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Bidirectional IL-31-driven communication between T cells and basophils during atopic skin inflammation

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I. Abstract

The novel T_H2-derived cytokine interleukin-31 (IL-31) has been implicated in the pathophysiology of atopic skin inflammation. Here for example, IL-31 has been identified to effectively elicit the cardinal AD symptom of pruritus (itch), relying on a synergistic cooperation of dysregulated immune cells and hyper-stimulated sensory neurons.

This study aims to systematically characterize the phenotype of IL-31-producing T cells and to elucidate their interaction with basophils in the context of atopic skin inflammation.

Results of the present study could identify the unique characteristics of IL-31$^+$ T_H2 cells presenting with a CRTH2$^+$ CCR4$^+$ CCR10$^+$ CXCR3$^-$ CCR6$^-$ phenotype and a distribution among the effector memory (CD62L$^-$ CD45RO$^+$) T cell subset. Furthermore, IL-31$^+$ T cells associate with a marked increase of the skin-homing chemokine receptors CCR4, CCR8, and CCR10 in AD patients. Notably, the abundance of IL-31$^+$ T cells but also the overall production of T cell-derived IL-31 was found increased in AD patients while frequencies of circulating basophils were not affected. Instead, AD patients presented with an increased population of cutaneous lymphocyte-associated antigen (CLA)$^+$ activated ST2$^+$ basophils that produce elevated levels of IL-4. Further, these basophils show increased survival upon IL-31 stimulation. Interestingly, in *Il-31ra*-knockout mice the presence of activated basophils, and also T_H2 cells, were decreased suggesting a dependence on the presence of *Il-31/Il-31ra* signaling. In line with these observations, AD-derived basophils present with an enhanced expression of IL-31RA/OSMRβ. The present study further describes a prominent communication loop from keratinocytes to basophils, supporting the importance of outside-inside signals for the perpetuation of AD. Mimicking an AD-like environment, circulating basophils from AD patients respond to IL-4/IL-13-treated keratinocyte supernatants by initiating antigen-independent T_H2 differentiation and production of IL-31. This observation implies an additional role for IL-31 signaling in T_H2 differentiation and probably expansion. Taken together, findings of the present study suggest a role of activated skin-homing CLA$^+$ basophils in the progression and/or perpetuation of AD based on their interaction with keratinocytes as well as skin-infiltrating T cells and strengthens the IL-31/IL-31 receptor complex as a therapeutic target in the treatment of mild to severe atopic dermatitis.

II. Zusammenfassung

Das neue T_H2-assoziierte Zytokin Interleukin-31 (IL-31) steht in Zusammenhang mit der Pathophysiologie von atopischen Hautentzündungen. IL-31 wurde als effektiver Auslöser von „Juckreiz“, ein Kardinalsymptom bei atopischer Dermatitis (AD), identifiziert. Dieser Juckreiz ist auf eine synergistische Kooperation zwischen nicht regulierten Immunzellen und überstimulierten sensorischen Nerven zurückzuführen ist. Diese Studie charakterisiert systematisch den Phänotyp der IL-31-produzierenden T-Zelle und durchleuchtet die Rolle der IL-31$^+$ T-Zelle in atopischer Hautentzündung. Im Focus steht die Interkation der IL-31$^+$ T-Zelle mit Basophilen. Die Ergebnisse dieser Studie beschreiben einmalige Charakteristika der IL-31$^+$ T_H2-Zelle, mit einem CRTH2$^+$ CCR4$^+$ CCR10$^+$ CXCR3$^-$ CCR6$^-$ Phänotypen und der Zugehörigkeit zu den Effektor-Gedächtnis-T-Zellen (CD62L$^-$ CD45RO$^+$). Darüber hinaus exprimiert die IL-31$^+$ T-Zelle von Patienten mit AD vermehrt die typischen Haut-migrierenden Chemokin-Rezeptoren CCR4, CCR8 und CCR10. Hierbei ist nicht nur ein erhöhtes Vorkommen von IL-31$^+$ T-Zellen bei Patienten mit AD zu vermerken, sondern auch eine insgesamt höhere Produktion von IL-31, wobei jedoch die zirkulierende Basophilen-Frequenz unverändert bleibt. Stattdessen weisen sie vermehrt aktivierte ST2$^+$ Basophile auf, die kutanes Lymphozyten-assoziiertes Antigen (CLA) exprimieren und vermehrt IL-4 produzieren. Des Weiteren weisen diese Basophilen unter IL-31 Stimulation ein erhöhtes Zellüberleben auf. Interessanterweise konnten in *Il-31ra*$^{-/-}$-Mäusen verminderte Frequenzen von aktivierten Basophilen, sowie T_H2-Zellen nachgewiesen werden, was die Rolle des *Il-31/Il-31ra* Signalweges in der Biologie von Basophilen und der Entwicklung von T_H2-Immunantworten weiter unterstreicht. Im Einklang mit diesen Ergebnissen, scheinen Basophile von AD Patienten auch eine erhöhte Expression des IL-31RA/OSMRβ Rezeptorkomplex zu zeigen. Darüber hinaus beschreibt die vorliegende Studie eine Kommunikationsschleife von Keratinozyten zu Basophilen. In einem AD-simulierenden Umfeld fördern zirkulierende Basophile nach Stimulation mit IL-4/IL-13-behandelte Keratinozyten-Überstände die T_H2-Differenzierung und die IL-31 Sekretion. Zusammengefasst unterstützten die Ergebnisse der vorliegenden Studie eine Rolle von aktivierten CLA$^+$ Basophilen in atopischen Hautentzündungsprozessen, weist auf das komplexe Zusammenspiel von Keratinozyten, Basophilen und T-Zellen hin und unterstreicht den Stellenwert des IL-31/IL-31 Rezeptor-Komplexes als therapeutisches Ziel in der Behandlung von AD.

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IV. Abbreviations

A

Ab	Antibody
Akt	Protein kinase B
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell

B

Bcl-6	B cell lymphoma 6 protein
BDC	Blood dendritic cells
BFA	Brefeldin A
BIR	Baculovirus inhibitior of apoptosis protein repeat
BSA	Bovine serum albumin

C

CARD	Caspase recruitment domain
cDNA	complementary DNA
CCL	C-chemokine ligand
CCR	C-chemokine receptor
CLA	Cutaneous lymphocyte-associated antigen receptor
CNS	Central nervous system
CNTF	Ciliary inhibitory factor
CT-1	Cardiotropin 1
CXCL	CX-chemokine-ligand
CXCR	CX-chemokine-receptor

D

DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxy-ribonucleic-acid
DNAse	Desoxyribonuclease
DRG	Dorsal root ganglia

E

EDTA	Ethylendiamintetraacetate
EGF	Epidermal growth factor
ECM	Extracellular matrix

F

Fab	Antigen-binding fragment
FACS	Fluorescence activated cell sorter
FAM	6-Carboxyfluorescein
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
Foxp3	Forkhead box P3

G

GATA3	GATA-binding protein 3
GPCR	G-Protein coupled receptor
GM-CSF	Granulocyte-macrophage colony stimulating factor

I

IFN	Interferon
Ig	Immunglobulin
IGF-1	Insulin like growth factor
Iono	Ionomycin
IL	Interleukin

IP3	Inositol-1,4,5-triphosphat
IRF	Interferon regulatory factor

J

JaK	Janus-kinase

K

KC	Keratinocyte
KO	Knockout

L

LN	Lymph node
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide

M

mAb	Monoclonal antibody
MACS	Magnetic adsorption cell sorting
MHC	Major histocompatibility complex
MIN	Mintues
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase mRNA Messenger-RNA
MyD88	Myeloid differentiation response gene 88

N

NF-kB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NLR	NOD like receptor
NTN	Neuropoitin

O

OSM	Oncostatin M
OSM-R	Oncostatin M receptor

P

pAb	Polyclonal Antibody
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinositol-3-Kinase
PKC	Proteinkinase-C
PLC	Phospholipase-C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorph nuclear cell
PRR	Pathogen recognition receptor

R

RA	Retinoic acid
RIP	Receptor interacting protein kinase
RLR	RIG-like receptors
RNA	Ribonucleic acid
RORa	RAR-related orphan receptor alpha
RORg	RAR-related orphan receptor gamma
RT	Room temperature
RT-PCR	Reverse transcription-PCR

S

SOCS	Suppressor of cytokine signaling
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
SD	Standard deviation

T

TCR	T cell receptor
TNF	Tumor necrosis factor
TF	Transcription factor
TGF	Transforming growth factor
TLR	Toll like receptor
TSLP	Thymic stromal lymphopoietin

V

VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth Factor

W

WT	Wild type

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1. Introduction

1.1 The adaptive immune system

The adaptive immune system is subdivided into a humoral response, mainly mediated by B lymphocytes, and a cellular response, driven by T lymphocytes. The major difference of the adaptive immunity over the innate immunity is its specificity in the recognition of invading pathogens. Once the adaptive immune system is activated, it ultimately aims to resolve the invasion of pathogens by secretion of pathogen-specific antibodies mediated by B cells (humoral response) or by cytokine secretion facilitated by effector cells, T lymphocytes in particular, which is known as the cellular response and a key process for proper pathogen clearance[1].

1.1.1 Cellular Response

The cellular immune response is mediated by T lymphocytes. This cell type matures in the thymus and spreads thereafter to all parts of the body. The immature T cells leaving the bone marrow are called pro- T cells or double-negative T cells since they express neither of the two characteristic co-receptors, CD4 or CD8. Upon VDJ (variable, diversity, joining gene segments) recombination, they express the complete T cell receptor (TCR) as well as both co-receptors and have been, therefore, termed double-positive immature T cells. Their maturation down the CD4 or CD8 lineage depends on the recognition of antigens presented by antigen presenting cells (APCs). While $CD8^+$ T cells develop upon recognition of antigens presented by class I MHC- (major histo-compatibility complex) expressing cells, $CD4^+$ cells require presentation of antigens *via* class II MHC-presenting cells. The hereby emerging two cell populations then engage in diverging immunological functions[2,3]. Similar to B cells, T cells recognize antigens by specific immunoreceptors expressed on their surface. These T cell receptors, however, recognize only peptidic antigens that are introduced by APCs. Through this process, T cells develop tight antigen-specificity and can distinguish different microbes[1].

The TCR consists of an antigen-recognition region, composed of an α and β chain. It is responsible for interacting with the antigen presenting MHC-I and MHC-II molecules of APCs. However, a complete T cell stimulation and subsequent signal transduction can only

take place upon interaction of TCR co-receptors, namely the transmembrane molecule CD3, the shared receptor of CD4 and CD8 T cells, and the ζ-protein. The TCR together with its co-receptors form the TCR-complex[4].

1.1.2 CD4$^+$ T lymphocytes

The subtype of T lymphocytes that expresses the co-receptor CD4 plays a crucial role in the defense against microbes. They orchestrate the immune response directed against intruding pathogens. They intensify as well as regulate the innate immune response by secretion of effector cytokines and chemokines, which in turn mediate the recruitment of further immune cells to the site of infection[1]. Antigen-specific T helper cell populations originate from naïve thymus derived CD4$^+$ T cells that are activated, differentiate and proliferate. Their differentiation into a particular subset of T helper cells is dependent on the antigen presented by MHC II molecules of dendritic cells and macrophages on the one hand and by the predominant cytokine microenvironment on the other hand.

Further, strength and duration of the antigen presentation to TCRs expressed on naïve T cells is crucial for the cell fate. In turn, the secretion of a particular set of cytokines by differentiated CD4$^+$ T cells leads to the activation of macrophages and the transcription of genes coding for lysosomal proteases that support eradication of phagocytosed pathogens[5]. The immune response mediated by CD4$^+$ T cells, however, needs tight regulation in order to prevent auto-immunity, an immune response directed against the host immanent molecular structures. Therefore, they consist of different subsets with distinct functions, including regulatory ones. An additional function of CD4$^+$ helper T cells is to promote the differentiation and proliferation of antigen specific B cells, and thereby encourage the production and secretion of antibodies[6]. Hereby, T cells upregulate transiently the chemokine receptor CXCR5, which leads to the migration of T cells towards the germinal center of B cell follicles[7].

1.1.2.1 CD4$^+$ T cell subsets

The CD4-expressing T lymphocytes are indispensable for the defense against pathogens, including bacterial, parasitical and fungal antigens[1]. The development of CD4$^+$ T cells begins with naïve T cells, circulating through secondary lymphoid organs (SLOs), where

they are activated by antigen presentation via APCs. This step triggers the T cell expression of the cytokine receptor IL-2R (CD25) and initiates their clonal expansion. However, antigen presentation alone is not sufficient for complete activation of T cells mediated by APCs[1]. Antigen presenting cells express additional co-stimulatory molecules such as CD80, CD86 and CD40 that bind to their respective ligands (CD28 and CD40L) expressed by T cells[2]. Upon activation *via* antigen stimulation and signaling through co-stimulators, T cells initiate their differentiation programs and can secrete cytokines that determine their diverse functions. The exact profile of secreted cytokines is in fact directly dependent on the activating pathogen-derived antigen and the pre-existing specific receptor on the surface of the T cells. The pathogen, which encourages antigen presentation on dendritic cells or macrophages, also leads to the establishment of a certain cytokine environment in the tissue, which in turn influences the differentiation of T cells towards a unique profile. Further, the strength and duration of the TCR signaling plays a crucial role in the development of the T cell towards an effector or a memory T cell[8].

1.1.2.2 Differentiation of CD4$^+$ T cells

The differentiation of naïve T cells into effector T cells is an essential step towards immunity and its capability to eliminate invading microbes. Several subsets of CD4$^+$ T cells exist and are distinguished by the patterns of cytokine secretion as well as chemokine receptor expression and expression of master transcription factors. Each effector CD4$^+$ T cell subset fulfills a distinct role and carries out a different function in the process of eradication of pathogenic invaders. The differentiation of T cells is also referred to as lineage commitment, since the maturation of cells towards specific subsets is thought to be an irreversible process. Recent studies, however, have shown that CD4$^+$ T cells demonstrate some extent of plasticity[9]. Based on their cytokine profile, the various CD4$^+$ T cell subsets can regulate and modify the immune response[10].

T_H1 cells play an essential role in the defense against intracellular pathogens, but are also involved in the initiation of several autoimmune diseases. T_H1-skewed differentiation occurs in an IL-12 environment and T_H1 cells are characterized by secretion of IFN-γ. The binding of both cytokines to their respective affinity receptors of naïve T cells, leads to the activation of signaling pathways, which determine their subsequent T_H1 lineage

commitment. IL-12 binding to IL-12Rβ initiates the phosphorylation of the adaptor protein STAT4, which induces the expression of IFN-γ and the transcription factor T cell specific T-box transcription factor (T-bet). T-bet expression is also induced and stabilized by the signaling of IFN-γ through its specific receptors. T-bet phosphorylates STAT1, which induces the expression of its target gene *Tbex*. STAT4 and STAT1 are, therefore, essential for naïve CD4 cells to differentiate towards the T_H1 subset (see Fig. 1). T-bet initiates the expression of CXCR3 on their surface and allows T cells to enter peripheral tissue[11]. T_H1 cells eradicate intracellular pathogens, by the secretion of the cytokine IFN-γ, which activates macrophages to eliminate phagocytized microbes by inducing the production of microbicidal substances[12,13] .

The $CD4^+$ T_H2 subset is known to mount the immune response against extracellular parasites and to be involved in atopic/ allergic reactions. This T cell subset develops upon IL-4 mediated signaling, which induces the transcription of the GATA-binding protein 3 (GATA3). The crucial pathway skewing T cells to a T_H2 phenotype, however, is STAT6-dependent signaling transduction leading to *il4* and *Gata3* transcription, where GATA3 constitutes the characteristic transcription factor guiding towards T_H2 differentiation [11,14,15]. GATA3 promotes the secretion of the T_H2 characteristic cytokines IL-4, IL-5 and IL-13, and induces the expression of the chemokine receptor CCR4 on receptor level. CCR4 expression is not T_H2 exclusive and also found on other subsets of $CD4^+$ T cells[12]. However, the prostaglandin D2 receptor (CRTH2) and the homing marker CRTH2 are considered to represent T_H2 specific receptors. The pronounced abundance of T_H2 cells in tissues of the respiratory and gastrointestinal tract is in fact based on their expression of CRTH2[16].

T_H9 cells have recently been identified as an individual cell subset that is closely related to T_H2 cells. T_H9 are main drivers in the eradication of parasitic worm infections. Their differentiation is initiated upon signaling with IL-4 and TGF-β, which leads to the expression of the transcription factor PU.1. T_H9 cells secrete the characterizing effector cytokine IL-9. This subset is preferentially described as a skin-homing subset since it predominantly expresses the skin-homing receptors CCR4 and CLA[17,18].

