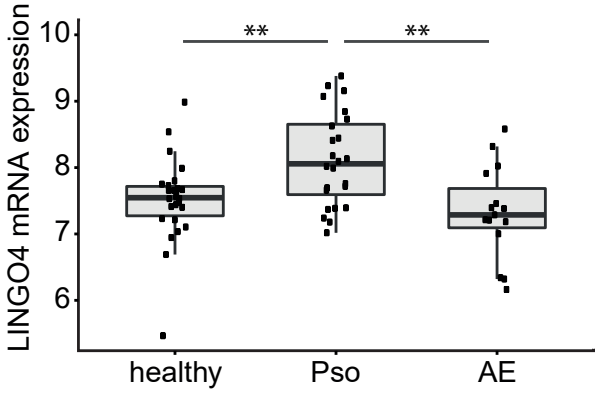

Figure 21 *LINGO4* mRNA expression is elevated in psoriatic skin lesions.
Whole genome expression arrays of skin biopsies from psoriasis, atopic eczema patients and healthy controls. Healthy n=26, psoriasis n=24, atopic eczema (AE) n=15. Expression of *LINGO4* was analyzed. Statistical significance was calculated by Welch 2-sample t-test and defined as $^{**}P<0.01$.

ferences to both healthy controls and atopic eczema (Fig 21). It was previously shown that keratinocytes of healthy donors and fibroblasts did not express relevant levels of *LINGO4* (Fig 6). However, to verify lymphocytes as the source of elevated *LINGO4* expression in the skin and moreover to analyze if this phenomenon was restricted to skin or also can be observed in the periphery, PBMCs from psoriasis patients and healthy donors were isolated and stimulated with PHA for 4 hours. *LINGO4* expression was analyzed on mRNA level (Fig 22). qRT-PCR analysis revealed induction of *LINGO4* expression upon PHA stimulation in both groups compared to unstimulated cells. In addition, a 2-fold higher *LINGO4* expression could be detected in psoriasis patients compared to healthy controls. The same was observed for expression levels of *RORC2*.

To further examine whether T cells of psoriasis patients themselves express more *LINGO4* on a basal level or if elevated levels are caused by higher numbers of Th17 cells in psoriasis patients, $CD4^+$ T cells were isolated from PBMCs of psoriasis patients and healthy donors. Defined cell numbers were cultured under Th17-polarizing conditions. As control, cells were stimulated with anti-CD3/CD28 mAbs only.

Cells from psoriasis patients and healthy donors cultured under Th17-polarizing conditions showed similar *LINGO4* expression levels. Th17 differentiation induced *LINGO4* expression compared to cells cultured under control conditions as previously described (Fig 23). As expected, *IL-17A* and *RORC2* expression was induced in Th17 differentiated versus control cells confirming efficient differentiation. However, cells of psoriasis patients showed significantly higher levels of *IL-17A* and *RORC2* in both Th17 differentiated and control cells compared to healthy donors.

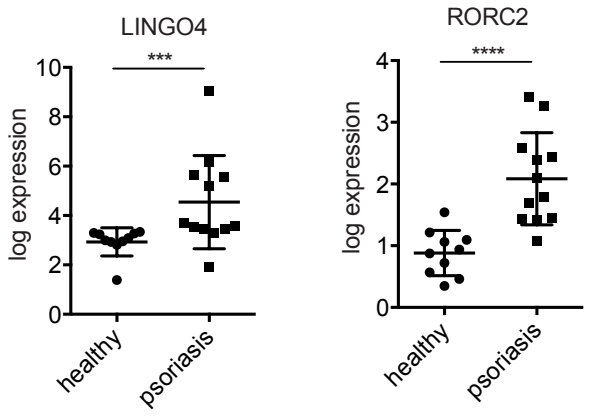

Figure 22 PBMCs of psoriasis patients show elevated *LINGO4* expression.
PBMCs of psoriasis patients (n=12) and healthy donors (n=10) were stimulated with PHA for 4 hours. *LINGO4* and *RORC2* mRNA expression was analyzed by qRT-PCR. Each patient was normalized to its unstimulated control. Statistical significance was calculated by Mann-Whitney test and defined as ***<0.001, ****$P<0.0001$.

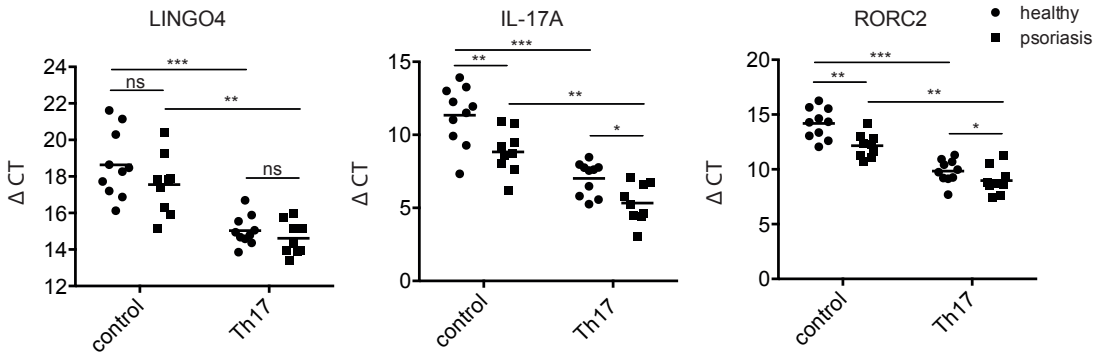

Figure 23 *In vitro* differentiated Th17 cells of psoriasis patients do not differ in *LINGO4* expression levels but *IL-17A* and *RORC2* levels compared to healthy controls.
$CD4^+$ T cells were isolated from PBMCs of psoriasis patients (n=11) and healthy donors (n=10) and cultured under Th17-polarizing conditions for 7 days or with anti-CD3/CD28 mAbs only (control). mRNA expression levels of *LINGO4*, *IL-17A* and *RORC2* were analyzed by qRT-PCR. Shown are Δ CT values normalized to housekeeping gene EF1α. Statistical significance was calculated by Mann-Whitney test and defined as *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

4.1.6 *Lingo4* expression in the thymus is diminished by Rorγt knock-out in mice

Even though gene knockdown by sh RNAs can lead to an efficient reduction of the target gene expression, results often cannot be compared with those of a complete knock-out achieved by genetic modification. Since a complete knock-out in human primary cells is difficult to achieve, mice offer the possibility to analyze the impact and underlying regulatory mechanisms under

a complete knock-out. Unfortunately, LINGO4 knock-out (KO) mice do not exist yet and breeding is complex and time-consuming. However, as data showed a strong correlation between *LINGO4* and *RORC2* expression, Rorγt knock-out (Rorγt -/-) mice were used to analyze a possible correlation between both genes also in mice. Thymus and small intestine (SI) were investigated for *Lingo4* mRNA expression compared to heterozygous (HET) mice. T cells mature in the thymus and Rorγt is expressed by nearly all double-positive T cells during this process. Therefore, Rorγt is an important factor for proper development of T cells and the adaptive immunity, while the small intestine is a Th17 cell rich site. For analysis, small intestine and thymus were used for RNA isolation and qRT-PCR analysis.

qRT-PCR analysis of *Rorγt* expression showed efficient gene knock-out (Fig 24A and B). Furthermore, heterozygous mice revealed strong *Rorγt* expression in the thymus (Fig 24A) while expression in small intestine was lower but still present (Fig 24B). *Lingo4* expression only differed significantly in the thymus, where *Rorγt* knock-out was associated with significantly decreased *Lingo4* mRNA levels (Fig 24A). Contrary to this, *Rorγt* knock-out did not show an impact on *Lingo4* expression in small intestine (Fig 24B) underlining that *Lingo4* was expressed independently from *Rorγt*.

4.2 FOXO4 in IL-22 producing CD4$^+$ T cells

FOXO4 was found to be upregulated in Th22 cells observed in whole genome expression arrays of distinct T cell subsets [Eyerich et al., 2009]. The finding led to the question whether FOXO4 regulates the phenotype of Th22 cells and is involved as novel factor in the regulation of IL-22 expression. Although, AHR, known to regulate IL-22 production in Th17 cells, was identified to play a role in IL-22 expression of Th22 cells as well, knockdown experiments showed that AHR was not exclusively responsible for the Th22 phenotype [Trifari et al., 2009].

4.2.1 Establishment of *in vitro* Th22 differentiation from naive T cells

To examine FOXO4 expression and its influence on IL-22 production, *in vitro* Th22 differentiation was established from naive CD4$^+$ T cells isolated from blood of healthy donors.

Isolated naive T cells were stimulated with Th22-polarizing cytokine cocktail consisting of IL-6 and TNF-α along with neutralizing anti-IL-4, anti-IL-12 and anti-IFNα mAbs and TCR stimulating anti-CD3/CD28 mAbs. Cells were differentiated in T cell medium containing 1 % human serum. Medium was changed after 5 days and cells were removed from the anti-CD3 coated well and cultured for two more days. As control, naive T cells were stimulated under the same conditions with anti-CD3/CD28 mAbs only. For analysis of successful Th22 differentiation, *IL-22* mRNA expression was measured after 7 days of culture. To induce cytokine expression, cells were further restimulated with anti-CD3/CD28 mAbs for 6 hours.