T_H17 cells have been described to defend the host against extracellular pathogens, like bacteria, but also fungi. Nevertheless, similar to T_H1 cells, this subset is involved in numerous chronic inflammatory and autoimmune diseases[19,20]. Upon stimulation with the cytokines IL-1β, TGF-β, IL-21 and IL-6, STAT3 will be phosphorylated, which in turn activates the transcription factors RAR-related orphan receptor gamma (RORγ) and RAR-related orphan receptor alpha (RORα). These transcription factors promote the secretion of T_H17 typical cytokines: IL-17A, IL-17F, IL-22 and IL-21. Furthermore, STAT3 facilitates the up-regulation of CCR6 and the IL-23 receptor (IL-23R). Of note, CCR6 is not expressed exclusively by this cell type[21]. A constant IL-6 stimulus is required to prevent the differentiation of these cells into the regulatory $Foxp3^+$ T cell lineage. IL-21 has been described to interact in a positive feedback loop with the differentiated T_H17 cells leading to the up-regulation of the IL-23R on its surface. However, T_H17 cells do not universally express the IL-23R. The absence of this receptor leads to a rather regulatory T_H17 phenotype, with secretion of anti-inflammatory IL-10[21,22]. IL-23 signaling has recently been shown to play a crucial role in the development of T_H17 cells and their effector function by the phosphorylation of STAT3 in the absence of TCR stimulation. It is not known, which exact gene expression IL-23 affects directly or indirectly, but it seems to more generally promote a pathological T_H17 response[19,20].

A more recently described subset of $CD4^+$ T cells is the population of T_H22 cells. These T cells are mainly characterized by production of IL-22 in the absence of IL-17. Some members of the IL-10 family of cytokines, such as IL-22 can initiate the secretion of IL-10 as well as TNFα from T_H22 cells, thereby mediating both anti-inflammatory and pro-inflammatory responses[23]. The differentiation towards this subset is achieved by the simultaneous signaling of IL-6, IL-23 and TNFα. However, it has yet not been clearly demonstrated which pathway is involved in driving naïve T cells towards a T_H22 fate. Several analyses hint to the participation of the T_H22 markedly abundant Aryl hydrocarbon receptor AhR, a transcription factor that might maintain the IL-22 production. T_H22 cells are chemokine receptor CCR4- and CCR10-positive as well as skin-homing receptor cutaneous leukocyte-associated antigen receptor (CLA) expressers and are, hence, predominantly distributed in the skin[24,25].

Regulatory T cells (T_{regs}) play an essential role in regulating the immune response of the various $CD4^+$ T helper cells subsets. The differentiation of these cells is promoted by TGF-β and IL-2-induced signaling, which leads to the phosphorylation of STAT5, a crucial factor for the induction of Forkhead box P3 (Foxp3), a transcription factor exclusively expressed by T_{regs}. Foxp3 governs the secretion of the cytokines IL-10, IL-35 and TGF-β, thereby establishing a positive feedback loop. IL-10 is an anti-inflammatory cytokine, responsible for maintaining the homeostasis of immune responses[26,27], whereas IL-35 as a member of the IL-12 cytokine family is known to mediate rather pro-inflammatory responses. Nevertheless, similar to IL-10, IL-35 specifically produced by T_{regs} is a strong suppressor of cell proliferation by initiating cell cycle arrest, but has also been shown to limit anti-tumor T cell responses based on similar pathways[28,29]

Another regulatory $CD4^+$ T cell subset, which only recently has been discovered, is the so-called Tr1 subset. Tr1 cells are characterized by emphasized IL-10 production in the absence of the transcription factor Foxp3 and exert a predominantly suppressive function. A continuously growing body of studies suggests that the Tr1 subset is strongly regulated by the master transcription factor AhR, suggesting a marker function for AhR. However, the differentiation of premature T cells towards a Tr1 character is essentially dependent on proper IL-10 signaling[30].

A $CD4^+$ T cell subset without any effector function is known as the follicular T cell (T_{FH}) population. Expression of the transcription factor B-cell lymphoma 6 protein (Bcl-6) and the elevated secretion of IL-21 are considered two main characteristics for this cell subset. The main role of T_{FH} cells is to support differentiation of naïve B cells upon cell-cell-contact into antibody producing plasma cells. Consistently T_{FH} cells expresses the chemokine receptor CXCR5, that promotes the migration of T_{FH} cells towards germinal centers[7].

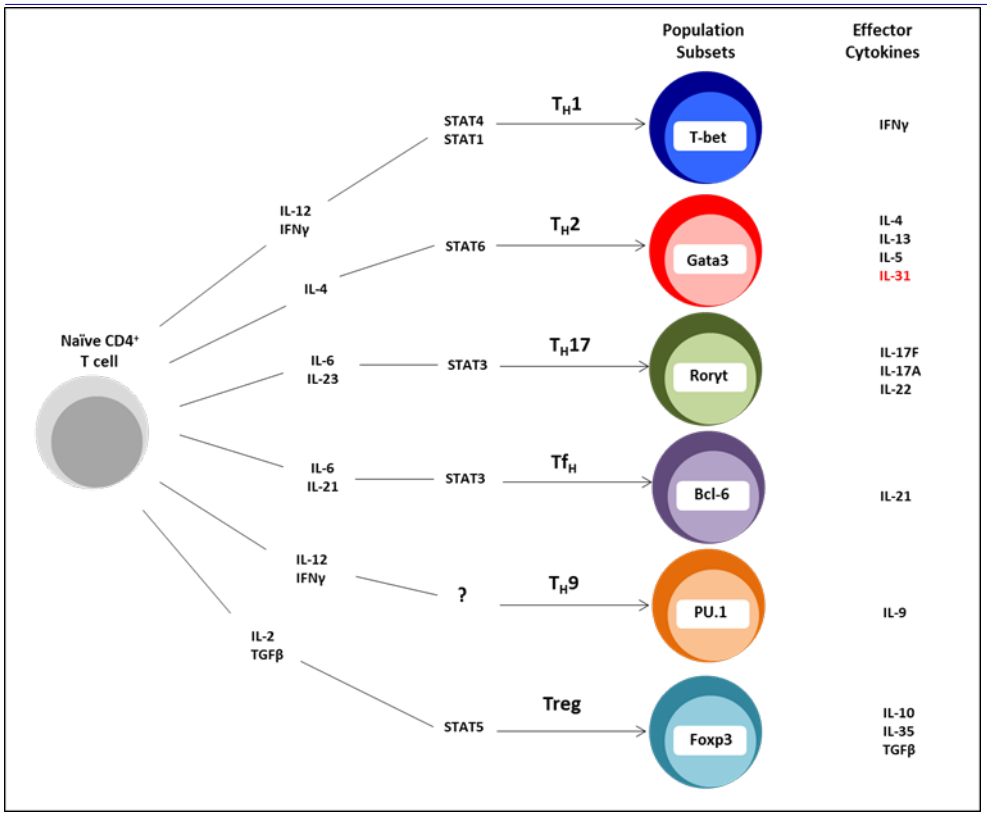

Figure 1: The classical T cell populations and their respective differentiation programs. Naïve T cells can differentiate into distinguished T cell subsets when exposed to a distinct cytokine microenvironment. The activation of different STAT-pathways leads to the expression of specific transcription factors in each subset (adapted from the master thesis of Angeliki Datsi).

1.1.2.3 Development of T cell memory

One of the key characteristics that separate adaptive from innate immunity is the development of memory upon specific activation through antigen stimulation. Memory cells require complete TCR signaling for their functional maintenance. Interestingly, to ascertain readiness and appropriate function and to distinguish self from non-self-antigen, memory T cells maintain a self-educating cross-talk with the "good" host microbiome[31]. Immunological memory is characterized by the expansion and differentiation of antigen-specific lymphocytes. In order to develop T cell memory, naïve T cells are activated and start proliferating after recognizing their specific antigen, thereby gaining specificity, a crucial step to effectively exert the function as effector T cells. Although a vast majority of these cells die with clearance of the infection, a small number of memory T cells remain alive, either circulating in peripheral blood or quiescently residing in various tissue sites. In this status, memory T cells are referred to as memory $CD4^+$ T cells. In the absence of specific antigen, $CD4^+$ T cells undergo homeostatic

expansion thereby constantly increasing the memory pool with each subsequent infection. Memory T cells are also capable of self-renewal and survive indefinitely, facilitating a faster and more intense immune response with exposure to redundant infection[32].

Several different subpopulations of memory cells can be distinguished based on the respective function. The two main populations are consisting of "effector memory (T_{EM})" and "central memory (T_{CM})" T cells. The latter is of the reactive type and migrates to the site of infection where they proliferate and differentiate into effector T cells in response to antigen stimulation. The characteristic surface marker pattern for T_{CM} cells is composed of $CD45RO^+$, $CCR7^+$ and $CD62L^+$. CD62L is the marker for L-selectin, which is typically down-regulated in activated memory cells. CD62L is shed upon TCR stimulation and, therefore, only weakly expressed on T_{EM} cells, which are also $CCR7^-$ [32,33].

1.2 Pro-inflammatory cytokines and gp130 receptors

One of the effector functions of CD4 T cells is the secretion of cytokines, once they have developed towards memory cells as naïve T cells lack the production of cytokines. Here we distinguish between the two different types of cytokines: pro-inflammatory and anti-inflammatory cytokines.

Pro-inflammatory cytokines, such as IL-1 and IL-6 family members are not limited to signal through their respective cytokine receptors, but also transduce their inflammatory impact via multiple further receptor combinations. The aforementioned concept is observable with the well-characterized IL-1 family of cytokines: The IL-1 family consists of 11 members, which mostly function as pro-inflammatory cytokines. Selected members are IL-1β, IL-18 and IL-33. Non-hematopoietic cells such as epithelial cells of the mucosal sites secrete these interleukins upon cell damage or pathogen invasion. Hence, some cytokines, e.g. IL-33, have also been named alarmins[34]. Another pro-inflammatory cytokine family comprises interleukins with the common feature to engage with the glycoprotein 130 (gp130). Among these are IL-6, IL-11, IL-27 and OSM. Although IL-31 dos not engage with gp130, it is still grouped into the IL-6 family due to the activation of the same downstream signaling cascades[35]. Upon activation of their cognate heterodimeric receptors, members of the IL-6 family have been described to play an essential role in

transducing signals of inflammation. This observation is based on numerous studies reporting of IL-6 families mainly to be secreted under inflammatory conditions by diverse cell types such as monocytes, T cells, fibroblasts and endothelial or epithelial cells. The latter cell types represent the first barrier either to the environment (keratinocytes on the skin; epithelial layer of the gastrointestinal tract) or to the blood stream (vascular endothelium)[36].

1.2.1 IL-31 and its receptor heterodimer IL-31RA/OSMRβ

IL-31 represents a relatively novel member of the IL-6 family of cytokines and its gene is located on chromosome 12q24.31. With a mere homology of 31% between the human and mouse IL-31 gene, there is no cross-species activity. Once the IL-31 gene is transcribed and translated, it is composed of 164 amino acids in the precursor state and encodes for a 141 amino acid containing mature form. These amino acids form into a four α-helix structure[37]. Although IL-31 itself shares similar structures with the proteins targeting gp130 receptors, it does not engage with gp130 itself, but rather with a heterodimeric receptor composed of the IL-31RA/OSMRβ subunits. In humans, IL-31RA exists in several different isoforms, but the main two variants are known as the long and the short isoform, where the short isoform resumes an inhibitory, non-signaling function. In mice and rats only the long signaling isoform has been detected so far[37].

Besides IL-31, this receptor heterodimer is activated with comparable affinities by oncostatin M (OSM)[38]. Although the OSMRβ is widely expressed throughout the mammalian body, current data supports a more exclusive expression of the IL-31RA subunit to epithelial and neuronal cell types. Engagement of OSM or IL-31 with the IL-31RA/OSMRβ heterodimer triggers the activation of JAK-STAT, Akt/PI3K and MAPK pathways. Of note, at least MAPK activity is not inducible by signaling through the IL-31RA alone, but seems to need both receptor subunits[39]. IL-31 signaling leads to the induction of cell survival, proliferation and differentiation machineries by activating a robust STAT3 phosphorylation, comparable levels of pERK and STAT5 phosphorylation as via OSM stimulation, but neither of both regulates any of the other “typical” IL-6 targets such as STAT1[38]. Within the IL-6 cytokine family, STAT3 signaling has been linked to roles in pro- but also anti-inflammatory responses and regenerative processes, such as proliferation

and inhibition of apoptosis of intestinal epithelial cells[40,41] . So far it is not known if IL-31 takes part in these functional roles via STAT3 activation.

1.2.2 Molecular source and regulation of IL-31

The source and molecular machinery of IL-31 expression and its up-regulation under particular conditions is still poorly understood, but several studies describe its preferential expression by $CD3^+$ $CD4^+$ type 2 T-helper cells (T_H2) in mice and humans[42–45]. Recently, a large-scale tissue bank screening has demonstrated that IL-31 expression is almost exclusive for this immune cell type [46]. However, the signals and mechanisms regulating its expression are not fully deciphered. Stott *et al*. proposed that the expression of IL-31 in the $CD3^+$ $CD4^+$ T_H2 subset is controlled in a dose-dependent manner by IL-4 signaling[46]. Further, IL-33 stimulation can increase the secretion of IL-31 via STAT6-dependent up-regulation of the T1/ST2 receptor perpetuating further secretion of IL-31, but not initiation of its production itself [42,47,48].

Furthermore, silencing of STAT6 in T_H2 cells does not affect the expression and secretion of IL-4, but of all other canonical T_H2 cytokines, including IL-31. These observations point out the necessity of STAT6 expression for IL-31 secretion[42]. In contrast to the enhancing impact of IL-33 on IL-31 production, TGF-β has a rather damping effect on IL-31 secretion via phosphorylation of SMAD2/3 molecules. This TGF-β property might explain why fully differentiated T_H9 cells seem to display only moderate IL-31 expression[46]. The differentiation of T_H9 is initiated by the stimulation with IL-4 and TGF-β. The signaling of IL-4 initiates IL-31 expression. However, the simultaneous stimulation with TGF-β dampens this IL-4 effect. T_H2 cells, in comparison, are not relying on the influence of TGF-β as they are generated by the influence of IL-4 alone and maintain their phenotype, and therefore their capability to express IL-31 by secretion of autocrine-acting IL-4[42]. Further mechanisms, which control the secretion of IL-31 have been described by Hwang *et al*. This group described that calcium dependent (NFAT1 and NFAT2) proteins are inclined to bind to the promotor region of the *Il-31* gene, thereby initiating gene transcription in T_H2 and mast cells[49]. Because cytokines induce the transcription and secretion of IL-31, its expression needs to be tightly regulated in order to restrict pathogenic disorders. As with multiple other interleukins, the protein family of suppressor of cytokine signaling (SOCS) takes a pivotal role in this by inhibiting an uncontrolled expression of IL-31[50].

1.3 Atopic dermatitis and its immunopathology

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by the development of highly pruritic eczematous lesions[51,52]. AD often begins in infancy and follows a chronic, relapsing course. Around 15-50% of children and 2-10% of adults are affected[53]. AD etiology is a complex of genetic, environmental, and neuro-immune factors that contribute to skin barrier dysfunction and inflammation. Its diagnosis is based on family and patient history as well as several sets of diagnostic criteria comprising essential, important, and associated features of AD[52]. Essential features include pruritus and eczematous lesions that can be acute, subacute, or chronic. Important features include early onset, dryness, and increased total and specific IgE levels, although about 20% of AD patients fail to demonstrate elevated total or specific IgE[52]. Patients with eczema or atopic dermatitis feature dermato-histopathological findings of cutaneous infiltrates dominated by $CD4^+$ T cells and elevated inflammatory cytokines. Recently, neuro-immune risk factors such as elevated skin expression of IL-4, IL-13 and IL-31, have been implicated in AD inflammation and pruritus[45,54]. Also, genetical studies have associated distinct genes as crucial players in the manifestation and early onset of AD by affecting the differentiation of the epidermis or being linked to the T_H2 cytokines IL-5 and IL-13[53]. However, the detailed molecular mechanisms underlying AD-associated pruritus are still largely elusive[45,55] Interestingly, the chronic stage of AD is characterized by a T_H1 dominated cutaneous infiltrate with a persisting T_H2 component[53].

Phenotypically, xerosis is a hallmark of AD patients, being linked to epidermal barrier dysfunction. The nervous system itself plays an important role in sensing incoming signals and mediate pruritus and pain to the central nervous system (CNS), initiating also vasodilatation and edema. Immune reactions in AD combine responses of the innate as well as adaptive immune system, including most of the above described skin resident cells. Keratinocytes are activated upon encounter with microbial peptides and secrete diverse cytokines and chemokines (e.g. TSLP). This way activated keratinocytes together with eosinophils, basophils and mast cells dominate the acute phase of AD, instructing the strong T_H2 response. However, the exact interactions between the innate immune cells with cells of the adaptive immune system are still not fully elucidated. Therefore, the transition between the acute to chronic stage is still under strong investigation[53,56].

1.3.1 Pruritus as a cardinal symptom of AD

While pruritus initially was characterized as a symptom, where patients have the desire to scratch, the perception about itch has modified towards a recognized sensory encounter. Based on the intensity and duration of the scratching-desire, clinicians distinguish between acute and chronic pruritus, whereas the latter is classified as such upon an existence for longer than 6 weeks. Itch, which is caused by distinct skin disorders, can be identified by the release of histamine, cytokines, prostaglandin and proteases[57]. As of lately, especially the patho-mechanism of the pruritus-scratch-cycle, which results in lesional skin, has become a focus of intense research. More precisely, scientists today assume that multiple mechanisms exist that lead to often intractable itch since chronic as well as acute pruritus is observed in patients suffering from miscellaneous diseases such as inflammatory skin disorders, bacterial infections, cancer or systemic/metabolic disorders. Pruritus itself in turn can lead to intense skin lesions that induce inflammatory processes, tissue destruction and even systemic body damage. The anatomical structure of the skin that senses pruritic agents is a delicately branched sensory nerve system originating from dorsal root ganglia (DRG) that connect the incoming signals from the periphery to the central nervous system[57,58]. DRG neurons not only perceive incoming pruritic signals from histamine, but also react on other soluble factors such as ions, proteases, peptides or cytokines. Multiple pruritogens, their cognate receptors, channels and interaction networks have been identified[59]. Single cell PCR in murine DRG neurons advanced our understanding to distinguish itch nerves on the molecular level[60–62].