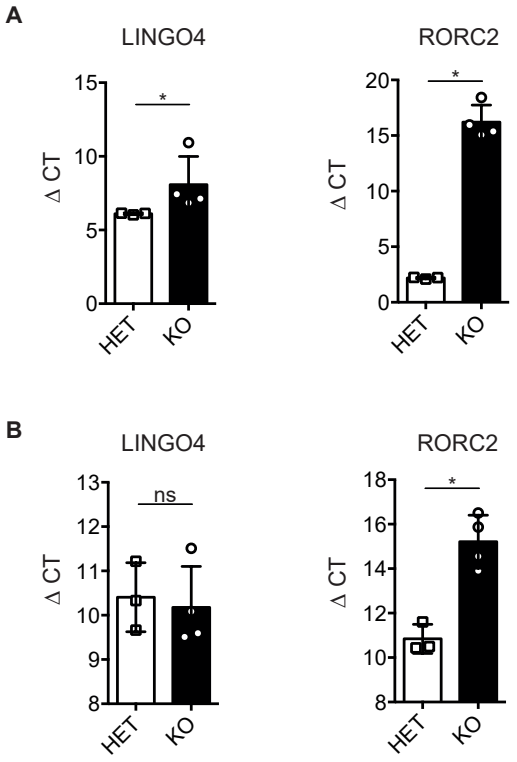

Figure 24 *LINGO4* expression is decreased in thymus of Rorγt KO mice but not affected in the small intestine.
Cells from thymus (**A**) and small intestine (**B**) of heterozygous (HET) and *Rorγt* -/- (KO) mice, respectively, were used for RNA isolation and *Lingo4* and *Rorγt* expression was analyzed by qRT-PCR for n=3 heterozygous and n=4 KO mice. Values are presented as Δ CT values and normalized to housekeeping gene *Gapdh*. Statistical significance was calculated by Mann-Whitney test and defined as **P*<0.05.

Stimulation of naive T cells with the Th22-polarizing cytokine cocktail resulted in elevated IL-22 mRNA expression compared to control cells (Fig 25A). Restimulation with anti-CD3/CD28 mAbs led to increased IL-22 expression in both control and Th22 differentiated cells. However, higher levels were observed for Th22 cells, confirming successful Th22 differentiation.

To verify successful differentiation on protein level, supernatants of differentiated cells were generated by restimulation with anti-CD3/CD28 mAbs for 48 hours. A clear induction of IL-22 in Th22 differentiated cells was observed (Fig 25B). TNF-α was only induced in one donor upon Th22 differentiation, while IFN-γ levels were also induced. IL-17A was only expressed at low levels by both control and Th22 cells (8.5$\pm$0.4 pg/ml and 16.6$\pm$4.8 pg/ml, respectively) as expected for Th22 cells. IL-4 was not detectable in any of the samples.

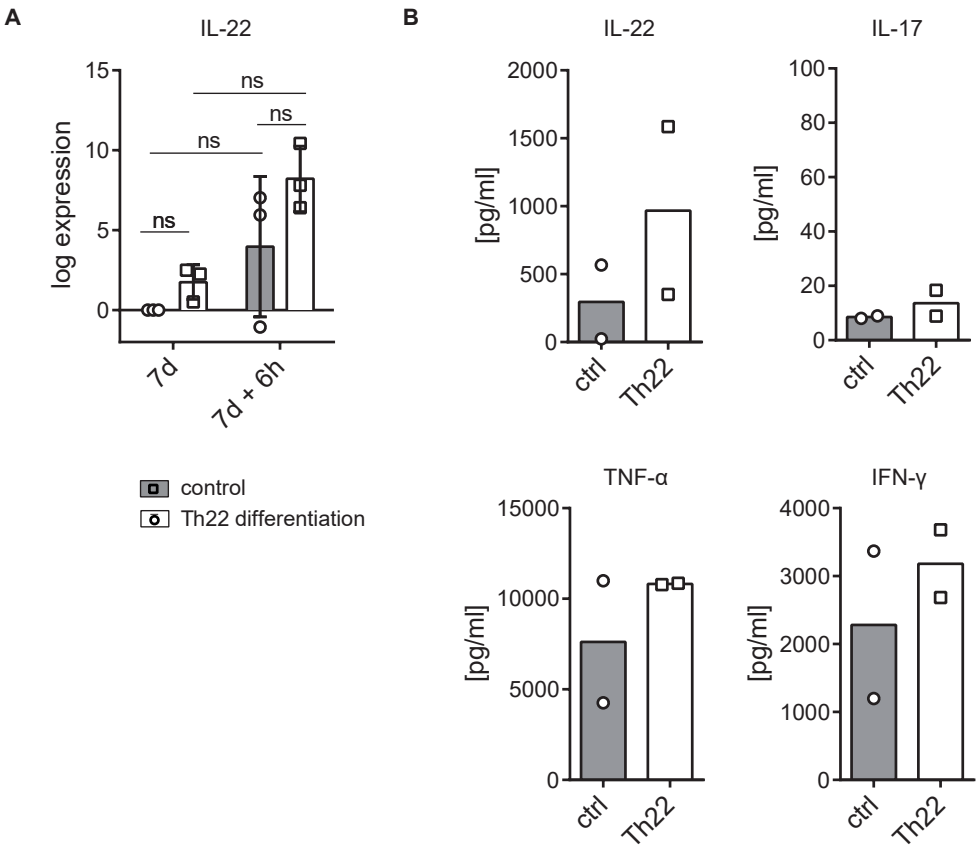

Figure 25 Naive T cells can be successfully differentiated *in vitro* into Th22 cells.
$CD4^+CD45RA^+$ T cells were differentiated into Th22 cells *in vitro* by adding a cocktail of IL-6 and TNF-α as well as neutralizing antibodies against IL-4, IL-12 and IFN-γ in the presence of plate-bound anti-CD3 and soluble anti-CD28 mAbs (each 0.75 μg/ml) for 7 days. After 5 days, cells were removed from anti-CD3 coated well and medium was changed. As control, naive T cells with TCR stimulus only were cultured for the same time. (**A**) qRT-PCR analysis of *IL-22* mRNA expression after 7 days of culture or after restimulation with anti-CD3/CD28 mAbs for 6 hours. Values were normalized to control cells at day 7. n=3 individual donors. (**B**) IL-22, IL-17, TNF-α and IFN-γ protein concentrations measured by ELISA in cell-free T cell supernatants generated by restimulation with anti-CD3/CD28 mAbs for 48 hours after 7 days of differentiation. n=2 individual donors. Statistical significance was calculated by Wilcoxon test and defined as $^*P<0.05$.

4.2.2 FOXO4 is induced upon *in vitro* Th22 differentiation and regulates IL-22 production

First, the role of FOXO4 in IL-22 producing cells was addressed. Therefore, naive T cells were incubated under Th22-polarizing conditions. Different time points during differentiation (0=naive, 60 min, 1 day, 5 days and 7 days) were investigated for FOXO4 expression in the cytosolic fraction by western blot analysis.

FOXO4 was induced upon Th22 differentiation (Fig 26). Already after 60 min of incubation an increase of FOXO4 was observed. FOXO4 levels further increased during the time of incubation confirming upregulated FOXO4 expression found in Th22 clones. To analyze a

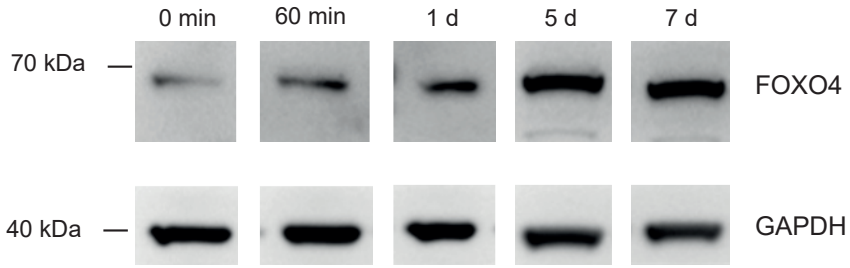

Figure 26 FOXO4 expression increases during Th22 differentiation.
Naive CD4$^+$ T cells were differentiated with Th22-polarizing cytokine cocktail in addition to stimulation with anti-CD3/CD28 mAbs. Cells were harvested at indicated time points and FOXO4 expression was investigated by western blot analysis. Blot was stained with anti-FOXO4 mAb and secondary anti-rabbit-HRP pAb. As loading control the housekeeping protein GAPDH was stained.

potential functional impact of FOXO4 on IL-22 production in T cells, CD4$^+$ effector T cells were isolated from blood of healthy donors and lentivirally transduced with sh RNA directed against a coding region sequence of FOXO4 or with unspecific sh RNA as control. Successful knockdown was confirmed by western blot analysis. FOXO4 expression was weak in control cells transduced with sh ctrl RNA, however, knockdown was still visible in cells expressing sh RNA directed against FOXO4 (Fig 27A). *IL-22* and *AHR* mRNA levels were investigated by qRT-PCR. Both *IL-22* and *AHR* expression showed decreasing trends with up to 30 % decrease in *IL-22* and up to 34 % decrease in *AHR* (Fig 27B). Cytokine concentrations were measured in cell culture supernatants generated by stimulation with anti-CD3/CD28 mAbs for 48 hours. The same decreasing trend as in mRNA expression was observed on IL-22 protein level in cell culture supernatants. Cells produced up to 50 % less IL-22 upon FOXO4 knockdown. A similar trend was observed for IL-17A, though decrease was only up to 30 %, while IFN-γ, TNF-α and IL-4 levels were not affected by FOXO4 knockdown (Fig 27C).

4.2.3 FOXO4 overexpression increases IL-22 production

The connection of FOXO4 to IL-22 production in T cells was shown by FOXO4 knockdown in CD4$^+$ T cells resulting in decreased IL-22 levels. FOXO4, therefore, might not only be involved in IL-22 regulation, but also in inducing the Th22 phenotype. This question was addressed using again the lentiviral overexpression system already described for LINGO4 overexpression in naive T cells (4.1.2). FOXO4 was overexpressed in naive CD4$^+$ T cells by lentiviral-mediated transduction with FOXO4-HA overexpression vector. As control, cells were transduced with empty vector. In the following, cells were differentiated into Th22 cells for 7 days using the Th22-polarizing cytokine cocktail in addition to anti-CD3/CD28 mAb stimulation (3.2.4 and Tab 20). qRT-PCR analysis of cells restimulated with anti-CD3/CD28 mAbs for 6 hours after 7 days of differentiation confirmed successful FOXO4 overexpression (Fig 28A). However, expression levels in qRT-PCR ranged from 1.8 - 8-fold induction revealing a high inter-individual variance between the different donors. On mRNA level, no clear induction

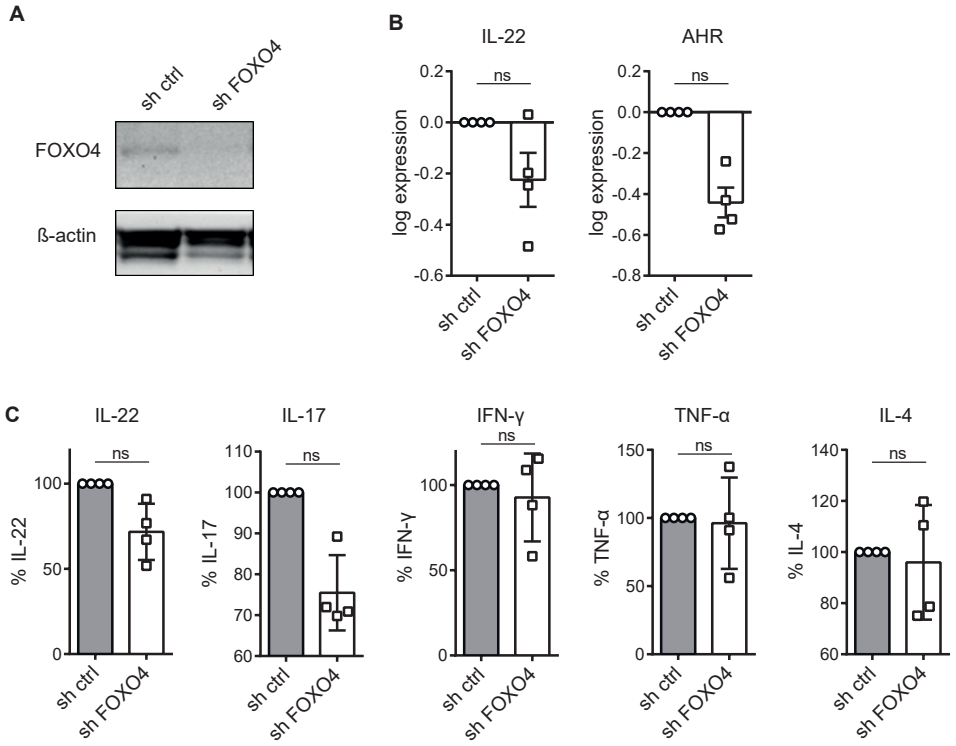

Figure 27 FOXO4 knockdown in effector T cells results in decreased IL-22 levels.
$CD4^+$ effector T cells were lentivirally transduced with sh RNA targeting FOXO4 to achieve gene silencing. As control, cells were transduced with unspecific sh RNA. n=4 individual donors. (**A**) FOXO4 knockdown was confirmed by western blot analysis in cells stimulated 24 hours after the second infection with anti-CD3/CD28 mAbs for 48 hours. Results of one donor are shown representatively. (**B**) qRT-PCR analysis of *IL-22* and *AHR* mRNA expression in transduced cells stimulated 24 hours after the second infection for 6 hours with anti-CD3/CD28 mAbs. (**C**) Protein concentrations in cell culture supernatants from cells stimulated 24 hours after the second infection with anti-CD3/CD28 mAbs for 48 hours were analyzed by ELISA. Values of sh ctrl cells were set as 100 % and sh FOXO4 values were normalized to them. Statistical significance was calculated by Wilcoxon test and defined as $*P<0.05$.

of *IL-22* and *AHR* expression could be detected. Low expression levels of IL-22 in FOXO4 overexpressing cells were observed in the same donors that showed only low FOXO4 overexpression. The same was observed for *AHR* expression levels. On protein level, a significant increase of IL-22 was observed upon FOXO4 overexpression confirming the hypothesis of FOXO4 influencing IL-22 levels and being involved in the induction of the Th22 phenotype (Fig 28C). In addition, TNF-α and IFN-γ showed an increasing trend while IL-17A and IL-4 were not detected (data not shown). Concerning the latter, future studies have to be performed to fully elucidate the function of FOXO4 in Th22 cells.

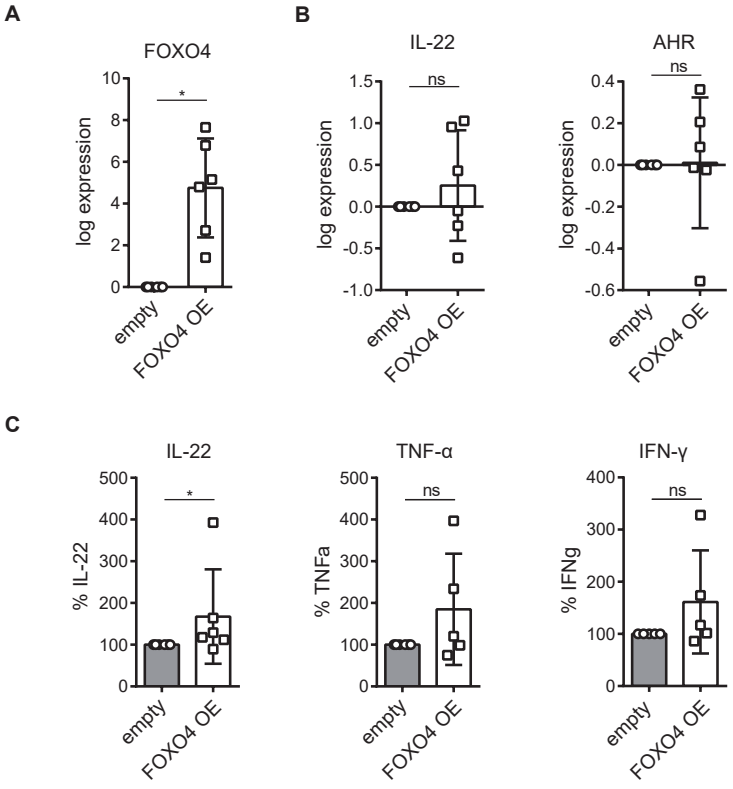

Figure 28 FOXO4 overexpression results in increased IL-22 levels.
FOXO4 was overexpressed in naive CD4$^+$ T cells by lentiviral transduction and cultured under Th22-polarizing conditions for 7 days. As control, cells were transduced with empty vector.(**A**) qRT-PCR analysis of Th22 differentiated, FOXO4/empty overexpressing cells. Cells were restimulated with anti-CD3/CD28 mAbs for 6 hours after 7 days of culture. Values were normalized to empty transduced Th22 differentiated cells. n=6 individual donors. (**B**) qRT-PCR analysis of *IL-22* and *AHR* expression levels in these cells. (**C**) IL-22, TNF-α and IFN-γ protein concentrations measured by ELISA in cell culture supernatants generated by restimulation with anti-CD3/CD28 mAbs of these cells for 72 hours. Statistical significance was calculated by Wilcoxon test and defined as $*P<0.05$.

5. Discussion

T cells are important actors of the immune system that specifically react to invasion of pathogens. Particularly $CD4^+$ T cells play important roles in the adaptive immune system by coordinating appropriate immune responses through activation and recruitment of other cells to sites of inflammation. On the other hand, they are main players in many autoimmune disorders when immunological tolerance fails. Therefore, it is critical to not only understand the contribution of different T cell subsets in different inflammatory and autoimmune diseases, but also to fully describe the exact regulation and differentiation process of T cell subsets in general. This knowledge might not only lead to a better understanding of pathogenic mechanisms but also to new therapeutic approaches in the long term.