Several prominent sensors of pruritic signals belong to the family of transient receptor potential channels (TRP)[63]. For example, the capsaicin receptor TRP vanilloid receptor 1 (TRPV1) and mustard oil-activated TRP ankyrin receptor 1 (TRPA1) are expressed by DRG neurons and are essential for the correct signal transmission of diverse pruritogens[64–66]. These studies also shed light on the fundamental importance of the interaction of neurons with resident (e.g. keratinocytes) and non-resident skin cells (e.g. T cells) to initiate and regulate pruritus.

Cytokines have been found to act as pruritic sensitizers (e.g. IL-4 in humans), acute pruritogens (e.g. IL-31 in mice) or at least as inductors of atopic dermatitis-like phenotypes accompanied with itch (e.g. IL-4/IL-31, IL-31 in mice)[66–68]. In a handful of genetically modified mouse lines, an overexpression of a single cytokine (IL-4, IL-13, IL-18,

IL-31, TSLP) appears to be sufficient for the development of AD-like skin disease[59]. In humans TSLP, IL-4 and IL-13 signaling pathways have been particularly addressed as major pathophysiological signaling traits urging development of atopic dermatitis and pruritus[69,70]. Dupilumab, a novel monoclonal antibody binding the alpha subunit of the interleukin-4 receptor (IL4Rα) and blocking the IL-4/IL-13 signaling pathways was successfully studied in phase 1 - 3 clinical trials with patients suffering from AD. Dupilumab induced a rapid and robust improvement in pruritus and eczema. Further, Oetjen *et al.* demonstrated that chronic itch is dependent on neuronal IL-4Rα and JAK1 signaling and that patients suffering from therapy-resistant chronic itch improve when treated with JAK inhibitors [70].

Although not much is known about the pathophysiological role in humans compared to a clearer picture in animal models, where overexpression of *Il-31* in transgenic mice leads to severe pruritus and a subsequent development of inflammatory skin lesions resembling atopic dermatitis[44], hence a critical role of the IL-31/IL-31RA axis in AD pathophysiology is anticipated. In fact, recent clinical studies target the IL-31RA pathway by blocking the receptor directly with a humanized anti-IL31 receptor alpha monoclonal antibody (CIM331, nemolizumab) and resulted in a significant reduction of pruritus after a single subcutaneous dose of CIM331 compared to placebo[71,72].

As mentioned above, overexpression of *Il-31* in transgenic mice leads to atopic-like dermatitis and severe itch, as observed in atopic dermatitis patients[44]. To investigate the role of the IL-31/IL-31RA axis in humans and subsequently in the pathophysiology of AD patients, IL-31 qPCRs were performed from skin samples of non-lesional and lesional regions of several AD patients revealing an abundant expression of *Il-31* transcripts in lesional regions of patients with pruritus[45]. Highest expression of *Il-31* mRNA was detected in patients with *prurigo nodularis*, a skin disease associated with high levels of pruritus therefore also connecting the expression of IL-31 with pruritus[45].

Also Kato *et al*. revealed an abundant expression of IL-31 immunhistochemicaly in patients with AD and the expression of the respective IL-31RA on keratinocytes, cutaneous nerve fibers and human DRG neurons[73]. As Kato *et al*. shows a co-expression of IL-31 with CD11b leukocytes, the expression of IL-31RA by cutaneous nerves implies the existence of a strong neuro-immune link between IL-31 and cutaneous sensory neurons that transmit itch signals[73].

Based on these initial results, further groups aimed to unravel the molecular mechanisms of the pruritic IL-31 cytokine and the relevance of its consecutive receptor heterodimer on neurons. Analysis on murine DRG neurons dissected IL-31RA expression in small-to medium diameter DRG neurons (<30 µM) while large-diameter DRG neurons (<50 µM) were mainly negative. Moreover, small-diameter DRG neurons were found to co-express IL-31RA and Trpv1. In fact, blockage of the capsaicin receptor reduced the induction of IL-31 mediated itch and stimulation with IL-31 also activate neuronal Ca^{2+}- influx through the Trpa1 and Trpv1 receptors, while IL-31-induced scratching was significantly diminished in Trpa1- and Trpv1-knockout mice. These findings suggest a direct effect of T_H2 cell-derived IL-31 on a subpopulation of sensory DRG neurons, which cause onset of pruritus in AD[68]. Further transcriptomic analysis of dorsal root ganglia that have been activated by IL-31 revealed an up-regulation of mitogenic genes that are essential for proliferation, survival and metabolism. Indeed, IL-31 pump-equipped mice show an increased abundance of the DRG neuron-associated gene *Prph* in the lesional skin compared to untreated control mice. IL-31 positively regulated distinct genes related to the quantity and extension of sensory neurons, like the neurotrophin nerve-growth factor NGF[74].

1.3.2 IL-31 and its role in AD

Several murine as well as human studies have not only implicated, but clearly demonstrated a role for IL-31 in AD patients by analyzing different parameters, e.g. it was discovered that in serum samples of AD patients enhanced levels of IL-31 and IL-33 were measured when compared to healthy volunteers. It was therefore hypothesized that IL-33 release from e.g. epithelial cells might lead to a subsequent increase of the secretion of IL-31 by T_H2 cells[48,75] or epithelial cells. Furthermore, *in vitro* studies have demonstrated the release of chemokines such as macrophage inflammatory protein 1β (CCL4), thymus-expressed chemokine (CCL25), TARC (CCL17), MDC (CCL22) and other lymphocyte recruiting molecules from IL-31 activated keratinocytes[44].

The release of CCL17 and CCL22 from activated keratinocytes results in the recruitment of cutaneous lymphocyte antigen (CLA) expressing $CCR4^+$ T cells to the epidermis. The CLA^+ $CCR4^+$ cell subset has become a focus of many studies and is discussed as an abundant circulating allergen-reactive T cell in atopic dermatitis patients[76]. In this context, HDM-

reactive T cells have recently been described to secrete IL-31 and be enriched in patients with AD[77]. This aligns with studies describing IL-31-expressing T_H2 cells to express CLA and home to the skin. These T cells have been detected in peripheral blood as well as skin biopsies of AD patients and might just be this HDM-reactive IL-31-expressing T cells. A co-expression of the proteins, however, has not been addressed yet. The involvement of these T cell populations in AD has been suggested, based on the further observation describing high abundance of IL-4 and IL-13 producing CLA^+ $CD3^+$ T cells when compared to healthy controls. This T cell subset is again found to be enriched in the epidermis of AD patients[43]. Of note, recruited $CD68^+$ macrophages and primary keratinocytes robustly express IL-31RA and the receptor is up-regulated in the epidermis of AD patients[43]. The exact role of the recruited macrophages as well as the role for IL-31RA on keratinocytes and the epidermis of AD patients has recently been described by Hänel *et al*., who describes a direct IL-31 dependent gene expression of genes involved in the organization of the physical skin barrier. In fact, they show an enhancement of trans-epidermal water loss and therefore a dysregulation of the skin barrier upon IL-31 signaling[78,79]. Further, up-regulation of IL-31RA expression on keratinocytes and the subsequent secretion of mediators recruiting IL-31 expressing T_H2 cells to the inflammatory site of AD patients points to a feedback-in loop and, hence, influence on disease progression based on this lymphocyte population. Which factors modulate the up-regulation of IL-31RA expression in AD patients is still to be investigated and might be of as much interest as the insight about the underlying “pathophysiological” signaling pathways of this receptor itself. It has been proposed that pro-inflammatory cytokines such as IFN-γ induce the mRNA expression levels of the IL-31RA in keratinocytes and DRG neurons[80]. Recent observations tend to link skin resident memory T cells ($CD3^+$ $CD45RO^+$ CLA^+) as primary players to disease onset of AD, as they reveal antigen specificity and high proliferative capacity upon activation[77]. Most of them are already antigen experienced as they express histocompatibility antigen-DR (HLA-DR) molecules on their surface. This means in turn, that a secondary challenge with the same antigen, e.g. bacterium-derived staphylococcal enterotoxins (SEB) or pyroglyphidae-derived Der9 p1, could enhance the effector phenotype of T_H2 cells[81,82]. Although the presence of *Staphylococcus aureus* has been described thoroughly in atopic dermatitis patients, SEB is known to be a general activator of all T cell subsets and therefore less specific[81,83]. Recently the role of house dust mite allergens (HDMs) on priming of T_H2 cells has been addressed, in particular in the focus of

atopic dermatitis. Here, Szegedi *et al.* have demonstrated an increased frequency of HDM-reactive T cells in the periphery of patients with chronic AD, which they refer to as a pre-existing T_H2-Tc2 or even T_H31-Tc31 programming[77]. Further, rising studies about the microbiome composition of mucosal sites could identify distinct detrimental as well as pathogenic microbes within patients with inflammatory disorders or even autoimmune diseases. Priming of T cells, however, is a complex process, which relies on a strong interplay between the innate and adaptive immune system. Patrolling phagocytes of the adaptive immune system recognize via their toll like receptors (TLRs) or pattern recognition receptors (PRRs) invading pathogens or allergens and present them to cells of the adaptive immune system, like $CD4^+$ T helper cells. The most effective antigen presenting cells are tissue resident dendritic cells (DCs), which capture the antigen and depending on the subsequent activation of the according TLR activate either effector or regulatory T cells[2]. Similar to keratinocytes, exposure of primary human $CD1c^+$ and monocyte derived dendritic cells to IL-31 leads to STAT1 activation and subsequent IL-31RA upregulation[84]. Although some of the upstream pathways leading to the expression of the IL-31RA are known, it still remains unclear, which promotor region and transcription factor is directly responsible for its production. However, dendritic cells further secrete high amounts of pro-inflammatory cytokines such as TNF-α, IL-6, CXCL8, CCL2, CCL5 and CCL22, when stimulated with IL-31 leading to an amplification of the inflammatory response. As mentioned above, these partly DC-derived cytokines induce a massive influx of distinct immune cells, among others, T_H2 cells and eosinophils. A recent study has described eosinophils to express stable amounts of IL-31RA, correlating its expression with a lower apoptosis rate under IL-31 treatment when compared to untreated controls[85]. This observation indicates that IL-31 triggered signaling might represent a survival advantage for eosinophils – a cell type found in early/acute lesions of AD patients – which might be responsible for an additional mechanism of enhancement of inflammatory processes. Activated dendritic cells do not only lead to the influx of eosinophils into the skin, but participate in the activation of $CD4^+$ T cells, among them the abundantly present T_H2 cells, by presenting either SEB antigen or HDM antigen to tissue resident or infiltrated naïve T cells. Uncontrolled clonal expansion of those effector T cells leads to the recruitment of additional eosinophils and $CD68^+$ macrophages[43]. A more recent study has implicated a role for the IL-31/IL-31RA axis also in basophils in inflammatory skin diseases[86]. Here, patients with chronic urticaria, a pruritic skin disease,

were found to have increased serum levels of IL-31 and basophils, which express the IL-31RA and OSMR. Further, IL-31 has been described to be released by IgE or IL-3 stimulated basophils, which in turn instructed the release of the classical T_H2 cytokines IL-4 and IL-13[86].

However, the role of IL-31 on basophils in AD and their interaction with other IL-31 producers in this setup has not been described so far.

1.3.3 Basophils

Basophils are the least common type of PMNs with an abundance of 0.1 - 1% in the peripheral blood, have a very short life span (approx. 60 h) and are in need of continuous replenishment from the bone marrow[87]. They contain segmented nuclei that are disguised by the vast metachromatic granules in the cytoplasm when stained with basic dyes, an observation that essentially influenced the naming of these cells by their discoverer Paul Ehrlich. In particular, their granules are one of many reasons, why basophils for so long have been mistaken for circulating mast cells. Another reason is their surface expression of the high affinity IgE receptor FcεRI. The lineage commitment of basophils is determined by the transcription factor GATA2 and C/EBPa. The serum IgE level crucially controls basophil hematopoiesis[87]. An increase in serum IgE ultimately results in basophil differentiation and proliferation in the bone marrow. During this process pre-mature basophils up-regulate their characteristic surface marker IL-3Rα (CD123), which is a prerequisite for IL-3 consumption[87,88]. Further, all matured basophils are expressing the chemokine receptor CCR3, independent of their activation status, while the CD63 surface marker determines the activation/degranulation status of matured basophils[89]. Basophil proliferation can also be stimulated via thymic stromal lymphopoietin (TSLP) stimulation resulting in a slightly differing phenotype to IgE-mediated proliferation, including an enhanced expression of the ST2 receptor. ST2 expression points towards an elevated cellular activation status[90]. A main distinguishing feature of basophils compared to mast cells is represented by their unique development-dependent tissue localizations. While basophils complete their maturation in the BM, mast cells mature upon antigen contact in the periphery. Furthermore, mast cells are capable of proliferating after proper stimulation, while basophiles do not exhibit this ability. The discovery of the high IL-4 production and secretion by basophils marked the

most significant difference to mast cell biology and placed basophils over the past decades into the pivotal space of immune modulators and activators of particularly T_H2 cells[90–92].

1.3.3.1 Basophils in comparison to professional antigen presenting cells

The main function of antigen presenting cells is to display antigens for example retrieved by the ingestion of microbes to naïve T lymphocytes in order to initiate their proliferation and differentiation and immune response. The classical APCs are the dendritic cells, also described as the linkers between innate and adaptive immunity (see also chapter 1.1.2). Only transiently present in the circulating blood stream, DCs are present in large numbers at the epithelial tissue site and express predominantly innate immune receptors (so called TLRs). DCs mature under the influence of pro-inflammatory cytokines and the cytokine Flt3 ligand, which binds to its cognate Flt3 tyrosine receptor, a receptor tryrosine kinase with high homology to c-Kit, expressed by DC precursor cells leading to DC maturation and differentiation. Upon phagocytosis of pathogens, dendritic cells will undergo activation, then migrate from the site of tissue inflammation to the closest draining lymph node (LN), where they present the antigen to LN-residing naïve T cells. This immune cell interaction then initiates the differentiation of naïve T cells to T cell subpopulations. Dependent upon the quality of the presented antigen, DCs induce a T_H1 response by the secretion of IL-12, a T_H2 response via IL-4 signaling or a T_H17 response when they release a IL-6/IL-23 cytokine cocktail[93]. Hereby, the process of antigen presentation is divided into two steps. Before antigen (Ag) is presented via MHC (major histocompatibility complex) class II molecules on a cellular surface, the antigen needs to be ingested by APCs and then be degraded in acidic endosomes. Loading of degraded antigen fragments to the appropriate MHC class molecules occurs during the trafficking progress of early to late endosomes to the endosomal/lysosomal compartment. The newly formed Ag-MHC-II complex then re-traffics to the cell surface, where it replenishes the (Ag)-MHC-II class stocking and is exposed to the T cells with their interacting CD4 receptor. The function of CD4 is to firmly stabilize the antigen presenting interaction of T cells and enhances the T cell immune response[2,3]. Another important cell type capable of antigen presentation are basophils[94,95]. The priming into T helper cell subsets originating from naïve T cells residing in the periphery or the secondary lymphoid organs (SLO) is a complex system, which involves several co-stimulatory molecules, receptors and cytokines. Different APCs are

responsible for the differentiation of naïve T cells into effector T cells. T_H2 cell differentiation e.g. can be driven by antigen presenting basophils[94,96].

There are different types of DCs known, all as derivate of $CD14^+$ monocytes, the immature phagocyte progenitors. Upon exposure to certain cytokines (GM-CSF and IL-4) monocytes first differentiate into immature DCs, which circulate continuously between the bloodstream and epithelial tissues (*i.e.* skin, lung as well as the gastro-intestinal tract), which are in contact with the external environment. These sites represent the first-contact locations of microbes with DCs that react upon exposure to microbial peptides with phagocytosis and in consequence have been described to function as professional APCs. Several types of DCs have been identified in the human and murine immune system with partially over-lapping function. Their nomenclature derives predominantly from the tissue they reside in[97]. Langerhans cells (LCs) are mainly found in the epidermis and reside in the follicular and intra-follicular epithelium. They are characterized by the expression of the surface markers CD1a, CD1c and CD207 (langerin). Initially, LCs have been grouped into the classical antigen presenting cell subset, but more recent findings identified an additional function for LCs as immune modulators[98]. A more common antigen presenting dendritic cell subset is represented by the dermal myeloid dendritic cells (mDCs), which share expression of the surface marker CD1c. mDCs differentiate directly from circulating monocytes that home to the dermis after exposure to an inflammatory environment. Once mDCs are fully differentiated and activated by antigen digestion, they will produce distinct cytokines and chemokines orchestrating the adaptive immune response against invading pathogens and infections[99]. In contrast to the mDCs, which differentiate from monocytes, the plasmacytoid DCs (pDCs) subset expresses the surface marker BDCA-2 and stems from plasmacytoid precursors. pDCs additionally express the toll like receptors TLR7 and TLR9, which recognize single stranded RNA and can sense viruses. In fact, pDCs are mainly involved in the combat of viral infections. Consequently, pDCs produce high amounts of IFN-α, a cytokine that is known to indicate and activate further cascades of the innate immune system in response to viral infections[97,100].