Th1 and Th2 cells are the best described Th cell subsets and their regulation and differentiation process is known in detail. More recently discovered subsets like Th17 cells and Th22 are less well defined and investigated. Although the major transcription factors STAT3 and RORC2 driving the Th17 phenotype are identified and many other factors, among them RORA, BATF, IRF4 and RUNX1 - to name some of them - were found to participate in the differentiation process, there still remain open questions regarding the regulation process of Th17 differentiation, especially as most data are based on murine studies [Ivanov et al., 2006, Zhou et al., 2007, Schraml et al., 2009, Zhang et al., 2008, Yang et al., 2008b, McGeachy and Cua, 2008]. Coming to Th22 cells, even less is known. The aryl hydrocarbon receptor (AHR) was shown to be an important transcription factor, but is not exclusively responsible for the Th22 phenotype as knockdown experiments have shown [Trifari et al., 2009]. Moreover, AHR is also expressed in Th17 cells as IL-22 producers and more related to IL-22 expression than specifically to Th22 cells [Veldhoen et al., 2008a]. Therefore, a major transcription factor defining the Th22 phenotype is still missing as well as detailed knowledge about the differentiation process.

In this study, two new potential players, LINGO4 and FOXO4, for Th17 and Th22/IL-22 producing T cells, respectively, were investigated for their expression profile and function in the differentiation processes.

5.1 LINGO4 as a novel factor regulating the Th17 phenotype

In whole genome expression arrays of T cell clones, obtained from skin or blood samples from psoriasis or atopic eczema patients, elevated LINGO4 levels were found in Th17 or IL-17-associated cells, such as the possibly pathogenic Th1/Th17 cells and Th22 cells, while Th1 and Th2 clones did not show *LINGO4* expression. These results indicate a possible role of LINGO4 in T cells, though, to our knowledge, expression of this gene has not been described in T cells, yet.

LINGO4, a protein of unknown function, is one of four members within the LINGO family that consists of LINGO1, LINGO2, LINGO3 and LINGO4. The secreted protein discovery initiative (SPDI) predicted LINGO4 on bioinformatic level as a single-pass membrane protein [Clark et al., 2003]. Existing data on LINGO4 are solely based on mRNA analysis [Haines and Rigby, 2008, Liang et al., 2012]. Two murine studies were performed in the field of neuronal development that related *Lingo4* expression to mouse embryogenesis. The first study investigated expression patterns of the four different LINGO family members during mouse embryogenesis identifying different patterns for all four members. The most prominent, highest expressed and best described member of the LINGO family is LINGO1 that is mainly known in the context of the central nervous system as a regulator/repressor of myelination [Mosyak et al., 2006, Llorens et al., 2008]. Although not expressed by T cells, LINGO1 was identified to play a role in the Th17-mediated autoimmune disease multiple sclerosis (MS) and discussed as potential therapeutic target due to its ability to prevent myelination of neurons and therefore leading to neuronal dysfunction [Mi et al., 2007, Rudick et al., 2008]. LINGO2 was associated with Parkinson's disease and essential tremor by two studies, while nothing is known about the function of LINGO3 [Vilarino-Guell et al., 2010, Wu et al., 2011].
LINGO4 was solely expressed in neural tubes close to motor neurons and expression increased during development, but decreased to low levels in adult mice [Haines and Rigby, 2008]. The second study showed LINGO4 mRNA expression during early mouse embryogenesis in rhombencephalon, spinal cord and nasal placode [Homma et al., 2009]. Another study investigated a possible relation between LINGO4 and essential tremor, but could not find any correlation although genetic variants of *LINGO1* and *LINGO2* were identified as potential risk factors for developing this disease [Liang et al., 2012].

However, the structural motifs of LINGO4 protein might give an indication on the function. LINGO4 contains 11 leucine-rich repeat (LRR) motifs that are important for protein-protein interactions. Further, these motifs were found to be present in a wide variety of proteins with different functions, among them signal-transducing proteins or adhesive proteins [Kobe and Deisenhofer, 1994, Kobe and Kajava, 2001, Wit et al., 2011, Dolan et al., 2007]. Also in innate immunity LRR containing proteins are represented by, e.g., the family of Toll-like receptors that recognizes common structures of pathogens and are prominently involved in host defense [Matsushima et al., 2007]. Besides LRRs, LINGO4 contains immunoglobulin-like domains represented in adaptive immunity by, e.g., the T cell co-receptors CD4 and CD8, but also by MHC-class proteins [Wang et al., 1990, Ryu et al., 1990, Leahy et al., 1992, Saper et al., 1991]. In general, this motif is important for protein-protein and protein-ligand interactions [Barclay, 2003]. Although the exact protein structure of LINGO4 is not identified yet, a role as receptor and signal transducer is conceivable concluding from the structural motifs.

LINGO4 expression in T cells identified based on our whole genome expression data set was not described before and led to the investigation of the role of LINGO4 in T cells. LINGO4 expression in T cells was verified by analyzing the expression pattern in different immune

and non-immune cells. Keratinocytes and fibroblasts, main cell types of epidermis and dermis, respectively, did not express *LINGO4*, and neither did monocytes. However, in PBMCs *LINGO4* expression was detectable. PBMCs consist of monocytes as well as lymphocytes, including NK cells, B cell and T cells. *LINGO4* expression could be assigned to $CD4^+$ T cells, although NK cells and B cells were not tested for *LINGO4* expression in this study. By analyzing distinct $CD4^+$ *in vitro* differentiated T helper subsets, *LINGO4* was found to be associated with Th17 cells confirming the findings from whole genome expression arrays of T cell clones. Nevertheless, expression levels revealed that *LINGO4* in general is lowly expressed.

Besides validating the previous observations from whole genome expression data of T cell clones, these experiments also showed that *in vitro* differentiation from naive blood-derived T cells is an appropriate tool to study LINGO4 in Th17 cells. Th17 cells are characterized by cytokine secretion of IL-17A, IL-17F and IL-22 as well as by their key transcription factor RORC2. While *in vitro* differentiated Th17 cells showed clearly increased *IL-17A* and *IL-17F* mRNA levels (data not shown) as well as *RORC2* induction, *IL-22* mRNA was not induced upon differentiation. Furthermore, IL-17A could be measured in T cell supernatants generated from Th17 differentiated cells by restimulation with anti-CD3/CD28 mAbs. However, IL-17A secretion was diminished by this way of restimulation after lentiviral transduction that was used to generate genetically modified T cells, while other cytokines were not affected. In addition, intracellular cytokine staining of *in vitro* differentiated Th17 cells was not possible. Neither IL-17, IL-22 nor IFN-γ or TNF-α positive cells could be detected after restimulation with PMA/ionomycin. Live/dead staining revealed a high number of dead cells after restimulation, indicating that these cells are too exhausted after 7 days of differentiation and restimulation, particularly when lentivirally transduced.
T cell exhaustion is mainly described in the context of cancer and infection with human immunodeficiency virus (HIV), hepatitis C virus (HCV) or chronic cytomegalovirus (CMV) [Antoine et al., 2012, Wherry and Kurachi, 2015]. Exhausted T cells often lose effector functions like cytokine secretion, alter transcription factor expression and upregulate inhibitory pathways [Wherry and Kurachi, 2015]. A long and persistent stimulation, e.g., by antigen, is thought to be responsible, a situation present in chronic inflammation and infection, but also during *in vitro* differentiation [Wherry, 2011, Wherry and Kurachi, 2015]. In addition, costimulatory molecules like CD28 are often downregulated [Antoine et al., 2012]. Therefore, it is conceivable that restimulation of *in vitro* differentiated Th17 cells led to a similar state, preventing intracellular cytokine staining or cytokine secretion.