Table 1: Human APC subsets in the skin (Modified from Kashem *et al.*, 2017)

	LC	CD141⁺ DCs	CD1c⁺ DCs	pDCs	CD14⁺
Location	**Epidermis**	**Dermis/Lymph node/blood**			**Dermis**
Murine equivalent	LC	cDC1	cDC2	pDC	Mo-Mϕ
HLA-DR	+	+	+	+	+
CD11c	low	Low	+	-	+
CD1a	++	-	+	-	-
CD14	-	-	-	-	+
CD1c (BDCA1)	+	-	+	-	-/+
CD303 (BDCA2)	-	-	-	+	-
CD304 (BDCA3)	-	+	+/-	+	-
CD141 (BDCA4)	-	++	-/+	-/+	-
XCR1	-	+	-	-	-
CD370 (CLEC9A)	-	+	-	-	-
CD207 (langerin)	++	-	-/+	-	-
CD326 (EpCAM)	+	-	-	-	-
CD324 (E-cadherin)	+	-	-	-	-
CD11b	Low	-	+	-	+
CX3CR1	+	-	+	-	+
CD172α (SIRPα)	+	-	+	-	+

Basophils do express abundant levels of several antigen-binding receptors as well as co-stimulatory receptors (MHC-II, CD80, CD86 and CD40) and secrete when activated high levels of IL-4 and TSLP, cytokines essential for skewing naïve T cells to a type 2 (high affinity) phenotype. Interestingly, the activation of these T cells is less based on the antigen presentation, but rather achieved antigen independent by cytokine release[101].

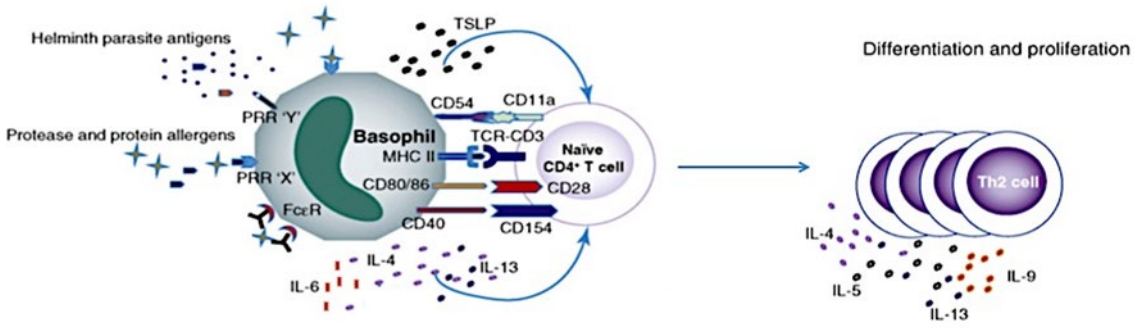

Figure 2: Basophils act as physiological antigen presenting cells that induce T_H2 responses. Through MHC class II-dependent cognate interactions with $CD4^+$ T cells in the context of co-stimulatory molecules (CD80, CD86, CD40 and CD54), and through secretion of IL-4, IL-13, and thymic stromal lymphopoietin (TSLP), basophils drive antigen-specific T_H2 responses. By endocytosing IgE-complexed antigens via FcεR, basophils can induce T_H2 responses. Consequently, FcεR has a prominent role in memory responses, but not in inducing primary responses (Modifed from Maddur *et al*., 2010).

1.4 Aim of the study

Evidence from previous studies links the IL-6 family member IL-31 to the pathogenesis of atopic dermatitis. However, the role of T_H2-associated IL-31 in onset, progression and resolution of atopic dermatitis is still not understood in detail. Therefore, this study is aiming to address the following items:

1) To identify the molecular source of IL-31 from different lymphocyte subsets and determine the frequency and the phenotype of IL-31-producing cells in healthy volunteers and atopic dermatitis patients.

2) To investigate the expression pattern of the heterodimeric receptor IL-31RA/OSMRβ on basophils and their presence as well as their phenotypically characteristics in healthy volunteers and atopic dermatitis patients.

3) To identify a potential role of the IL-31/IL-31RA axis for basophils as well a potential interaction with T cells in human and mouse.

2. Material and Methods

2.1 Patients

For the purpose of this study, several human samples were collected and analysed. The majority of the results is generated by whole blood samples from either healthy volunteers or patients diagnosed with AD according to the inclusion and exclusion criteria (table 2 and 3). Extracted whole blood (see chapter 2.2.1) was used to characterize the different subtypes of immune cells, especially of T cells.

All patients were consent by the medical staff of the "Dept. of Dermatology, University Hospital Düsseldorf" and all of them provided written informed consent before participation according to the guidelines of the Declaration of Helsinki and the ethical committee of the University clinic Düsseldorf (study number: 5908R).

Table 2: Key inclusion and exclusion criteria for healthy volunteers

Key inclusion criteria	• Capable of giving informed consent • Individuals having no personal or family history of atopic dermatitis or any other systemic (cutaneous) disorder • Individuals not suffering from any chronic or acute inflammatory disease
Key exclusion criteria	• Previously treated with an immune-modulating/or another systemic therapy • Any oral antibiotic within 28 days prior to baseline. • Pregnancy • < 18 years of age • Major psychiatric illness

Table 3: Key inclusion and exclusion criteria of AD patients

Key inclusion criteria	• Capable of giving informed consent. • Naïve to previous systemic treatment (e.g. Cyclosporin A) • Diagnosis of atopic dermatitis for at least 1 year prior to baseline. • AD lesions must be present in at least two distinct anatomical areas • Subject must have stable AD for at least 60 days prior to screening visit and at baseline visit

Key exclusion criteria	• Previously treated with Cyclosporin A or another systemic AD therapy • Subject suffers from an additional acute or chronic (cutaneous) inflammatory disorder • Any oral antibiotic for AD within 28 days prior to baseline. • Pregnancy • < 18 years of age • Major psychiatric illness

2.1.1 Blood sampling

Blood samples of healthy blood donors were primarily obtained from individuals, which voluntarily undertook a blood donation at the Blood Donation Center (DRC) of the University Hospital Düsseldorf (UKD, Moorenstraße 5, 40225 Düsseldorf). At the DRC facility, standard buffy coats (also called lymphocyte reduction systems) are generated and available by request from their affiliated blood bank department. Buffy coats were obtained within 4h of fabrication.

The collection of venous blood samples from consented AD patients was performed according to standard protocols implemented at the department of dermatology at the university clinic Düsseldorf by the medical staff. Of all enrolled participants, no harmful event was registered due to sampling techniques.

2.1.2 Primary human keratinocytes

Primary human keratinocytes were obtained from skin specimens of healthy donors. Fresh biopsies were cleaned of subcutaneous fat using a scalpel. The skin was then cut in pieces of 2-3 x 2-3 mm using a razor blade and transferred to a 24 well plate (Greiner, Germany) with 8 pieces per well. The pieces were desiccated for approximately 1h at room temperature upon open plate cover. Afterwards, the skin pieces were covered with 1 mL DMEM containing 10% FCS (fetal calf serum, GIBCO) and CellShield (ThermoFisher) and placed in a humid cell incubator keeping an ambient temperature of 37°C with a standard atmosphere containing 5% CO_2. After one week skin pieces were removed and the primary keratinocytes that grew from the skin tissue into the well were harvested by trypsinization using a 0.05% / 0.03% trypsin/EDTA-mix (Gibco, Germany) for 5 min at 37°C. Trypsinized keratinocytes were then re-suspended in a stop solution (Gibco,

Germany) inhibiting trypsin activity, transferred to a 15 mL falcon tube (Sarstedt, Germany) and pelleted in a centrifuge (Heraeus, Germany) for 10 min at room temperature (RT) with 1200 rpm (rounds per minute). Supernatant was discarded, the pellet re-suspended in 10 mL keratinocyte medium (Kera-SFM; Gibco, Germany) supplemented with Human Keratinocyte Growth Supplement (Gibco, Germany) and transferred to a 75 cm^2 flasks (T75, Greiner, Germany) pre-coated with coating matrix (Gibco, Germany). Keratinocytes were maintained in the cell culture incubator (100% humidity, 5% CO_2) until a confluent monolayer was formed and passaged 1:3 into new keratinocyte medium containing pre-coated T75 culture flasks for maximally three times using the indicated trypsinization procedure. Keratinocyte medium was replaced every second day. For the experiments in this study, keratinocytes of third passage were seeded into a 6-well plate (Greiner, Germany) and when reaching 80% confluence incubated with 30, 50 or 100 ng/mL of a particular single cytokine (either IL-4, IL-13, IL1-β, TNFα or IL-17), or combinations of these cytokines, for 6h and 24h and supernatants obtained. Supernatants were freshly subjected to analysis or stored at -80°C in sterile 2 mL Eppendorf tubes (Eppendorf, Germany) until performance of the appropriate analysis technique.

2.2 Isolation of leukocytes

2.2.1 Isolation of leukocytes from peripheral blood

A characterization of lymphocytes of the provided blood material requires an isolation step. The procedure of lymphocytes isolation from whole blood is based on physical differences of the distinct blood components, e.g., red blood cells (RBCs) possess a higher density than PBMCs (polymorph blood mononuclear cells) (Fig. 3).

Utilizing these varying physical properties, 50 mL of whole human blood were infused to a Ficoll tube (GE Healthcare, Germany) providing a density gradient (sodium diatrizoate and calcium disodium ethylenediamintetraacetic acid) and placed in a centrifuge for 30 min with 900 g at RT. Red blood cells (RBCs) as well as granulocytes were pelleted upon centrifugation, while lymphocytes and platelets accumulate in the interface between serum on top and Ficoll solution. Lymphocytes were harvested from the interface by pipetting technique and transferred to a 50 mL falcon tube (BD Biosciences, Germany), then washed twice with 1x phosphate buffered saline (PBS), each washing step followed

by centrifugation for 8 min at 4°C with 450 g. The pellet was re-suspended in 30 mL of PBS and cell density assessed using a counting chamber (Neubauer Counting Chamber). Contamination with platelets was removed via the indicated washing steps. The isolated PBMCs were freshly subjected to the respective experimental analysis or stored at -20°C with freezing medium consisting of 90% FCS with 10% DMSO.

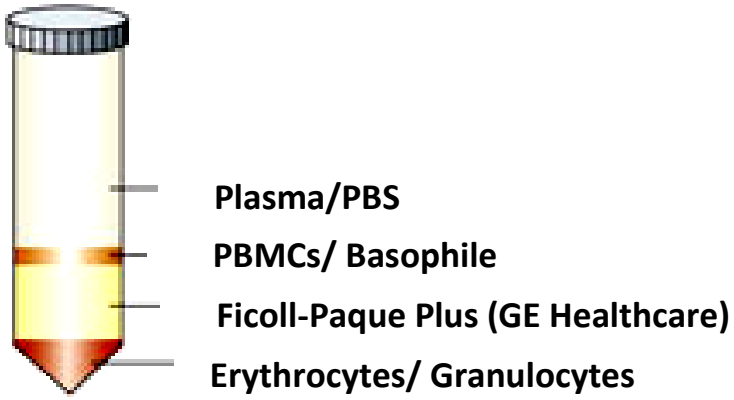

Figure 3: Schematic figure of the different layers after ficoll density gradient preparation

2.3 Magnetic cell sorting

2.3.1 Enrichment of $CD4^+$ T cells

The focus of this study was the identification and characterization of the IL-31 producing $CD4^+$ T cell subset. Hence, it is important to obtain a pure $CD4^+$ cell population. Ficoll gradient purification yields PBMCs that are comprised of diverse cell populations, e.g., containing numerous types of leukocytes and basophils. In order to achieve an enrichment of $CD4^+$ T cells, an additional enrichment procedure is necessary. One approach is to enrich the desired cell type in the cell suspension by depletion of all other leukocyte populations i.e. $CD8^+$, $CD14^+$, $CD16^+$, $CD11c^+$, $CD19^+$, $CD56^+$, $CD25^+$, $NKp44^+$, $CD123^+$, $CD1a^+$, $CD34^+$ immune cells).

Here, depletion was achieved by incubating PBMCs with biotin-labelled antibodies (dilution see table 5; 500 µL antibody solution/$1x10^8$ cells) for 15 min on ice followed by washing with MACS buffer (1x PBS; 0.2% BSA; 2 mM EDTA) with 10 times of the staining volume (450 g; 8 min). The T cell suspension was then incubated with anti-biotin magnetic beads (1:70 dilution; 1 mL/$1x10^8$ cells) for 15 min at 4°C.

Table 4: Antibody list for biotin antibodies

Surface marker	Fluorochrome	Clone	Company	Dilution
CD1a	biotin	HI149	BioLegend	1:500
CD34	biotin	581	BioLegend	1:500
NKp46	biotin	9E2	BioLegend	1:500
CD19	biotin	HIB19	BioLegend	1:500
CD11c	biotin	3.9	BioLegend	1:500
CD16	biotin	3G8	BioLegend	1:500
CD14	biotin	HCD14	BioLegend	1:500
CD56	biotin	HCD56	BioLegend	1:500
CD25	Biotin	BC96	BioLegend	1:750
CD45RA	biotin	HI100	BioLegend	1:1200
CD45RO	biotin	UCHL1	BioLegend	1:750
CD8	biotin	HIT8α	BioLegend	1:500
CD123	biotin	6H6	BioLegend	1:500

The cell suspension was again washed with 10 times of the staining volume with MACS buffer and in the end re-suspended in 2 mL MACS buffer. The sorting of the cells is performed with the *DepleteS* program of the AutoMACS (Milteny biotec), which contains magnetic columns and keeps in the first step the labelled cells within the column and releases them in a second step in a separate tube. This procedure produces two separate samples. The first one is containing the negative population, meaning all unlabeled cells and therewith the $CD4^+$ population, the second sample contains all depleted cells, which are labelled with the magnetic beads (Fig. 4).

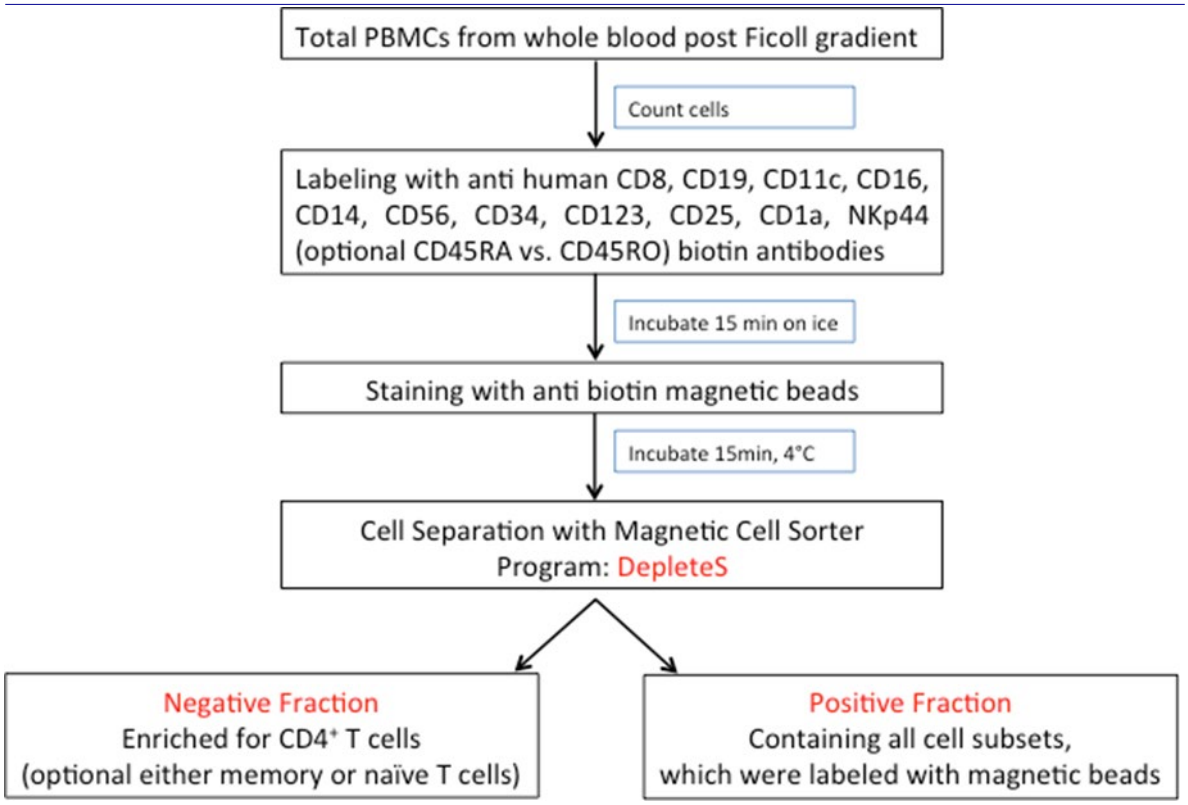

Figure 4: Enrichment of CD4$^+$ T cells via magnetic cell sorting. Isolation and enrichment of CD4$^+$ T cells is achievable by either negative fractioning or positive fractioning.

2.3.2 Enrichment of CD123$^+$ cells

Another part of this study focused on the characterization of basophils (CD123$^+$) and their interaction with T cells. CD123$^+$ basophils were enriched from whole PBMCs obtained from Ficoll gradient purification of whole blood samples (see also chapter 2.2.1). Because the frequency of basophils in the blood is low, a slightly different enrichment approach was implemented as compared to the CD4$^+$ T cell approach.