Still, *in vitro* Th17 differentiation is an important tool, both in human as well as murine T cell studies, particularly in regard to autoimmune disorders. While in mice *in vitro* Th17 differentiation is well established, human Th17 cells are more difficult to obtain. Murine cells from, e.g., spleen or lymph nodes can be differentiated by a cytokine cocktail of IL-6 and TGF-β in addition to anti-CD3/CD28 stimulus [Bedoya et al., 2013]. Differentiated cells showed induction of *Rorγt* as well as IL-17A in qRT-PCR and ELISA analysis. Further, intracellular cytokine staining of differentiated cells resulted at least in a frequency of 4.5 % IL-17A positive

cells [Bedoya et al., 2013]. However, *in vitro* differentiated Th17 cells were not stable and could easily be transdifferentiated into other T helper subsets [Nurieva et al., 2009]. Upon transfer into lymphopenic hosts a conversion into IFN-γ producing Th1 cells was observed, while cells that were transferred into normal mice retained their Th17 phenotype revealing limitations of *in vitro* differentiation already in mice [Nurieva et al., 2009].
Human naive T cells are more difficult to differentiate as a combination of IL-6 and TGF-β is not sufficient for differentiation and rather depends on IL-6, IL-1β as well as IL-23 that is important for Th17 maintenance [Acosta-Rodriguez et al., 2007, Wilson et al., 2007]. Other studies described preferential differentiation of FOXP3$^+$ naive Tregs into Th17 cells under polarizing conditions with IL-2, IL-1β, IL-23 and TGF-β highlighting the variability between differentiation methods [Valmori et al., 2010, Gagliani et al., 2015]. Different protocols exist, and the role of TGF-β during differentiation is critically discussed to be either suppressive or necessary for differentiation [Acosta-Rodriguez et al., 2007, Wilson et al., 2007, McGeachy and Cua, 2008, Volpe et al., 2008, Hakemi et al., 2011]. For verification of *in vitro* Th17 differentiation, mainly qRT-PCR and ELISA are used, while intracellular cytokine analysis by flow cytometry is missing, confirming our observation that intracellular cytokine staining is difficult to achieve [Hakemi et al., 2011, Wilson et al., 2007, Volpe et al., 2008, Hiller and Traidl-Hoffmann, 2012]. Although a comparison of *in vitro* differentiated and physiological Th17 cells regarding detailed gene expression profiles and cytokine secretion is missing, Th17 signature can be achieved by *in vitro* differentiation and offers an easier access to Th17 cultures than working with Th17 *ex vivo* clones. In addition, *in vitro* differentiation enables insights into the differentiation process that cannot be achieved by fully differentiated *ex vivo* clones. Therefore, *in vitro* Th17 differentiation is an important and valuable tool to gain insight into the exact differentiation process, although it has to be kept in mind that this technique has its limitations and might not represent full reality.

A strong correlation between *LINGO4* and *RORC2* expression was identified in kinetics of *in vitro* Th17 differentiation as well as in T cell clones underlining a potential role of LINGO4 in Th17 cells. The expression pattern of *LINGO4* during *in vitro* Th17 differentiation over 7 days revealed an early upregulation of *LINGO4* compared to control cells already at day 1. Further, the expression pattern showed an oscillatory course that was shared by the key transcription factor *RORC2*, which showed nearly identical relative expression levels as *LINGO4*. In contrast, mRNA levels of *IL-17A* remained constant once induced. Oscillatory expression correlated with medium changes during cultivation that caused corresponding anti-cyclic oscillation in IL-17A protein concentrations in supernatants. Therefore, a potential autocrine feedback loop regulated by IL-17A was analyzed.
IL-17A is a member of the IL-17 cytokine family further consisting of IL-17B, IL-17C, IL-17E, IL-17F and IL-25 with highest homology to IL-17F that binds to the same receptor as IL-17A [Li et al., 2000, Lee et al., 2001, Starnes et al., 2001, Hurst et al., 2002]. IL-17A signals through IL-17RA (IL-17RA). This receptor is ubiquitously expressed and found in cell types like T cells, fibroblasts and endothelial cells [Yao et al., 1995]. In mice, IL-17RA is sufficient for IL-17A signaling, while in humans a complex of IL-17RA and IL-17RC is required for effi-

cient downstream signaling [Toy et al., 2006]. This might explain why, despite the ubiquitous expression of IL-17RA in humans, not all cell types show a high responsiveness to IL-17A [Toy et al., 2006].

A feedback loop of IL-17A on Th17 cells is described in mice [Smith et al., 2008]. Stimulation of splenocytes from wild type mice with IL-17A or IL-17F resulted in decreased levels of IL-17A and IL-17F in the contained fraction of IL-17-expressing T cells. In cells of IL-17RA deficient (*Il17ra-/-*) mice this effect was lacking, suggesting a controlling function of IL-17A on its producer cells by a short feedback loop critically dependent on IL-17RA expression [Smith et al., 2008]. However, it could not be clarified if this effect was of autocrine or paracrine nature, as splenocytes were used instead of pure T cell populations [Smith et al., 2008].

Also, other studies investigated possible regulatory functions of IL-17RA in T cells or feedback loops in Th17 differentiation. A negative feedback loop controlled by STAT3, an early transcription factor in Th17 differentiation, can for instance limit human Th17 differentiation and might represent a safety mechanism against an overshooting Th17 response under inflammatory conditions [Purvis et al., 2014]. Furthermore, IL-17A secreted by, e.g., Th17 cells led to STAT3 activation and phosphorylation in downstream cells like endothelial cells via IL-17RA signaling, inducing pro-inflammatory cytokine secretion and recruitment of neutrophils [Yuan et al., 2015]. The fact that IL-17A was shown to act on STAT3 by IL-17RA binding, though in non-immune cell types, combined with the fact that a self-regulation in Th17 cells was already detected depending on STAT3 opens the possibility of an autocrine regulation in human Th17 differentiation.

To investigate a potential feedback loop by IL-17A, IL-17RA expression was measured in in vitro differentiated Th17 and control cells. For comparison of expression levels, different cell types known to respond to IL-17A stimulation and to express IL-17RA like fibroblasts, keratinocytes and monocytes were used, but also $CD4^+$ T cells [Paulissen et al., 2013, Shahrara et al., 2009, Shi et al., 2011]. Indeed, all these cells, including *in vitro* differentiated Th17 cells, showed *IL-17RA* expression, although at different levels. Cells known to respond to IL-17A showed a stronger IL-17RA expression than *in vitro* differentiated T cells. However, stimulation of *in vitro* differentiated Th17 cells and control cells with different concentrations of rIL-17A did not show any impact on *LINGO4*, *IL-17A*, *RORC2* and *IL-17RA* expression, disproving the hypothesis of an autocrine feedback loop. Nevertheless, another underlying feedback mechanism mediated by other factors secreted by Th17 cells is still possible. It is even possible that IL-17A only affects *LINGO4* and *RORC2* expression during differentiation of Th17 cells and not at the fully differentiated stage that was used in this experiment.

The strong correlation between *LINGO4* and *RORC2* relative expression levels was striking. Further, analysis of genomic organization revealed direct proximity of both genes on the minus strand of chromosome 1. Since protein data for LINGO4 are not existing and a function could not be assigned to this protein yet, the question emerged whether *LINGO4* codes for a functional protein at all or rather is a bystander product of *RORC2* expression. Another possibility would be a common transcription of both genes on one mRNA. However, nested PCR clearly showed that mRNAs of *RORC2* and *LINGO4* were not expressed as a common transcript. In fact, by now it was found that the human genome is organized and genes are

not randomly distributed as at first hypothesized. Instead, clustering of genes with similar functions was identified [Caron et al., 2001, Lercher et al., 2002]. For instance, expression of housekeeping genes was found to cluster, while a tissue-specific expression clustering could not be identified so far [Lercher et al., 2002]. However, organization of genes in close proximity is suggested by their often similar expression levels [Woo et al., 2010]. Woo *et al.* showed that co-expression was higher when distance between genes was in the sub-megabase range, as it is the case for *RORC2* and *LINGO4*, than in genes with a higher distance in between. Further, tandem (-/-) oriented genes in the sub-megabase range showed a lower co-expression than divergent genes which lie on different strands (-/+) [Woo et al., 2010]. Another study tested three groups of genes (protein-protein interactions, complexes and pathways) for clustering upon their function. Indeed, genes of all three groups showed a spatial concentration both intra-chromosomal as well as inter-chromosomal that was significant, meaning genes are organized in close distance when having the same function or interacting with each other [Thévenin et al., 2014]. Therefore, chromosomal organization of *LINGO4* and *RORC2* supports a functional common role.

5.1.1 T cells suppress LINGO4 protein overexpression

The function of LINGO4 in T cells was addressed by lentiviral-mediated overexpression. Naive T cells are difficult to transfect as common techniques using lipofectamine, electroporation or calcium phosphate result in low efficiency or a high toxicity for the cells [Chicaybam et al., 2013, Kim and Eberwine, 2010, Ebert et al., 1997]. Therefore, lentiviral transduction was used, having the advantage of stable integration of the transfer gene and the possibility to transfect all kinds of cell types, even non-dividing cells like naive human $CD4^+$ T cells [Frimpong and Spector, 2000, Bilal et al., 2015].

However, in naive T cells LINGO4 overexpression could be solely detected on mRNA level, while protein overexpression failed. Interestingly, this phenomenon seemed to be T cell specific as in HEK cells, transfected with the same overexpression vector as proof of concept, the protein was detected. This finding suggested a strict regulation of LINGO4 protein in T cells. In general, detection of LINGO4 protein was difficult as commercially available antibodies all resulted in various and unspecific staining patterns. Mass spectrometry analysis was the only possibility to detect endogenous LINGO4 protein proving that the gene is protein-coding. For this approach, also overexpressed LINGO4-HA protein was isolated from HEK lysates by immunoprecipitation via HA-tag to build up a library of LINGO4-specific peptides. Only based on this library endogenous LINGO4 could be detected in *in vitro* differentiated T cell subsets with the sensitive and conservative data-independent analysis (DIA) method. Surprisingly, no differential protein abundance between the different T cell subsets was detectable although *LINGO4* mRNA expression was elevated in Th17 cells. Possible reasons might be that significant differences on mRNA level are too small to be mirrored on protein level or that protein translation is strictly regulated by post-transcriptional modifications influencing, e.g., mRNA turn-over rate, stability or degradation [Machnicka et al., 2013, de Sousa

Abreu et al., 2014, Prabakaran et al., 2012].

In general, gene expression does not necessarily show a high correlation between mRNA and protein levels. Different studies showed various and inconsistent results regarding this correlation. Some studies showed a positive regulation between mRNA and protein while others did not observe a correlation at all or a negative one, depending on investigated genes and cell types [Anderson and Seilhamer, 1997, Guo et al., 2008, Chen et al., 2002, Lichtinghagen et al., 2002]. Additionally, protein and mRNA levels have to be compared at different time points as translation of proteins takes longer than transcription of mRNA. Moreover, transcription and subsequent translation of the same gene can be regulated independently by different pathways [Cheng et al., 2016].
Regarding the discrepancy of *LINGO4* mRNA upregulation, but identical protein levels in Th17 cells compared to other T helper subsets, this might indicate strict regulation of protein levels by post-transcriptional mechanisms leading to a fast protein turn-over rate or different protein activities of LINGO4 in Th17 cells compared to other T helper subsets. This phenomenon was already assumed for AHR, a transcription factor of Th17 cells [Trifari et al., 2009]. While *AHR* mRNA expression was induced by Th17-polarizing conditions, activity of AHR protein measured by the expression of its downstream target CYP1A1 was not altered compared to non-polarized cells showing even lower mRNA level [Trifari et al., 2009]. As the structure of LINGO4 is not fully understood, nothing is known about the function of this protein or its potential post-translational modifications. However, missing protein overexpression in T cells specifically might indicate a critical regulatory function of this protein in T cells, particularly Th17 cells, that needs to be strictly controlled.

Direct degradation of protein by the proteasome might be another possible reason for missing LINGO4 protein overexpression despite the high amounts of mRNA in naive T cells. Proteasomal degradation is an important mechanism of protein regulation. Unfolded or misfolded proteins that accumulate in the ER induce the unfolded protein response (UPR) and are degraded by ER-associated degradation (ERAD), a process also induced upon ER stress by , e.g., protein overexpression maintaining capacity and homeostasis of the ER [Raden et al., 2005, Shen et al., 2004, Brodsky and McCracken, 1999]. Misfolded or unfolded proteins are relocated to the cytosol and ubiquitinylated for recognition and subsequent degradation by the proteasome [Goder, 2012]. Central components of these complexes are E3 ubiquitin ligases that mark proteins with ubiquitin for proteasomal recognition and degradation [Hirsch et al., 2009].
However, proteasomal degradation was not responsible for missing LINGO4 protein overexpression, as treatment of HA-tagged *LINGO4* overexpressing Jurkat T cells with MG-132, a proteasome inhibitor, did not enhance presence of LINGO4 protein. Upon proteasome inhibition, ubiquitinylated LINGO4 should, if present, accumulate in the cells and should be detectable via the HA-tag. In general, compared to empty transduced cells, a higher accumulation of ubiquitin was detected in LINGO4 overexpressing cells already without inhibitor treatment possibly representing degraded LINGO4. However, it could not be identified by anti-

HA mAb meaning that either the antibody epitope was masked or the HA-tag was cleaved. In addition, treatment with proteasome inhibitor did not lead to accumulation of LINGO4 protein, although quantification of ubiquitin showed efficient proteasome inhibition. It is known that RNAs can have functional roles as noncoding (nc) RNA, also referred to as long non-coding (lnc) if they exceed length of human mRNA or intergenic RNAs [Palazzo and Lee, 2015]. However, a regulatory function of *LINGO4* mRNA itself was ruled out. *LINGO4* overexpression in naive T cells followed by Th17 differentiation did not show any impact, neither on Th17 differentiated cells nor on control cells. Moreover, a functional role of *LINGO4* as RNA is unlikely since mass spectrometry confirmed expression of the protein.

5.1.2 The role of LINGO4 in Th17 differentiation

Overexpression of LINGO4 did not reveal any functional insight as protein could not be overexpressed. Therefore, gene silencing using small hairpin (sh) RNAs was used to generate stable LINGO4 knockdown. Gene knockdown was shown to be successful on mRNA level during Th17 differentiation over a period of 7 days and indeed suggested a regulatory function of LINGO4 within this process. Th17 differentiation of sh RNA transduced naive T cells was critical for detection of *LINGO4* knockdown as T cells express *LINGO4* at a very low level, too low for knockdown detection without transcriptional induction by Th17-polarizing conditions. Interestingly, the knockdown of LINGO4 in Th17 differentiated cells resulted in decreased *RORC2* and *IL-17A* mRNA levels, while *RORA* showed elevated, hence not significant, expression and IL-17A protein levels remained unchanged. Further, expression analysis of different cytokines and factors associated with the GO-term “Th17 differentiation” showed significant downregulation of *IL-22*, *TBET* and *NLRP3*, while *REL* expression showed clear upregulated levels. Other factors remained unchanged.

Regulation of RORC2/RORγt, key transcription factor of Th17 cells, is quite complex and the mechanism still not completely understood [Ivanov et al., 2006]. Further, most knowledge is based on murine studies and it is unclear how much that is resembled in the human system. RORγt directly induces IL-17A and IL-17F transcription, thereby regulating Th17 differentiation [Ivanov et al., 2006, Manel et al., 2008]. However, Rorγt deficient mice are still capable of producing IL-17 and generating Th17 cells, but at lower extend. It was shown that RORα, a member of the same protein family, also controls IL-17 expression [Yang et al., 2008b]. This factor is upregulated in Th17 cells and regulated by STAT3. Similar to RORγt deficient mice, RORα deficiency resulted in decreased levels of IL-17 production, while a deficiency in both factors led to complete suppression of Th17 differentiation [Yang et al., 2008b]. STAT3 regulates RORγt and therefore Th17 differentiation. STAT3 is induced by TCR signaling via involvement of NF-κB and IL-6 signaling [Durant et al., 2011, Tripathi et al., 2017, Jin et al., 2009] and binds directly to the *Rorc* gene locus to initiate transcription. Further, it induces Th17 relevant transcription factors like *Rora*, *Batf*, *Irf4*, *Ahr* and *Maf* and binds directly to promotor regions of the Th17-cytokines *Il17a*, *Il17f* and *Il21* [Durant et al., 2011]. Contrary to STAT3, RORγt itself has a regulatory role in Th17 differentiation by controlling only a few

genes, e.g., *Il17a*, *Il17f* and *Il23r* [Ciofani et al., 2012]. RORγt expression is further controlled by c-Rel, a member of the REL/NF-κB family that binds directly to the *Rorγt* promotor, but does not activate *Rora* or *Il17a* [Ruan et al., 2011].

Due to decreased RORC2 levels upon LINGO4 knockdown LINGO4 can be assumed to be an upstream, and therefore, positive regulator of RORC2. As *STAT3* expression was not affected by knockdown, LINGO4 is probably acting downstream of STAT3. However, since LINGO4 is predicted to be a transmembrane protein, it is also possible that LINGO4 initiates a signaling pathway leading to *RORC2* activation independent of STAT3. Decreased *IL-17A* levels are more likely resulting from decreased *RORC2* levels and were not directly affected by LINGO4. It is described that also environmental factors contribute to Th17 differentiation. One example is the level of endogenous nitric oxide (NO), regulating the expression of the nitric oxide synthase-2 (NOS2)[Obermajer et al., 2013]. In this context, LINGO4 as a receptor in the cytoplasma membrane would be conceivable to regulate RORC2 expression, especially as the ligand is not known, yet [Obermajer et al., 2013].

Compensatory effects might explain why IL-17A protein amounts were not altered despite decreased mRNA levels. As mentioned before, IL-17 expression can also be regulated by RORA. Elevated *RORA* mRNA levels, therefore, could compensate decreased *RORC2* levels, thereby keeping IL-17 protein level unchanged. Moreover, mRNA and protein level do not always correlate, as discussed before. Further, IL-17A protein concentration was only measured in cell culture supernatant accumulated over 7 days, while mRNA levels represent expression at a defined time point, making comparison of these two parameters difficult.