First, the lymphocyte subsets, which are not expressing any basophil-characteristic surface markers, were depleted utilizing an antibody cocktail (surface targets: CD1a$^+$, CD3$^+$, CD4$^+$, CD8$^+$, CD14$^+$, CD15$^+$, CD16$^+$, CD11c$^+$, CD19$^+$, CD56$^+$, CD25$^+$, NKp44$^+$, CD34$^+$) and then subjected to magnetic cell separation. Hereby, labelled cells are fished from the heterogeneous PBMC suspension. The cell content of the un-labelled fraction, also called “negative fraction”, was counted using a cell-counting chamber (Neubauer Chamber; Roth) and incubated for 15 min at 4°C with the biotinylated anti-human CD123 antibody (see table 4). Following, anti-biotin beads were added, incubated for 15 min at 4°C and the PosselD program (positive selection running over two magnetic columns) was applied

in order to positively sort out the CD123 expressing cells using Miltenyi AutoMACS. The purity of the MACS-Sort was analyzed via flow cytometry (see chapter 2.4).

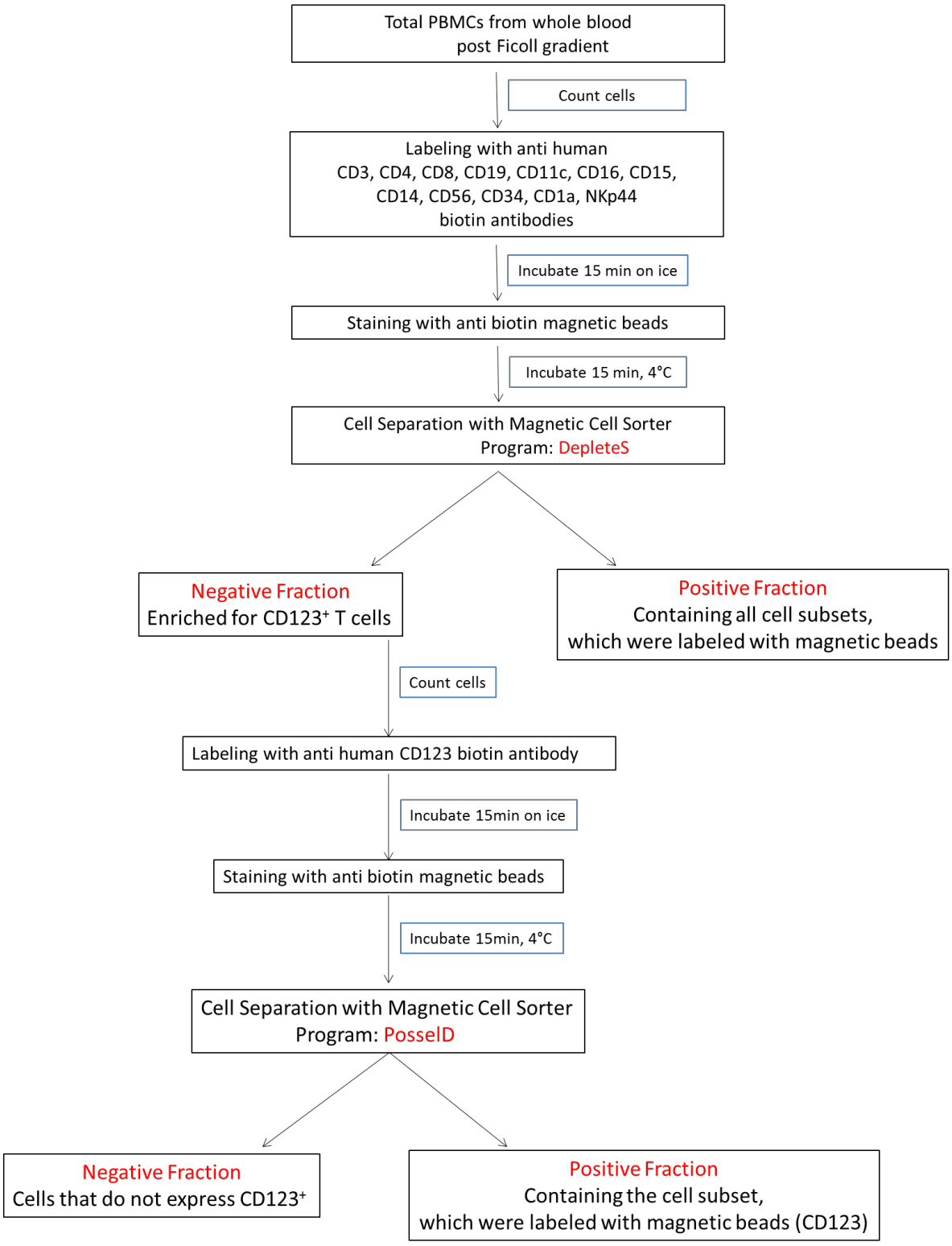

Figure 5: Enrichment of CD123+ basophils via magnetic cell sorting. **Isolation and enrichment of CD123+ cells is obtained in a two-step approach.**

2.3.3 Enrichment of CD14$^+$ cells

CD14$^+$ monocytes are often used as feeder cells for the setup of single cell cloning approaches. In contrast to the above described indirect enrichment approaches of memory or naïve CD4$^+$ T cells (chapter 2.3.1) and CD123$^+$ basophils (chapter 2.3.2), whole blood PBMCs are incubated directly with a magnetic-bead coupled anti-CD14 antibody (Miltenyi Biotec; 1:50 dilution) for 15 min on ice. Afterwards they are washed twice with MACS-buffer and separated via the PosselD program (positive selection running over two magnetic columns) using the Milteny AutoMACS. The positive fraction contains the CD14$^+$ monocytes.

2.4 Principle of multicolor flow cytometry and cell sorting

Multicolor flow cytometry was used in order to identify/characterize immune cells based on their size and surface markers. The principle underlying this method is the recognition of labelled cells based on their size as well as the fluorochrome conjugated to an antibody. The antibodies deployed for multicolor flow cytometry in this study are directed against either surface markers or intracellular components, such as secreted cytokines and transcription factors (see table 8; 9 and 10). Thereby different lasers are stimulating the fluorochromes with distinct wavelengths, which then emit signals in form of a novel, converted particular wavelength. The intensity of the emission and the quality of wavelength is determining the quality and quantitative expression of the targeted marker. This approach is also utilized to sort single cells or whole cell populations based on expression and recognition of a specific surface marker. Flow cytometry connects cell size to fluorochrome and based on manually applied settings automatically selects cells of interest from a heterogeneous whole cell suspension. For this study the multicolor flow cytometric analysis was performed with the CytoFlex flow cytometer (Beckmann Coulter, Germany).

2.4.1 Titration of antibodies

In order to distinguish precise cell subsets by surface marker labeling, the binding of the antibodies and the emission of their conjugated fluorochrome must be specific and devoid of generation of background signal (noise). Antibodies used in this study were predominantly monoclonal and obtained from particular hybridoma clones, a system that

ensures antibody conservation and specific epitope binding. To evaluate the specific epitope (complementary antigen) affinity and the appropriate antibody concentration creating acceptable background noise, each fluorescence-activated cell sorting (FACS)-deployed antibody of this study was tested in dilution series. Figure 6 shows the outcome received from an exemplary antibody titration. In order to determine optimal antibody dilution, FACS data plots need to be compared under the same experimental conditions and identical gate settings. The calculation of the geometrical mean of the positive populations (red square in Fig. 6) divided by the geometrical mean of the negative population (blue square in Fig. 6) results in a ratio that reflects specificity of the deployed antibody and gives an objective value for the assessment of the appropriate dilution of the antibody in an experimental setting.

The calculation of the geometrical mean of the positive populations (red) divided with the one of the negative populations (blue) results in the use of the antibody in a 1:75.

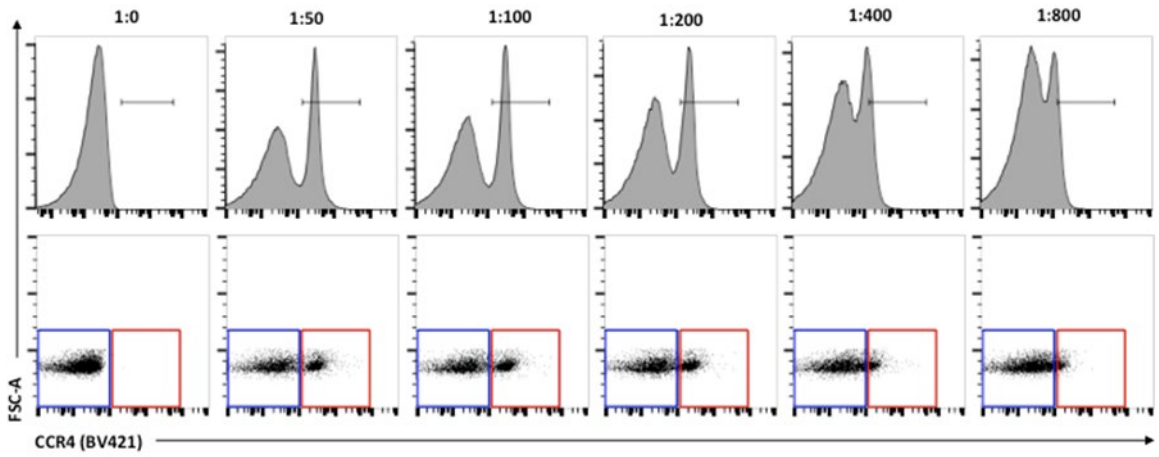

Figure 6: Exemplary titration of the antibody directed against human CCR4. The chemokine receptor CCR4 is conjugated with the fluorochrome BV421. Detection of $CCR4^+$ cells is achieved with all dilutions in comparison to the control (1:0), where no antibody was added. However, with rising dilution factor, the detected cell numbers decrease. In this case, the optimal antibody dilution lies between 1:100 and 1:200 dilution of the master antibody solution. FSC-A – forward scatter-A.

2.4.2 Surface staining

T cells are characterized by the expression of the co-receptors CD3 and CD4 on their surface membrane. Subpopulations are distinguished upon distinct expression patterns of surface markers such as CD45RO (memory T cells) or CD45RA (naïve T cells). In order to differentiate T cell (sub-) populations, distinct staining panels were designed utilizing combinations of antibodies conjugated with distinct fluorochromes. For FACS staining

purpose, the heterogeneous T cell mix was transferred into a 1.5 mL Eppendorf tube Sarstedt, Germany) and pelleted by centrifugation at 450 g for 8 min.

Table 5: Antibody list for human surface staining

Surface marker	Fluorochrome	Clone	Company	Dilution
CD3	APC-Cy7	OKT3	BioLegend	1:100
CD3	PE-TxR	UCHT1	eBioscience	1:75
CD4	BV650	OKT4	BioLegend	1:100
CD4	PE-Cy7	OKT4	BioLegend	1:100
CD4	PerCP	OKT4	eBioscience	1:100
CD8	PerCP	RPA-T8	BD Horizon	1:100
CD8	APC	RPA-T8	BioLegend	1:100
CD45RO	BV570	UCHL1	BioLegend	1:75
CD45RO	BUV395	UCHL1	BD Pharmingen	1:50
CD45RA	BV780	HI100	BioLegend	1:75
CD45RA	BUV737	HI100	BD Pharmingen	1:50
CD62L	AlexaFluor 700	DREG-56	BioLegend	1:50
Beta7	PerCP	FIB504	BD Bioscience	1:50
CCR6	PE	11A9	BD Pharmingen	1:75
CCR4	PE-Cy7	1G1	BD Pharmingen	1:150
CCR7	PE	G043H7	BioLegend	1:50
CD25	PE-Cy5	BC96	BioLegend	1:75
CD127	e421	A019D5	BioLegend	1:75
CCR9	PE	L053E8	R&D System	1:75
CLA	e421	HECA-452	eBioscience	1:150
CXCR3	PerCP	G025H7	BioLegend	1:150
CCR10	PE	FAB3478p	R&D System	1:50
IL33R	biotin	B4E6	BioLegend	1:100
CD56	PerCP	HCD56	eBioscience	1:75
CD14	PerCP	M5E2	BD Bioscience	1:100
CD14	PE	M5E2	BioLegend	1:100
CD16	PerCP	3G8	BD Bioscience	1:100
CD11c	PerCP	3.9	BD Bioscience	1:75
CD11b	PerCP	ICRF44	BD Bioscience	1:75
CD19	PerCP	HIB19	BD Boscience	1:75
HLA-DR	AF700	L243	BioLegend	1:100
HLA-DR	APC	L243	Biolegend	1:75
CD123	PcP	G46-6	BD Bioscience	1:100
CD123	FITC	7G3	BD Bioscience	1:75
CD63	PcP	7G3	BD Pharmingen	1:75
CD69	APC	H5C6	BD Pharmingen	1:75
FcERI	FITC	FN50	Biolegend	1:100
CD203c	PE	AER-37	BD Pharmingen	1:100
CCR8	PE	433H	BD Pharmingen	1:50
OSMRb	PE	FAB4389A	BD Pharmingen	1:50
OSMRb	APC	FAB4389A	R&D Systems	1:50
IL-31RA	FITC	FAB2796F	R&D Systems	1:50
CCR3	PE	5E8	BD Bioscience	1:100

Primary antibodies were diluted in 50 µL of MACS buffer for each sample (see Table 5 for dilution factors), then added to the cell pellet, re-suspended and kept for 15 min at 37°C, RT or 4°C, respectively. Cells were washed twice with 500 µL MACS buffer, each washing followed by centrifugation at 450 g for 8 min. The pellet was re-suspended with the appropriate fluorochrome-labelled secondary antibody diluted in 50 µL of MACS buffer. Cells were again incubated for 15 min at RT. In order to prevent unspecific binding, mouse IgG was added in excess to bind the residues of soluble free secondary antibody.

Two washing steps using MACS buffer followed, intermitted by centrifugation (450 g for 8 min). Cells were fixed in 100 µL Fix/Perm Buffer diluted 1:10 in ddH_2O (BD Lysis Buffer, Germany) for 10 min at RT. Following a last washing step with MACS buffer and subsequent centrifugation (450 g for 8 min), the cell pellet was re-suspended in 100 µL MACS buffer and directly subjected to flow cytometric analysis (CytoFlex, Beckman Coulter, Germany) utilizing various settings.

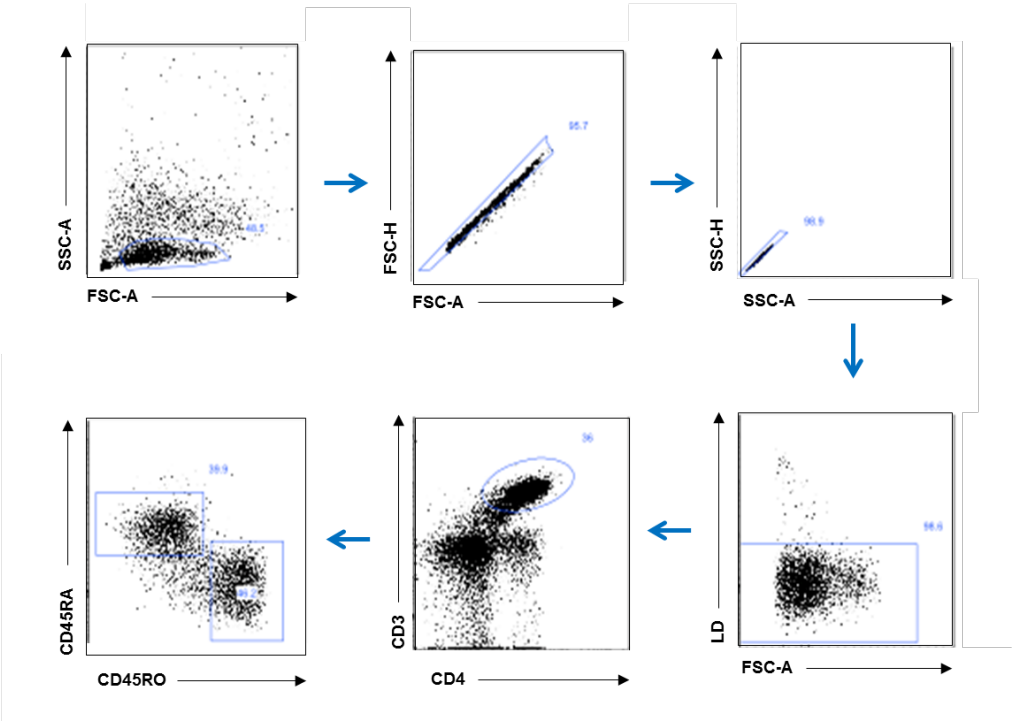

Figure 7: Gating strategy for naïve and memory CD4⁺ T cell populations.

Acquired flow cytometry data is analyzed *via* FlowJo 9.8 and requires several steps in order to investigate the right cell populations. Therefore, different gating strategies are performed and regarded to in several parts of the following data. Figure 7 shows the six main steps that are common in almost all of the experiments regarding T cell analysis. The FSC-A vs. SSC-A gate is set upon the size of T cells, while the following two gates are

placed in order to exclude duplicates. As the extraction process and the staining conditions can lead to cell death, all samples are stained for live dead (LD) with a cell viability dye (BioLegend). The gate is set on all fluorochrome negative cells, as the LD dye stains only in dead cells. Setting a gate for the $CD3^+$ $CD4^+$ marks the population of interest and excludes $CD8^+$ as well as T cells lacking both mentioned surface markers. Regarding the question of the analysis a further subdivision into naïve ($CD45RA^+$) and memory ($CD45RO^+$) T cells can be made. This particular surface marker staining protocol for FACS analysis was deployed with murine epitope targeting antibodies as well as human epitope targeting antibodies.