However, the effect of LINGO4 knockdown on *RORC2* expression hints to a function of LINGO4 as positive regulator of Th17 differentiation. Further, unchanged *STAT3* expression upon LINGO4 knockdown indicates that regulation by LINGO4 is either independent of STAT3 or LINGO4 is a downstream regulator of STAT3. Moreover, *IL-22* and *TBET* were significantly downregulated in response to LINGO4 knockdown in Th17 cells. However, IL-22 expression in Th17 cells is not regulated by RORC2, but rather by AHR [Veldhoen et al., 2008a, Effner et al., 2017]. AHR is a receptor that is activated by metabolites and functions both as transcription factor by translocating to the nucleus upon activation to induce gene expression, but also as E3 ubiquitin ligase to regulate proteasomal protein degradation [Sogawa and Fujii-Kuriyama, 1997, Ohtake et al., 2007]. However, how LINGO4 knockdown affects IL-22 expression and if there is a connection to AHR is unclear and needs further investigation. Regarding decreased *TBET* expression in LINGO4 silenced Th17 cells, murine studies showed that T-bet can suppress Th17 differentiation and RORγt expression [Lazarevic et al., 2011]. The fact that LINGO4 knockdown resulted in decreased *TBET* levels indicates it as a positive regulator as well, although mechanistic insight have to be addressed in future studies. Moreover, in humans, especially under inflammatory conditions, Th17 cells co-expressing IL-17 and IFN-γ as well as the corresponding transcription factors for Th17 and Th1 cells, RORC2 and TBET, are common, but regulatory mechanisms leading to this phenotype are

not known. LINGO4 knockdown further resulted in significant decrease of NLRP3 that is mainly described in connection with the inflammasome to induce host defense [Pétrilli et al., 2007]. Mutations in the gene were however connected with autoimmune disorders and an increase in Th17 response, as NLRP3 cleaves pro-IL-1β into active IL-1β which then initiates Th17 response [Pétrilli et al., 2007, Meng et al., 2010]. Further, NLRP3 expression is described for murine and human CD4$^+$ T cells, however, in the context of Th2 differentiation [Bruchard et al., 2015]. Details about NLRP3 expression in Th17 cells or the exact regulation are not known, but NLRP3 appears in the GO-term "Th17 differentiation" as regulator of the upstream differentiation process. However, as an interaction between NLRP3 and IRF4, a transcription factor also important in Th17 cells, was identified it is conceivable that NLRP3 indeed plays a direct role in Th17 cell differentiation and is potentially regulated by LINGO4.

Finally, evidence for a role of LINGO4 in Th17 cells was supported by elevated *LINGO4* mRNA expression in skin lesions of psoriasis patients compared to atopic eczema patients and healthy controls. This finding was confirmed by higher expression levels of *LINGO4* in stimulated PBMCs from psoriasis patients compared to healthy controls, showing that this finding was not restricted to skin but a systemic phenomenon. Since keratinocytes and fibroblasts, main cell types of the skin, did not express LINGO4, while lymphocytes did it is most likely that elevated LINGO4 levels observed in skin lesions originate from T cells. Moreover, psoriasis is known to be a Th17-mediated disease with strong infiltration of these cells into the skin [Bos et al., 1989, Ferenczi et al., 2000, Blauvelt, 2008]. In addition, PBMCs of psoriasis patients are known to produce a higher amount of IL-17A matching the findings of elevated *LINGO4* and *RORC2* levels and indicating a pathogenic Th17 phenotype in psoriasis [Benham et al., 2013]. Cultivation of CD4$^+$ T cells under Th17-polarizing conditions allowed some conclusions about *LINGO4* expression in psoriasis. *LINGO4* expression in CD4$^+$ T cells polarized under Th17-inducing conditions of psoriasis patients and healthy controls showed similar levels with a slight increasing tendency in psoriasis patients, while IL-17A and RORC2 expression was significantly increased. From these data, it can be concluded that CD4$^+$ T cells or Th17 cells of psoriasis patients do not express *per se* more *LINGO4* per cell, but that elevated levels in skin lesions and PBMCs likely were derived from a higher cell number of Th17 cells in this patient group, underlining that LINGO4 is upregulated in Th17 cells.

5.1.3 LINGO4 in Th17 cells - conclusion and outlook

The presented data point to a role of LINGO4 in Th17 cells as a potential positive regulator in their differentiation process as shown in Fig 29. This is further supported by the finding of elevated *LINGO4* levels in psoriasis patients. However, it was difficult to address the functional role of LINGO4 in this T cell subset as studies were limited to mRNA analysis. Endogenous LINGO4 protein could only be detected by mass spectrometry due to missing specific antibodies and overexpression of protein was suppressed in T cells. This fact as well as the low expression levels in none-Th17 cells indicated a strict regulation of LINGO4 important for cell

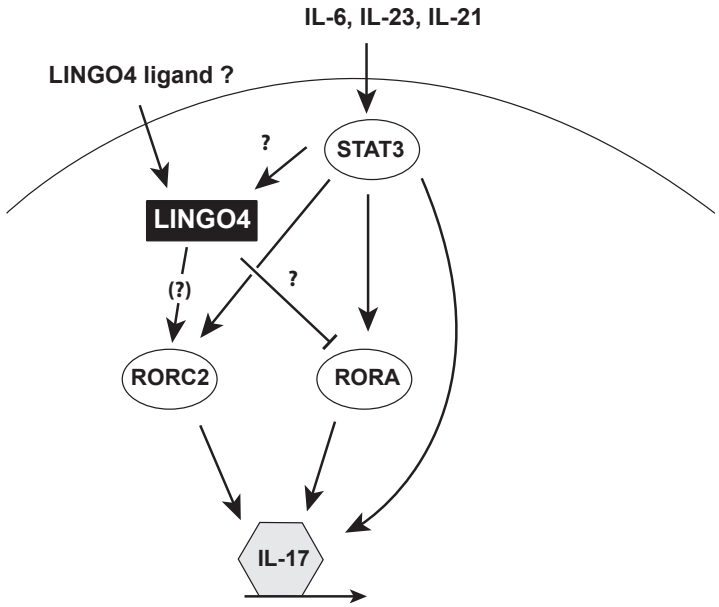

Figure 29 Possible regulation mechanism of LINGO4 in Th17 cell differentiation.
LINGO4 expression was associated with Th17 cells and might contribute to the differentiation process as shown in the scheme. The activator of LINGO4 is unknown, it might be an external LINGO4 ligand, but also induction by STAT3, itself activated by IL-6, IL-23 and IL-21, is conceivable. STAT3 was only excluded as a downstream target of LINGO4. LINGO4 was identified as a positive regulator of *RORC2* and might represent a negative regulator of *RORA*, both inducers of IL-17A. If the regulatory effect is of direct nature or indirect by activating so far unknown mediators has to be addressed in future studies.

homeostasis. However, knockdown experiments shed light on a functional role, indicating LINGO4 to be a possible positive regulator of RORC2 and thereby consecutively influencing IL-17 expression. Further studies need to address remaining open questions that critically depend on the generation of a specific antibody against LINGO4. Future murine studies can be helpful as well to gain mechanistic insight into LINGO4 function. Although Th17 differentiation in mice is not identical with the human process, most studies identifying new transcription factors and their mechanistic function in Th17 cells were conducted in mice by gene knock-out or overexpression. Complete knock-out might be more efficient for functional analysis than the incomplete knockdown in primary human cells. Preliminary experiments with *Rorγt* deficient mice revealed *Lingo4* expression in thymus and small intestine. In both organs, *Lingo4* was detectable despite *Rorγt* knock-out, ruling out that *Lingo4* is regulated by *Rorγt* and supporting the hypothesis of LINGO4 being an upstream regulator of RORC2.

Finally, increased LINGO4 expression in psoriasis patient samples does not give information about a pathogenic role of this protein in the disease. However, it proves again that LINGO4 is related to Th17 cells and broadens the knowledge about Th17 cells and associated diseases.

5.2 FOXO4 as novel regulator of IL-22 expression in T cells

Upon discovery and characterization of Th22 cells in humans, elevated FOXO4 expression levels were found in Th22 clones when compared to other T cell subsets by Eyerich *et al.* [Eyerich et al., 2009, Trifari et al., 2009, Duhen et al., 2009]. This led to the question of a possible regulatory function of FOXO4 in these cells, particularly as a unique transcription factor for Th22 was not identified, yet.

FOXO4 is a transcription factor of the FOXO family. These proteins are described to have a wide variety of functions, ranging from cell cycle regulation, DNA repair, participation in metabolic processes to response to cellular stress, tumor suppression and - in some organims - longevity [Martins et al., 2016, Calnan and Brunet, 2008]. To fulfil these diverse functions, FOXO4 proteins are regulated by post-translational modifications, e.g., phosphorylation, ubiquitination, methylation, acetylation and metabolites or oxidative stress stimuli [Martins et al., 2016, Calnan and Brunet, 2008]. However, a definite role of FOXO proteins, in particular FOXO4, in T cells is not known so far. Some data exist that show participation of FOXO proteins, in particular FOXO1 and FOXO3, in the induction and development of Tregs, in the differentiation process of effector cells as well as naive T cell survival [Kerdiles et al., 2010, Oh et al., 2012]. For regulation of naive T cell survival and T cell activation, STAT3 was identified as regulator of FOXO1 and FOXO3a proteins. In its unphosphorylated state, STAT3 restrains phosphorylated and inactive FOXOs in the cytoplasm during T cell activation. FOXOs translocate to the nucleus when STAT3 is phosphorylated upon IL-6 or IL-10 stimulation and get activated to participate in T cell quiescence [Oh et al., 2012]. In addition, knock-out of FOXO1 in mice showed a participation of this protein in regulation of T cell activation as well as prevention of Tfh cell development and connected B cell autoimmunity [Ouyang et al., 2009, Kerdiles et al., 2009, Kerdiles et al., 2010]. Further, FOXO1 is important for development of Tregs as FOXO1 deficient mice showed a decreased number of natural occurring Tregs [Kerdiles et al., 2010]. FOXO4 was described in the context of Tregs upon stimulation with progranulin (PGRN) in mice [Fu et al., 2016]. PGRN is known to mediate anti-inflammatory responses, and IL-10, secreted by Tregs, was identified as main downstream mediator of this anti-inflammatory effect [Fu et al., 2016]. Besides JNK-dependent signaling, the mechanism revealed a participation of FOXO4 and STAT3 in IL-10 regulation upon stimulation with PGRN. Both FOXO4 and STAT3 deficient mice showed decreased IL-10 levels after stimulation with PGRN [Fu et al., 2016]. However, this finding has not been confirmed in humans, yet.

To gain further insight into the role of FOXO4 in human T cells and to confirm the findings from whole genome expression arrays of different T cell subsets [Eyerich et al., 2009], naive $CD4^+$ T cells were *in vitro* Th22 differentiated and FOXO4 expression was investigated. However, as already described for Th17 differentiation and *in vitro* differentiations in general, it is unclear to what extend *in vitro* differentiated Th cells resemble naturally occurring ones. In addition, Th22 cells and their differentiation process are still not fully understood. Th22-

polarizing conditions are described to be achieved by addition of IL-6 and TNF-α, while TGF-β has an inhibitory effect on IL-22 production [Duhen et al., 2009]. Further, cytokines like IL-1β, IL-12 and IL-23 are discussed to enhance Th22 differentiation or IL-22 production in T cells, however, also opposite observations were made [Duhen et al., 2009, Volpe et al., 2009]. Additionally, human and mice Th22 cells seem to differ critically. While the existence of human Th22 cells is confirmed, in mice the existence of a stable Th22 subset is still discussed [Eyerich et al., 2009, Duhen et al., 2009, Trifari et al., 2009, Ahlfors et al., 2014]. One study used reporter mice to track IL-22 producing cells *in vivo* in inflammatory conditions by virus infection and skin challenge but could not dedicate IL-22 production in these settings to Th22 cells. Rather, ILC3s and IL-22 producing Th17 cells were the source of it [Ahlfors et al., 2014]. Others achieved specific Th22 differentiation in mice *in vitro*, but also observed plasticity and transdifferentiation into Th1 or Th2 phenotypes [Plank et al., 2017]. So again, this has to be kept in mind when interpreting the data.
However, analysis of IL-22 expression on mRNA and protein level confirmed successful *in vitro* Th22 differentiation in this study. Th22 differentiation was further confirmed by increased production of TNF-α and absence or low secretion of IL-17, IFN-γ and IL-4. *In vitro* generated Th22 cells, thereby, resembled physiological Th22 cells in their main cytokine expression profile. Further, western blot analysis of different time points during Th22 differentiation revealed an induction and increased expression of FOXO4, confirming a relation of FOXO4 to Th22 cells. Early upregulation after already 60 min indicated a potential funcional role during differentiation.

To gain functional insight into possible effects of FOXO4 on IL-22 production, lentiviral-mediated knockdown of FOXO4 in effector cells was performed. Indeed, the knockdown resulted in decreased levels of *IL-22* and *AHR* on mRNA level as well as IL-22 and IL-17 on protein level whereas IFN-γ, TNF-α and IL-4 production was not affected. However, effects showed no statistical significance despite a clear decrease of the stated factors within each donor. Nevertheless, data point to a potential regulation of IL-22 by FOXO4 and, therefore, to a role of this transcription factor in T cells. These experiments permit no conclusion regarding the mechanism, i.e., if FOXO4 directly regulates IL-22 production by binding to the promotor region or whether the effect is more indirect.

The observed effects of FOXO4 knockdown in effector T cells clearly pointed to an involvement of FOXO4 in IL-22 regulation leading to the question whether FOXO4 might also be involved in the regulation of Th22 differentiation. This question was addressed by lentiviral-mediated FOXO4 overexpression in naive T cells that were consecutively differentiated *in vitro* into Th22 cells. This setup offered an insight into potential regulatory functions during *in vitro* Th22 differentiation. In addition, the usage of Th22 cells represented a better tool to specifically investigate the role of FOXO4 in IL-22 expression than the usage of effector cells containing all T cell subsets.
Overexpression in naive, *in vitro* differentiated Th22 was efficient, however efficiency strongly varied in different donors. Differences in the efficiency of *FOXO4* overexpression between

the donors had some impact on *IL-22* and *AHR* mRNA expression. Donors with low *FOXO4* overexpression also showed lower *IL-22* or *AHR* expressions than those with high overexpression, explaining the increasing, but not significant trends, as effects on expression levels were in general smaller than in *FOXO4* expression. However, IL-22 protein levels were significantly increased in FOXO4 overexpressing cells compared to empty transduced cells. This finding showed again an effect of FOXO4 on IL-22 production. TNF-α and IFN-γ protein amounts were not affected significantly by FOXO4 overexpression, but both showed an increasing trend, while IL-17 and IL-4 were not detectable. As Th22 cells are described to express TNF-α, but not IL-17 or IL-4, these findings comply with the Th22 phenotype and therefore confirm successful Th22 differentiation. IFN-γ levels might result from cells that were not efficiently differentiated into the Th22 phenotype.

In general, not much is known about IL-22 regulation. IL-22 is produced by different Th cell subsets. Th17 cells are defined by IL-22 production besides IL-17A/F, but also Th1 cells can co-produce IL-22 next to IFN-γ, while Th22 cells secrete IL-22 and TNF-α, but no IL-17 or IFN-γ [Gurney, 2004, Ivanov et al., 2006, Chung et al., 2006, Eyerich et al., 2009, Trifari et al., 2009, Duhen et al., 2009]. As mentioned before in the context of Th22 differentiation, IL-22 is induced by different cytokines like IL-6 and IL-23, promoting also Th17 differentiation, TNF-α but also IL-12, important for Th1 differentiation [Volpe et al., 2009]. However, the exact regulation is still unclear. STAT3, activated by IL-6 and IL-23, seems also to play a role in the regulation of IL-22 production, as addressed in an infectious colitis mouse model by $CD4^+$ with a defect in STAT3 [Backert et al., 2014]. IL-22 regulation by STAT3 was further shown to be regulated by IL-21, a cytokine also involved in Th17 differentiation [Yeste et al., 2015]. Contrary to this, TGF-β, a Th17 inducer, leads to IL-22 inhibition at too high concentrations. Suppression is mediated downstream by c-Maf, a transcription factor binding directly to IL-22 promotor and repressing transcription [Rutz et al., 2011]. In addition, environmental factors influence IL-22 expression by T cells. Mouse studies showed the participation of transcription factor HIF-1α in the IL-22 upregulation upon hypoxia in T cells [Budda et al., 2016]. Interestingly, FOXO4 was identified as a negative regulator of HIF-1α in hypoxia and connection with tumor growth [Tang and Lasky, 2003]. Another factor regulating IL-22 is AHR, a ligand-dependent transcription factor [Rutz et al., 2013]. Before the discovery of Th22 cells, AhR was already described to regulate IL-22 production in murine Th17 cells [Kimura et al., 2008]. One study showed that IL-22 production of Th17 cells crucially depends on AhR by using AhR deficient mice [Veldhoen et al., 2008a]. Agonists known for this receptor are, e.g., the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Interestingly, FICZ was shown to induce IL-22 production in Th17 cells, while TCDD led to differentiation of T cells into Tregs, showing how different ligands mediate varying functions of AhR [Quintana et al., 2008]. In mouse studies, AhR was further identified to regulate STAT1 and STAT5, both known to be negative regulators of Th17 differentiation, to enable efficient Th17 cells [Kimura et al., 2008]. In addition, activation of AhR can partially overcome the c-Maf mediated suppression of IL-22 by also binding directly to its promotor [Qiu et al., 2012, Apetoh et al., 2010]. In human $CD4^+$ T cells, AHR stimulation by FICZ resulted in

increased IL-22 levels and a decrease of both IL-17 and RORC2, while other master transcription factor like TBET, GATA3 and FOXP3 remained unaffected [Ramirez et al., 2010]. Further, they observed an increase in only IL-22 producing cells. Similar observations were made by Trifari *et al.* . AHR was found to be upregulated in $CD4^+$ T cells only producing IL-22 without co-expression of IL-17 or IFN-γ [Trifari et al., 2009]. They also described an effect of RORC2 on IL-22 production in Th22 cells, though without clear effect. However, AHR is so far the only master transcription factor described for Th22 cells.
Since our data clearly point to a regulatory function of FOXO4 in IL-22 production, future studies should address the question of a possible interaction of AHR and FOXO4 as a collective regulation of IL-22 production and the Th22 phenotype by both factors is conceivable.

5.2.1 The role of FOXO4 in IL-22 production of T cells - conclusion and outlook

The obtained results showed a connection of FOXO4 with IL-22 expression, both in effector T cells as well as naive, *in vitro* differentiated Th22 cells. As knockdown in effector T cells resulted in decreased IL-22 levels and overexpression in elevated levels, FOXO4 seems to positively regulate IL-22 production.
However, open questions remain that could not be addressed in the current work due to time restrictions. These have to be addressed in future studies and may contain functional assays after FOXO4 overexpression or knockdown to gain insights into its physiological role and define an exact regulatory mechanism of FOXO4 in IL-22 production.

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