Table 6: Antibodies for surface staining of murine T cells and basophils

Surface marker	Fluorochrome	Clone	Company	Dilution
CD123	PerCP	6H6	BioLegend	1:100
FcERI	AF700	MAR-1	BioLegend	1:100
CD73	PB	AD2	BioLegend	1:75
MHCII	BV780	M5/114.15.2	BioLegend	1:150
CD69	BV605	H1.2F3	BioLegend	1:75
CCR3	APC	5E8	BioLegend	1:75
CCR4	PB	L291H4	BioLegend	1:75
CCR8	FITC	SA214G2	BioLegend	1:75
CLA	PcP	HECA-452	BioLegend	1:100
ST2	biotin	DIH9	BioLegend	1:100
CD4	PE-Cy7	RM4-5	BioLegend	1:150
CD4	BV605	GK1.5	BioLegend	1:150
CD4	BV780	GK1.5	BioLegend	1:150
CD44	AF700	IM7	BioLegend	1:75
FcERI	biotin	MAR-1	BioLegend	1:100
CD123	biotin	5B11	BioLegend	1:100
CD4	biotin	GK1.5	BioLegend	1:100
CXCR3	PerCP	CXCR-173	BioLegend	1:75

2.4.3 Intracellular staining for cytokines

The different T helper subsets are distinguished by their specific pattern of cytokine expression. The production of cytokines, however, is only initiated upon T cell activation via TCR stimulation. Hence, prior to assessment of cytokine expression via FACS analysis, T cells have to be stimulated with an appropriate signal. Here, addition of 5 ng/mL Phorbol Ester (PMA) and 500 ng/mL Ionomycin (Sigma-Aldrich) for 6h to complete medium served as master T cell stimulation. Application of 5 µg/mL Brefeldin A (Sigma-Aldrich) and 5 µg/mL Monensin (BD Bioscience) 30 min after initial stimulation was

utilized to prevent the release of cytokines from the intracellular compartment. Abrogating stimulation, cells were pelleted, washed and stained for distinct surface marker patterns and fixed by methodologies described above (see sub-chapter 2.4.2).

Table 7: Antibodies for intracellular cytokine staining of human lymphocytes

Cytokine marker	Fluorochrome	Clone	Company
IL-4	PE-Cy7	MP4-25D2	BioLegend
IL-5	APC	TRFIC5	BioLegend
IFN-γ	AF700	4S.B3	BioLegend
IFN-γ	PacificBlue	B27	BioLegend
IL-17F	PerCP	033-782	BD Pharmigen
IL-4	FITC	MH9A4	BD Pharmigen
IL-13	FITC	PVM13-1	eBioscience
IL-31	APC	MAb11	eBioscience
IL-10	BV421	JES 3-9 D7	BioLegend

The fixed cells were then washed once with 100 µL of 0.05% saponin containing PBS (equivalent to 500 mL 1x PBS; 0.25 g saponin; Sigma-Aldrich, Germany). Saponin is a detergent that permeabilizes the cell membrane leading to access of exogenously applied antibodies to intracellular located cytokines (dilutions see Table 7). All antibody dilutions in this intracellular staining protocol contained 0.05% saponin. Cells were incubated dependent on antibody identity for either 15 min or 12h at 4°C. Subsequently, cells were washed with 100 µL of the 0.05% saponin-containing PBS solution, centrifuged for 8 min at 450 g, the supernatant discarded and the cell pellet re-suspended in 100 µL of FACS buffer. Following a final centrifugation, the cell pellet was re-suspended in FACS buffer (100 µL) and readily subjected to flow cytometric analysis.

Table 8: Antibodies for intracellular cytokine staining of murine lymphocytes

Cytokine marker	Fluorochrome	Clone	Company	Dilution
IL31	APC-Cy7	Q6EBC2	MyBioSource	1:100
IL4	APC	11B11	Biolegend	1:75
IL13	PE	JES10-5A2.2	Milteny	1:75
IFNγ	PB	XMG1.2	BD Pharmingen	1:100

2.4.4 Intracellular staining for transcription factors

Analogue to the intracellular staining of cytokines, staining of transcription factors prerequisites permeabilization of the cell's plasma membrane. Consequently, staining of transcription factors was performed as described in sub-chapter 2.4.3 with a final re-

suspension of the cell pellet in 200 µL of freshly prepared fixation/permeabilization working solution (1:3 dilution; Foxp3 staining Kit, eBioscience, Germany). The cell suspension was incubated for 45 min at 4°C omitting direct light. Following, cells were washed with 200 µL of a permeabilization buffer (1:10 dilution in ddH_2O; part of Foxp3 staining Kit, eBioscience, Germany) and centrifuged at RT for 8 min at 450 g. The supernatant was discarded and the cell pellet re-suspended in 1x permeabilization buffer containing an intracellular staining antibody mix (respective antibody dilution see table 9). Epitope binding was performed for 45 min at RT in the dark. Of note, cells were not washed, but centrifuged directly (400 g, 8 min at RT), supernatant discarded and cell pellet re-suspended in 200 µL of FACS buffer for direct flow cytometric analysis.

Isotype controls were carried out with antibody types corresponding to the isotype directed against the epitope of the respective transcription factor (e.g. IgG1 isotype control for anti-Foxp3) and conjugated with the identical fluorochorme. IgG Isotype-controls were performed on the same cell population that was used for analysis of transcription factor staining intensity as calculated by mean fluorescence intensity (MFI). The MFI is therefore reflecting a comparing ratio within one cell population. Consequently, the MFI value rises with increase in positive staining of the targeted transcription factor.

Table 9: Antibody list for human and murine intracellular transcription factor staining

TF staining	Fluorochrome	Clone	Company	Dilution
Foxp3	FITC	259D/C7	BD Pharmingen	1:100
IgG Control	FITC	MOPC	BD Pharmingen	1:400
RoRgt	PE	Q21-559	BD Pharmingen	1:600
IgG Control	PE	27-35	BD Pharmingen	1:400
Gata3	APC	L50-823	BD Pharmingen	1:100
IgG Control	APC	MOPC-21	BD Pharmingen	1:400
T-bet	BV421	O4-46	BD Pharmingen	1:100
IgG Control	BV421	X40	BD Pharmingen	1:400

2.5 Molecular biology

2.5.1 RNA extraction

RNA was extracted from *ex vivo*-sorted basophils according to the above stated protocol (see chapter 2.3.2). $1x10^6$ cells were subjected to RNA extraction. Cells were pelleted by centrifugation (400 g for 8 min at RT and re-suspended in 350 µL RLT buffer containing β-Mercaptoethanol (1:100) for RNA stabilization (QIAGEN RNeasy® Mini Kit, Germany). Cell suspension was mixed with 1 volume 70% Ethanol, applied to an RNeasy Mini spin column (QIAGEN RNeasy® Mini Kit, Germany) placed in a centrifuge (Heraeus, Germany) for 15 sec at 4°C and 8000 g. The flow-through was discarded, the column washed with 350 µL RW1 buffer and 10 µL DNase I diluted in 70 µL RDD buffer was added to and incubated onto the column for 15 min at RT (all components of QIAGEN RNeasy® Mini Kit, Germany). The enzymatic DNAse activity was terminated by addition of 350 µL RW1 buffer (QIAGEN RNeasy® Mini Kit, Germany) and subsequent centrifugation of the column (15 sec at 4°C, 8000 g). To remove cellular debris, the column was washed twice (intermitted by centrifugation for 15 sec at $\geq$ 8000 g) with 500 µL RPE buffer. RNA was eluted by 30 µL of RNase-free water and subsequent centrifugation for 1 min at 13000 rpm. RNA concentration was assessed by NanoDrop (Life Technologies) analysis.

2.5.2 cDNA synthesis

The synthesis of cDNA from RNA samples was performed in a four-step approach. 4 µg of the RNA in a total volume of 10 µL were mixed with the volumes of reagents indicated in table 10 (MIX I to MIX III) in 3 consecutive steps as follows:

Step 1: addition of 6 µL of DNAse MIX I to RNA (4 µg/10 µL), and placement of samples to an amplification cycler (Biorad) programmed as follows: 20 min at 37°C, 10 min at 70°C, 4° ad infinitum.

Step 2: MIX II (4 µL) was added and incubated for 10 min at 70°C,

Step 3: Addition of 9 µL of MIX III, incubation for 2 min at 42°C.

Step 4: Addition of polymerase (1 µL, Superscript (Invitrogen, Germany)) to the whole mix and amplification using the following cycle steps:

50 min at 42°C, 10 min at 70°C, 4°C ad infinitum.

The final product, cDNA, is diluted in DNAse-free H_2O to receive a final concentration of 25 ng cDNA per 100 µL. cDNA was stored at -20°C until further analysis.

Table 10: Procedure for cDNA reverse transcription of mRNA

MIX I:

Reagent	Volume (in µL)
First strand buffer (Invitrogen)	1.5
Rnasin (Promega)	1
Dnase Mix (Roche)	1
Aqua	2.5

MIX II:

Reagent	Volume (in µL)
Oligo dT Invitrogen)	1
Random Hexamer (Promega)	0.4
Aqua	2.6

MIX III:

Reagent	Volume (in µL)
First strand buffer (Invitrogen)	4.5
dNTP (Bioline)	1.5
DTT (Invitrogen)	1
RNasin (Promega)	0.5
Aqua	1.5

2.5.3 Quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) analysis was performed to determine gene expression levels of sorted basophils. RNA was extracted basophils as described in chapter 2.5.1. Subsequent to reverse transcription of isolated mRNA (see chapter 2.5.2.), cDNA products were amplified via real-time polymerase chain reaction using various Gene expression assays (Table 11; AB Applied Biosystems, Germany). The transcription levels of target genes were assessed in relation to the expression of the referencing "house-keeping" gene 18S. The qPCR reaction mix consisted of 1 µL cDNA (0.25 ng/µl), 5µL of SYBR Green reagent (AB Applied Biosystems, Germany) and 2 µL ddH_20. Probes were transferred to a 96-well-plate (Greiner, Germany) and 2 µL of a respective primer pair (2 mM each, forward and reverse primer) mix added. The 96-well plate was sealed with optical adhesive films (AB Applied Biosystems, Germany) and centrifuged (Heraeus, Germany) for 1 min at 13.000 rpm and placed in a StepOnePlus™ Real-Time PCR System (ThermoFischer, Germany) programmed with the following cycling protocol:

10 min at 95°C followed by 60°C for 2 min and 95°C for 45 sec, in a total of 40 cycles and 4°C ad infinitum. Melting curves were used to assess primer qualities. For gene expression analysis, ΔCT- as well as the ΔΔCT- values were calculated utilizing Microsoft Excel

software. Values were adjusted to house-keeping expression of *18S*.

Table 11: Selected primers for SYBR Green human gene expression assays

Gene	Dye and Size		Company	Sequences
Bcl-2	Sybr;	360rxns	AB Applied Biosystems	fwd: 5' CTTCGTGCCTGCCTGTACC 3' rev: 5' CGFGCCCAGAAAGTGGACG 3'
Bax	Sybr;	360rxns	AB Applied Biosystems	fwd: ATG GAC GGG TCC GGG GAG rev: ATC CAG CCC AAC AGC CGC

2.6 Cell culture

Isolated and sorted T cells were kept in cell culture at 37°C, 100% humidity and an atmosphere containing 5% CO_2 in culture flasks (Greiner, Germany) containing general lymphocyte medium for up to 7 days. Clonal expansion was achieved by supplementation of T cell medium (composition see below) with IL-2 (2000 ng/mL) or TCR stimulation by CD2/CD3/CD28 coated Dynal beads (T cell expansion kit Milteny, Germany), or they can differentiate from naïve T cells towards T helper subsets or regulatory T cells through stimulation with distinct cytokines (see chapter 2.6.1 and table 12).

Special T cell medium

500 mL	RPMI
5 mL	non-essential AA (1%)
5 mL	L-glutamine (2 mM)
5 mL	sodium pyruvate (1%)
5 mL	Penicillin/Streptomycin (1%)
10 mL	Human Serum (5%; Sigma-Aldrich)

General Lymphocyte medium

500 mL	RPMI
5 mL	L-glutamine (2 mM)
5 mL	Penicillin/Streptomycin (1%)
50 mL	Fetal Calf Serum (10%; Sigma-Aldrich)

2.6.1 T cell differentiation

In order to establish T cell lines for a particular T helper subset, naïve T cells were stimulated with the appropriate conditioned medium. As described in chapter 1.3.2, every T cell lineage reacts on specific cytokines that initiate their differentiation and drives them towards a particular fate. Hence, these defined cytokines (see table 12;

purchased from R&D systems, Germany) in combination with a CD2/CD3/CD28 dynal beads containing in a TCR stimulation kit (Milteny, Germany) were used to firstly stimulate the proliferation and secondly achieve the differentiation of naïve T cells.

Table 12: Differentiation conditions for naive T cells

	Final-con. (ng/mL)	Stock conc. (µg/mL)	Volume Medium [mL]	T_H1-diff.	T_H2-diff.	T_H17-diff.
CD2/CD3/CD28 beads	/	/	**/**	**50 µL**	**50 µL**	**50 µL**
IL-2	500	1000	5	2.5	2.5	2.5
IL-4	20	100	5		1.0	
IL-12	10	10	5	5.0		
IL-1β	10	5	5			10
IL-23	25	50	5			2.5
IL-6	20	100	5			10
TGF-β	2.5	10	5			1.25
TNF-α	10	100	5			0.5
α-IFN-γ	2.5	1	5		12.5	12.5
α-IL-4	2.5	1	5	12.5		12.5

The exact conditions for T cell differentiation is shown in table 12 and based on the special T cell medium formulation. T cell cultures were maintained for 7 days. Subsequently, differentiation and proliferation efficiency was assessed by FACS analysis monitoring intracellular cytokine as well as transcription factor expression.

2.6.2 Co-cultures of naïve T cells and basophils with stimulated NHEK-supernatants

In order to determine the influence of distinct inflammatory cytokine environments, which play a role in different skin disorders, such as atopic dermatitis and psoriasis, and are mediated by the secretome of normal human epidermal keratinocytes (NHEK) and subsequently on basophils, and basophil-elicited priming of T cells, a co-culture experiment with *ex vivo* sorted basophils and autologous naïve T cells under the influence of stimulated NHEKs was established. Therefore, in a first step the NHEKs were seeded on a 6-well plate until they reached 80% confluence, afterwards keratinocyte-medium was replaced with 6 mL of fresh keratinocyte medium containing either IL-4 and IL-13 (50 ng/mL, each), IL-17A (100 ng/mL) or a combination of TNFα and IL-1β (30 ng/mL, each) for either 6h or 24h (Fig. 8A). Subsequently, supernatants of these cytokine-treated NHEKs were collected after this time points and 2 mL of the secretome-medium was subjected to co-cultures of basophils ($2x10^4$ cells/well) and naïve T cells ($2x10^5$ cells/well)

in a 24-well plate (Fig. 8B). These cells were then incubated for 8 days at 37°C and 5% CO_2. After the proliferation and differentiation period for the naïve T cells passed, the differentiation statuses as well as cytokine profile of the T cells under distinct microenvironments was determined (Fig. 8).

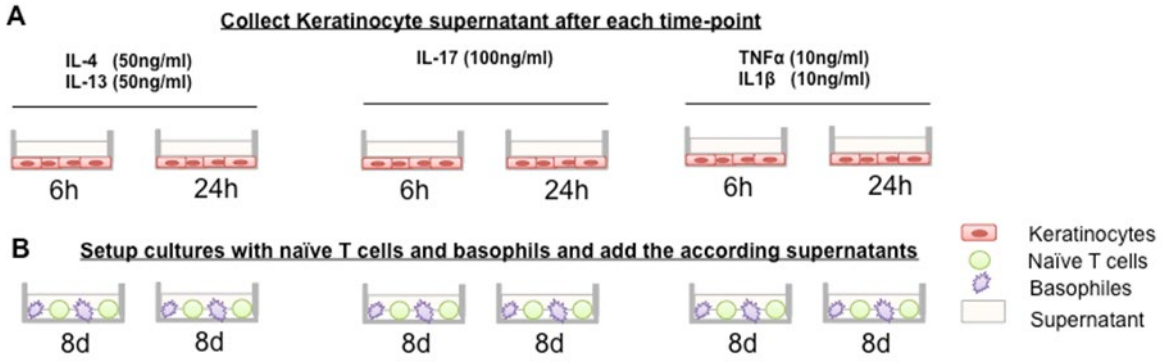

Figure 8: Schematic illustration of a co-culturing approach of autologous naïve T cells with ex vivo basophils under the influence of distinct keratinocyte supernatants.

2.6.3 Single cell cloning

Whole PBMCs consist of several distinct immune cell populations. Within the $CD4^+$ T cell compartment further variations of subsets exist. In order to identify IL-31 producing $CD4^+$ T cells in this heterogeneous cell mix and subsequently characterize IL-31^+ cells we aimed to purify single IL-31^+ cells by flow cytometric procedure. Therefore, PBMCs were isolated from 10 mL peripheral blood and enriched for $CD4^+$ T cells (see subchapter 2.3.1). These cells were then stained for specific surface markers (CD3, CD4, CD45RA, CD45RO and CCR7). As the focus of this study was based on *ex vivo* cells, which are capable to secrete IL-31, the population of interest is the memory T cell population ($CD3^+$ $CD4^+$ $CD45RO^+$ $CD45RA^-$ $CCR7^-$), since only memory T cells secrete cytokines. The single cells were sorted directly into a 96well plate, which contained feeder cells, using a MC MoFlo XPD flow cytometer (Beckman Coulter, Germany). Feeder cells consisted of irradiated antigen presenting cells from 2 donors, which was achieved by isolation of $CD14^+$ monocytes (see chapter 2.3.3). The irradiation was performed in complete RPMI (cRPMI; RPMI containing 1% non-essential amino acids; 2 mM L-glutamine; 1% sodium pyruvate; 1% Penicillin/Streptomycin; 5% human serum) at 45 Gy. The irradiated APCs were washed twice with cRPMI medium and intermittently centrifuged with 400 g for 8 min. APCs of the 2 donors were mixed in a 1:1 ratio. Cells were then plated (100 µL per well containing

1x10^5 cells) into a 96 U-bottom well plate (Greiner, Germany). To stimulate the TCR of sorted T cells, the feeder solution contained 1 µg/mL phytohemagglutinin A (PHA; Sigma Aldrich, Germany) and human recombinant (rh) IL-2 (500 U/mL). The sorted cells were kept in a cell culture incubator (36°C, 100% humidity, 5% CO_2) and were after 5-6 days stimulated with 100 µL of newly prepared cRPMI containing rhIL-2 (200 U/L).

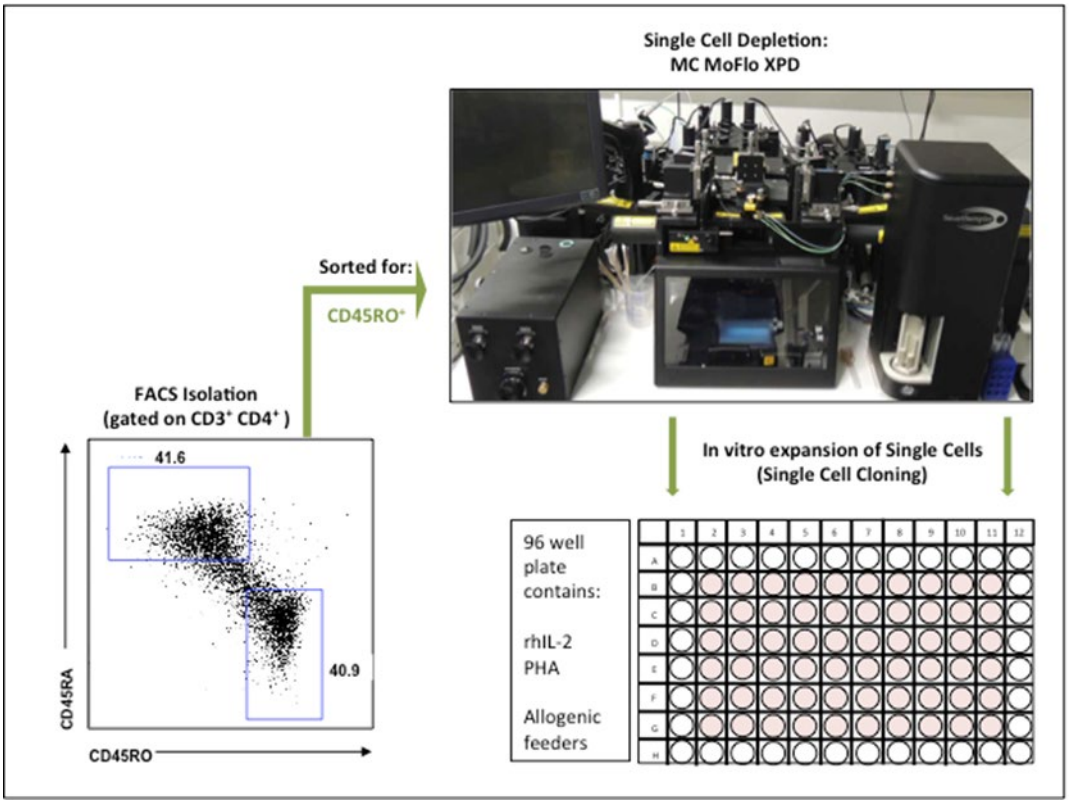

Figure 3: Single cell cloning process. After the characterization of naïve vs. memory CD4$^+$ T cells, the different populations are determined and single cell cloning is performed with the MC MoFlo XPD. Hereby, single cells of the above mentioned populations are sorted into each well of 96 well plates containing the irradiated allogenic feeders, rhIL-2 and PHA.

With feeding, cells will clonally expand. The clonal populations will build up a dense cellular aggregate at the round bottom of the 96well plate, which was monitored daily using an inverse cell culture microscope (Zeiss, Germany). Clones, which expanded successfully, were harvested 14 days after the initial single cell sort by breaking the T cell clusters upon pipetting up and down and were then transferred into a new 96 well plate for the analysis via flow cytometry for intracellular cytokine expression (see also subchapter 2.4.3).

2.7 Mouse experiments

2.7.1 Mice

In this study, the wild type mouse strain C57BL/6 and *Il-3ra*$^{-/-}$ (knock-out) mice were used. All mice were kept by the internal breeding facility of the "Zentrale Einrichtung für Tierforschung und Tierschutzaufgaben" (ZETT) of the Heinrich-Heine University Düsseldorf and overseen and provided for by staff of the ZETT. Mice were housed under germ free conditions with free access to food and water in excess. When aged 8 to 16 weeks, healthy mice were sacrificed and their organs harvested for isolation of T cells and basophils. Animal based procedures of this study were approved by the „Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen"(LANUV). (study number: O56/06).

2.7.2 Genotyping of *Il-31ra*$^{-/-}$

Il-31ra$^{-/-}$ mice were routinely genotyped. Amputated tail tips of approx. 2 mm size were lysed for 10 min at 75°C in 50 µL of nuclease free-water (Carl Roth GmbH, Germany), containing diluted 10x KAPA Express Extract Buffer (1:10; KAPA Biosystems, Inc, Germany) and 1 U/mL KAPA Express Enzyme (KAPA Biosystems, Inc, Germany). 1 µL of the lysate containing whole genome DNA of the *Il-31ra*$^{-/-}$ mouse strain was then added to 14 µl of DNAse free water (Carl Roth GmbH, Germany), 4 µL of 5x PCR-Buffer (Promega, Germany), 0.5 µL of dNTPs (Invitrogen, Germany), 0.1 µL Go-Taq (Promega, Germany) and 0.2 µL of sense and anti-sense primer (20 µM) of the mIL-31RA (reverse primer; 43416: 5'-GATACCACTAATGTTTCATGG-3') and the mIL-31RA (forward primer; 43419: 5'-CAGGTCTGCAACCTCACAGG-3') mixed with the Puro primer (43420: 5'-CGCAAGCCCGGTGCCTGA-3'). The subsequent polymerase chain reaction was performed on a Applied Applied Biosystems 7000 System/ Quantstudio 6 PCR cycler (ThermoFisher, Germany) under following cycling conditions:

1. 94° - 1 min
2. (94° - 15 sec, 59° - 25 sec, 72° - 45 sec) 40x
3. 72° - 3 min, 4° - ad infinitum

The product size of the amplicon is determined via agarose gel-electrophoresis, using 2 g peqGOLD Universal Agaorse (Peqlab Biotechnology GmbH, Germany), which is dissolved

in 100 mL of 1x Tris-Acetate-EDTA buffer (TAE, Carl Roth GmbH, Germany) by boiling in a microwave for 3 min with 1000 Watt or until full dissolution, cooled down to lukewarm temperature, substituted with 5 µL of Red Safe Nucleic Acid Staining Solution (Stock: 20.000x; Intron Biotechnology, Germany) and poured into a casting chamber (BioRad Laboratories, Germany). Gel-pockets of defined size were created by inserting a pocket crest and the gel was left for cooling in the open. For analysis of DNA probes, the gel was placed in an electrophoresis chamber (Mini Sub Cell GT, BioRad Laboratories, Germany) containing 1x TAE buffer, and pockets were loaded with Gene Ruler 100 bp DNA ladder (Thermo Fisher Scientific) and 15 µL of each PCR product, respectively. Electrophoresis was performed for 30 min at 100 V and abrogated. UV trans-illumination with a Cell-Cycler (BioRad Laboratories, Germany) using a 280 nm filter was utilized to visualize the separated Red safe intercalating DNA fragments. Approximate size of the *Il-31ra*$^{+/+}$ wild type amplicon was 290 bp and of the homozygous *Il-31ra*$^{-/-}$ 552 bp. Fragment sizes were determined by comparison to the Gene Ruler ladder of known fragment sizes.

2.8 Statistics

Data were expressed at mean +/- standard deviation (SD) or +/- standard error of the mean (SEM). Statistical significance was assessed by either paired or unpaired Student's t-test, Mann-Whitney-U-test, 1-way as well as 2-way ANOVA using GraphPad Prism software (version 5.03, GraphPad Software, La Jolla, CA, USA).

P-values below 0.05 were considered as statistically significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

3. Results

3.1 IL-31 is predominantly expressed by the CD4⁺ T cell population

Recent preclinical and clinical studies underscore that IL-31 plays a crucial role in the onset of atopic inflammation associated itch in mice[44,102] and humans[71,77,103]. Current findings suggest a predominant IL-31 expression in adaptive immune cells, CD4⁺ T cells in particular, linking T cell activity to pruritus elicitation[104]. Hence, the aim of this study was to identify the leukocyte cell subset, which expresses IL-31 upon stimulation.

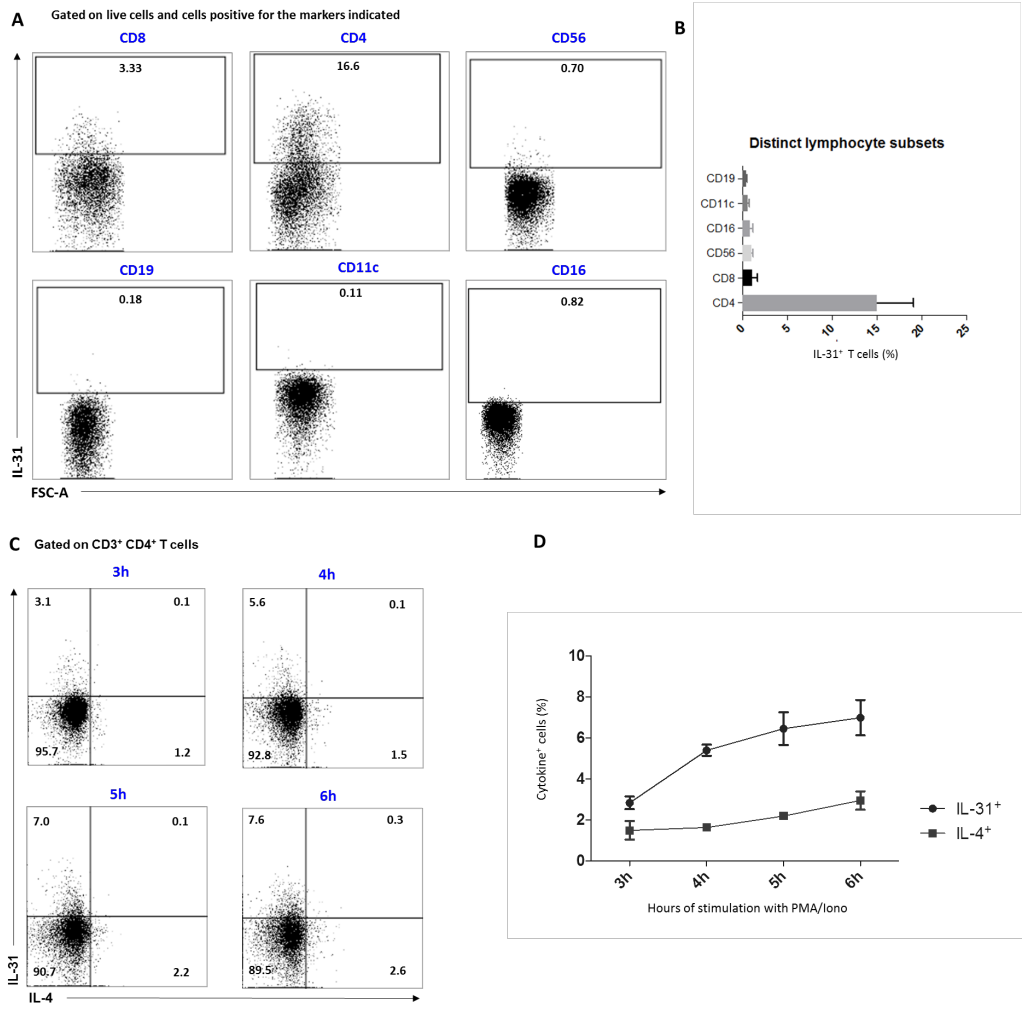

Figure 10: IL-31 expression in distinct leukocyte populations is predominantly expressed in the CD4⁺ T cell population after 6h of stimulation. (A) Representative dot plots of intracellular IL-31 expression in different leukocyte populations (CD8, CD4, CD56, CD19, CD11c and CD16) after stimulation of PBMCs with PMA/Iono (6h, 100 ng/mL for each), derived from living leukocytes of healthy individuals (n=6). The respective cells, which stained positive for IL-31 and the indicated markers, are identified by rectangles and the respective frequencies are stated. **(B)** Frequencies of intracellular IL-31 in the different leukocyte populations as indicated in (A). **(C)** Representative dot plots of IL-31 and IL-4 expression in $CD3^+$ $CD4^+$ $CD45RO^+$ T cells following stimulation with PMA/Iono for 3h to 6h; (n=6 independent healthy donors). **(D)** Time-course of IL-31 and IL-4 expression of $CD3^+$ $CD4^+$ $CD45RO^+$ T cells. Data are representative of independent experiments with six donors and presented as mean ± SD. Abbreviations: FSC-A, forward scatter.

In a first step, the IL-31 expression by different immune cell subsets was determined. PBMCs were isolated from peripheral blood of healthy volunteers (n=6) and stimulated with the "artificial" cytokine expression activators PMA and Ionomycin (100 ng/mL each) for 6h, then co-stained for characteristic immune cell surface markers (CD4: T helper cells, CD8: cytotoxic T cells, CD11c: mature dendritic cells and APCs, CD16: neutrophils and macrophages, CD19: B cells, CD56: natural killer cells) to delineate IL-31 expression in the different leukocyte subsets (Fig. 10A).

Flow cytometric analysis revealed predominant expression of IL-31 in $CD4^+$ T cells, while IL-31 appeared almost absent in $CD8^+$ cytotoxic T cells, $CD11c^+$ mature dendritic cells, $CD16^+$ neutrophils and macrophages, $CD19^+$ B cells and $CD56^+$ natural killer cells. Plotted frequencies show an approximately 5 times higher capacity to express IL-31 in $CD4^+$ T cells compared to $CD8^+$ cytotoxic T cells (Fig. 10B).

To monitor in more detail the expression of IL-31, PBMCs were stimulated *in vitro* for up to 8h using the T cell activator PMA/Iono and intracellular IL-31 was detected by flow cytometry. IL-31 expression was detected within 3h of PMA/Iono stimulation with a frequency of 2.4 ± 1.0% of total $CD3^+$ $CD4^+$ T cells and doubled after 5h of treatment (5.8 ± 2.5%) (Fig. 10C, D; representative plots). The frequency of IL-$31^{single+}$ cells increased only slightly (7.0% ± 0.9) 6h after PMA/Iono stimulation, but decreased already after 7h (5.3 ± 0.2%) and even more so after 8h (5.2 ± 0.2%) of stimulation. IL-4 frequencies of $CD3^+$ $CD4^+$ $CD45RO^+$ T cells followed a similar pattern as IL-31 over the time course of stimulation, but frequencies of IL-$4^{single+}$ cells were overall lower than IL-31^+ $CD3^+$ $CD4^+$ $CD45RO^+$ T cells with a maximal frequency of IL-4^+ T cells of (2.9 ± 0.4%) after 6h of stimulation (Fig. 10C, D).

The frequencies of healthy individuals display highest values after 6h of stimulation for both single cytokine-producing subsets, but also for IL-31^+ IL-4^+ double producers, despite very low frequencies for the healthy individuals (0.3 ± 0.1%) (Fig. 10C, D). Therefore, the maximal frequencies for all three sub-populations (IL-31^+ IL-4^+ double producers, IL-$31^{single+}$ and IL-$4^{single+}$) is reached after 6h of stimulation and hence used for the following experiments.

3.2 IL-31 is expressed by CD4$^+$ memory T cells

The pre-activation state is considered as the naïve T cell state and is marked by the expression of the surface markers CD45RA and chemokine receptor 7 (CCR7)[32]. Upon contact with its TCR specific antigen, the naïve T cell will get activated and differentiate towards a, e.g., CD4$^+$ T effector cell. This is the state, where T cells also establish the immune memory. Immune memory T cells are marked by the surface marker CD45RO[32].

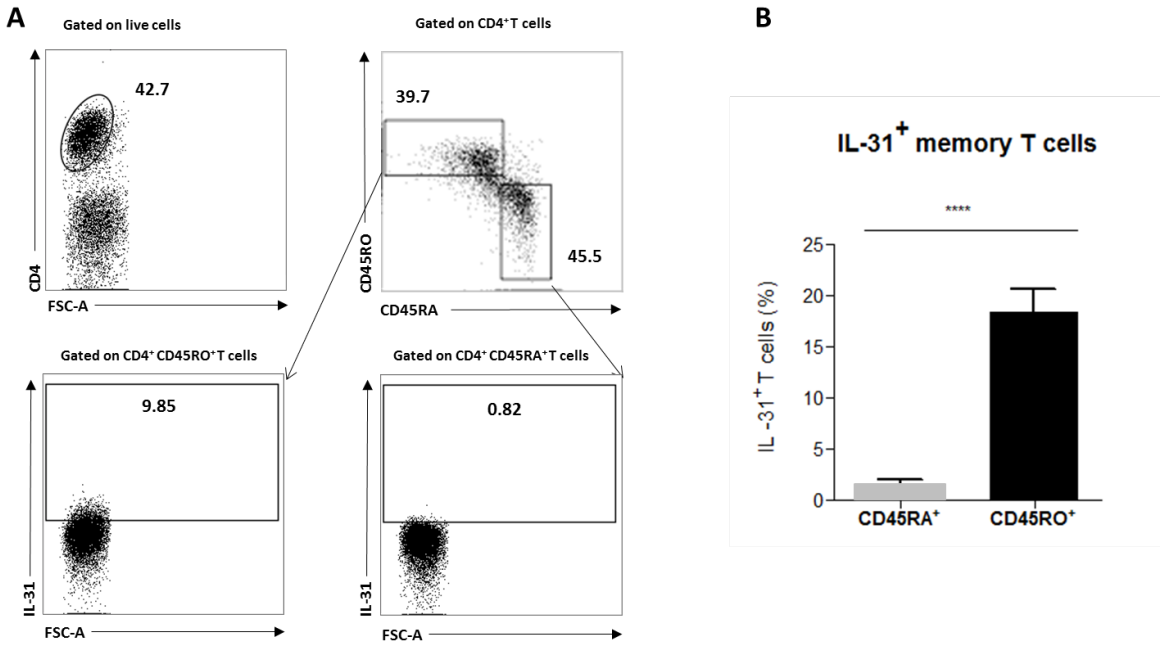

Figure 11: IL-31 is expressed by CD4$^+$ memory T cells. (A) IL-31 expression was assessed among CD4$^+$ T cells for the memory (CD45RO) and naïve (CD45RA) compartments by multicolor flow cytometry after a 6h stimulation of PBMCs with PMA/Iono. CD3$^+$ T cells were gated before assessing CD4 expression. The respective populations are identified by rectangles and frequencies are indicated in these representative dot plots. **(B)** CD4$^+$ T cells derived from healthy donors' exhibit an overall higher frequency of IL-31 expressing cells within the memory (CD45RO) compartment (black column) compared to the naïve population (grey column). Gates and quadrants were set according to negative controls. Data are representative of 17 independent experiments with pooled healthy donors (n=9) and AD patients (n=8) and presented as mean ± SD. Mann-Whitney-U Test, ****$P \leq 0.0001$.

Because PMA/Iono stimulated CD4$^+$ T cells produce IL-31, the next question was directed to whether the *in-vivo* antigen-primed memory T cell subset is also capable of producing IL-31. PBMCs (n=17 healthy and AD-donors) were co-stained for the surface markers CD4, CD45RO and CD45RA as well as for intracellular IL-31 after stimulation with PMA/Iono for 6h. Indeed, flow cytometric analysis revealed the predominant expression of IL-31 in activated CD4$^+$ T cells of the memory compartment (CD45RO$^+$/IL-31$^+$: mean frequency 18.5%) and an absence of IL-31 in naïve T cells (CD45RA$^+$/IL-31$^+$: mean frequency 1.5%) (Fig. 11A, B).

3.3 IL-31 is expressed by the effector memory compartment of CD4$^+$ T cells

T cells establish an immunological memory upon contact with their specific antigen. The memory compartment, however, can be subdivided into distinct subpopulations characterized by the combination of the following surface markers: (I) CD103$^+$ CD69$^+$ CD62L$^-$ tissue-resident memory T cells (T_{RM}), (II) CD103$^-$ CD62L$^-$ CCR7$^-$ effector memory T cells (T_{EM}) and (III) CD103$^-$ CD62L$^+$ CCR7$^+$ central memory T cells (T_{CM}).[31,32] As the surface marker CD103 is mainly utilized to identify the memory pool of tissue resident memory cells (T_{RM}), the predominant surface marker to differentiate the two T cell memory compartments in the peripheral blood is the integrin CD62L. Here, a distinction between effector memory (CD62L$^-$) and central memory T cells (CD62L$^+$) was made.

To explore if IL-31 is preferentially expressed by effector memory or central memory T cells, whole blood PBMCs (n=23 independent healthy donors) stimulated with PMA/Iono were co-stained with antibodies directed against the surface markers CD3, CD4, CD45RO and CD62L, and intracellular IL-31. Figure 12A shows a clear separation of both memory T cell sub-populations and documents co-expression of IL-31 in the absence of CD62L (T_{EM} mean frequency: 15.2 ± 2.4%), but barely any IL-31$^+$ CD62L$^+$ (T_{CM} mean frequency: 1.0 ± 0.5%) T cells, suggesting exclusive IL-31 production by the effector memory T cell compartment (Fig. 12B).

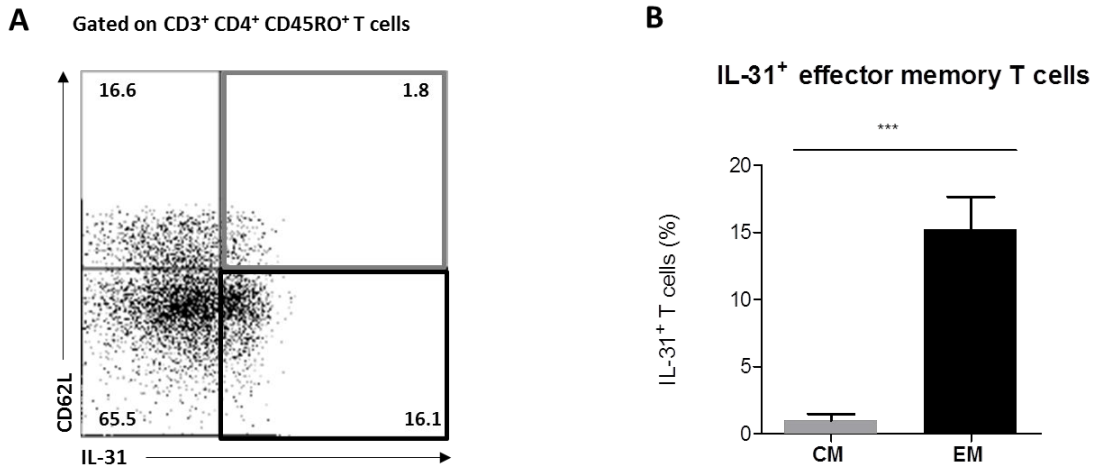

Figure 12: IL-31 is expressed by CD62L$^-$ effector memory T cells (T_{EM} cells). (A) Representative example of IL-31 and L-selectin (CD62L) expression in CD3$^+$ CD4$^+$ CD45RO$^+$ T cells. PBMCs were pre-gated for CD3$^+$ CD4$^+$ CD45RO$^+$ memory T cells. **(B)** Frequencies of CD62L/IL-31 co-expression; CD62L$^+$ central memory (grey column); CD62L$^-$ effector memory (black column). Gates and quadrants were set according to negative controls. Data are representative of independent experiments with 23 donors and presented as mean ± SD. Mann-Whitney-U Test, ***$P \leq 0.001$. Abbreviations: CM, central memory; EM, effector memory.

3.4 IL-31$^+$ T cells express skin-homing receptors

Several studies have described a role for distinct adhesion molecules, integrins and chemokine receptors (CCRs) in the control of organ specific migration of lymphocytes (summarized in[105]). Interestingly, it has recently been reported that the expression of IL-31 is linked to the expression of the skin-homing receptor CLA in T cells[43]. Therefore it was examined if IL-31$^+$ T cells feature a more distinct skin-homing receptor expression pattern, and the expression of the skin-homing markers CLA, CCR4, CCR10 and CCR8 within IL-31$^+$ T cells was investigated. Flow cytometric analysis of blood-derived PMA/Iono stimulated CD3$^+$ CD4$^+$ CD45RO$^+$ IL-31$^+$ T cells indeed revealed the expression of all investigated skin-homing receptors on IL-31$^+$ T cells (Fig. 13A). However, the skin-homing phenotype of IL-31$^+$ T cells appears to be mainly driven by CLA with a frequency of 39.0 $\pm$ 8.1% and CCR4 with a frequency two times lower (16.1 $\pm$ 7.1%). Finally, 9.9% $\pm$ 2.8% of IL-31$^+$ T cells express CCR10 and 3.2% $\pm$ 0.9% express CCR8 (Fig. 13B).

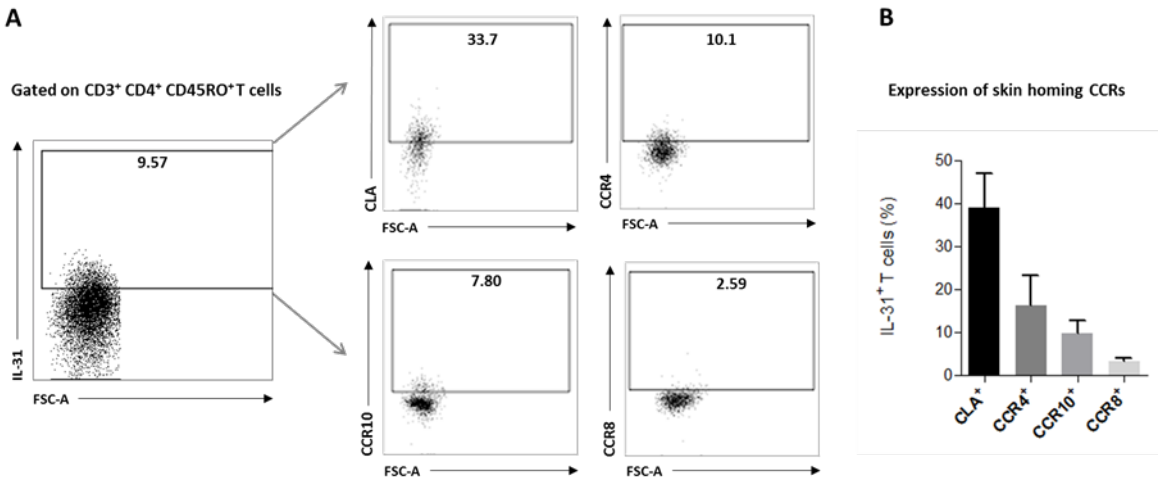

Figure 13: IL-31$^+$ T cells express a distinct pattern of skin-homing receptors. (A) Representative plots of co-expression of the skin-homing markers CLA, CCR4, CCR10 and CCR8 in pre-gated CD3$^+$ CD4$^+$ CD45RO$^+$ IL-31$^+$ memory T cells within PMA/Iono stimulated PBMCs **(B)** Within IL-31$^+$ memory T cells, CLA (black column) is the most abundantly expressed skin-homing receptor. Gates and quadrants were set according to negative controls. Data are representative of independent experiments with ≥7 healthy donors and presented as mean $\pm$ SD. Abbreviations: FSC-A, forward scatter; CCRs, CC chemokine receptors; CLA, cutaneous lymphocyte antigen.

Interestingly, chemokine receptors not only identify the organ-homing behavior of lymphocytes, but can also characterize the distinct T helper cell subsets of CD4$^+$ immune cells. While CXCR3 is, for example, predominantly expressed by T_H1 cells, the receptor

CRTH2 is exclusively expressed by T_H2 cells[16]. CCR6 however, follows a biased expression. Under inflammatory conditions, its expression indicates a skin-homing potential of immune cells, but more generally CCR6 expression marks the T_H17 T cell subset[24]. Therefore, in a next step the aim was to determine the expression of subset defining markers of memory T cells in the pool of IL-31$^+$ CD3$^+$ CD4$^+$ CD45RO$^+$ T cells. Figure 14A shows a representative flow cytometric analysis of the three major T cell subset-defining CCRs, CXCR3, CCR6 and CRTH2, in IL-31$^+$ T cells after stimulation with PMA/Iono.

This study describes an intermediate expression of CCR6 (3.7 ± 2.0%), a higher frequency of CRTH2 (8.0 ± 0.2%), but an almost absent expression of CXCR3 (1.9 ± 0.3%) by IL-31$^+$ CD3$^+$ CD4$^+$ CD45RO$^+$ T cells (Fig. 14A, B). Thus, the frequency for CRTH2 exceeds the frequencies of CCR6 and CXCR3 4-fold and 2-fold, respectively (Fig. 14B).

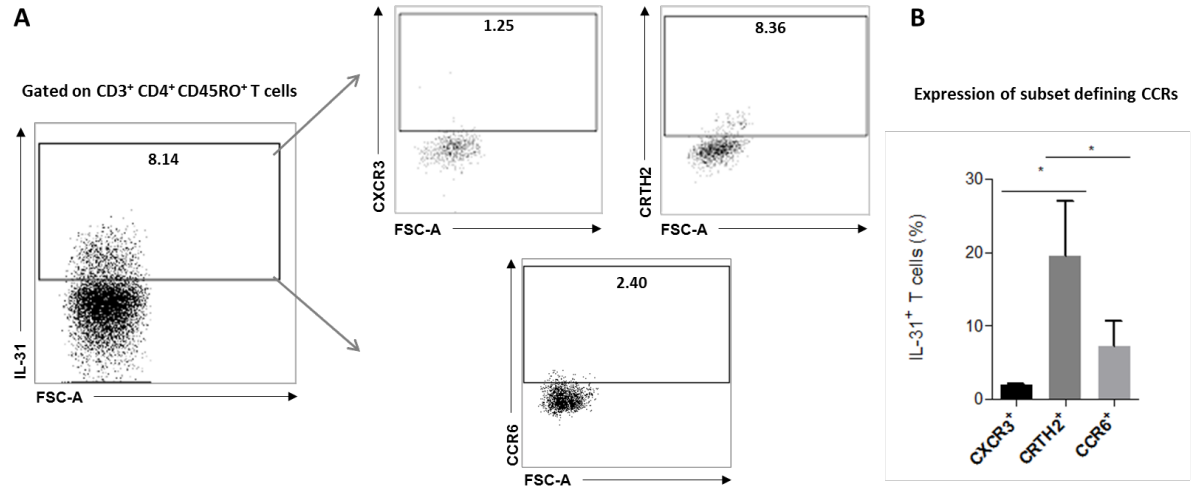

Figure 14: IL-31$^+$ T cells express the subset defining chemokine receptor CRTH2. (A) Representative dot plots for the co-expression of IL-31 with the subset-defining chemokine receptors CXCR3, CCR6 and CRTH2 in T cells derived from a healthy volunteer. Populations were pre-gated for CD3$^+$ CD4$^+$ CD45RO$^+$ T cells. **(B)** Flow cytometric analysis of IL-31$^+$ memory T cells shows highest frequencies for CRTH2. Gates and quadrants were set according to negative controls. Data are representative of independent experiments with ≥7 healthy donors and presented as mean ± SD. Kruskal-Wallis Test, 1-way Anova for nonparametric analysis**,** *$P \leq 0.05$. Abbreviations: FSC-A, forward scatter; CCRs, chemokine receptors.

3.5 IL-31 is co-expressed with classical T cell cytokines, but marks a unique T cell population

Different types of host infections induce differentiation of T cells into various T cell effector subsets. The status of differentiation is linked to expression of key transcription factors such as GATA3 and a shaping cytokine environment. Functional T effector cell

subsets express distinct cytokines, with e.g. IL-4/IL-13 typically expressed by T_H2 cells. To further elucidate the identity of IL-31$^+$ and T_H2 cells, as indicated by CRTH2 and IL-31 co-expression, the potential of IL-31$^+$ cells to co-produce IL-4 and IL-13 was investigated. PBMCs derived from whole blood of healthy donors (n=up to 12) were stimulated with PMA/Iono for 6h, and cytokine expression was analyzed in CD3$^+$, CD4$^+$, CD45RO$^+$ T cells. Flow cytometric analysis revealed distinct subsets of T cells producing e.g. either IL-4 or IL-31 (IL-4$^{single+}$, IL-31$^{single+}$) alone or being positive for both cytokines (IL-4$^+$/IL-31$^+$) (Fig. 15A, B). With regard to the expression of IL-13, flow cytometric analysis again marked three distinct T effector cell populations, namely, IL-13$^{single+}$, IL-13$^+$/IL-31$^+$ and IL-31$^{single+}$ populations (Fig. 15C, D). Interestingly, the frequency of T_H2 cell populations co-producing IL-31 with either classical T_H2 cytokine with an expression of 0.7 $\pm$ 0.2% for IL-4$^+$/IL-31$^+$ and 0.7 $\pm$ 0.3% for IL-13$^+$/IL-31$^+$, was markedly lower than the frequencies of T_H2 cells producing one of the cytokines (IL-4$^+$: 9.9 $\pm$ 2.6%; IL-13$^+$: 7.1 $\pm$ 3.0%; IL-31$^+$: 6.5 $\pm$ 2.0%). Moreover, the IL-31 producing cells do not co-produce the T_H1 characteristic cytokine IFN-γ or the T_H17 characteristic one IL-17 either (Fig.15 C,D).

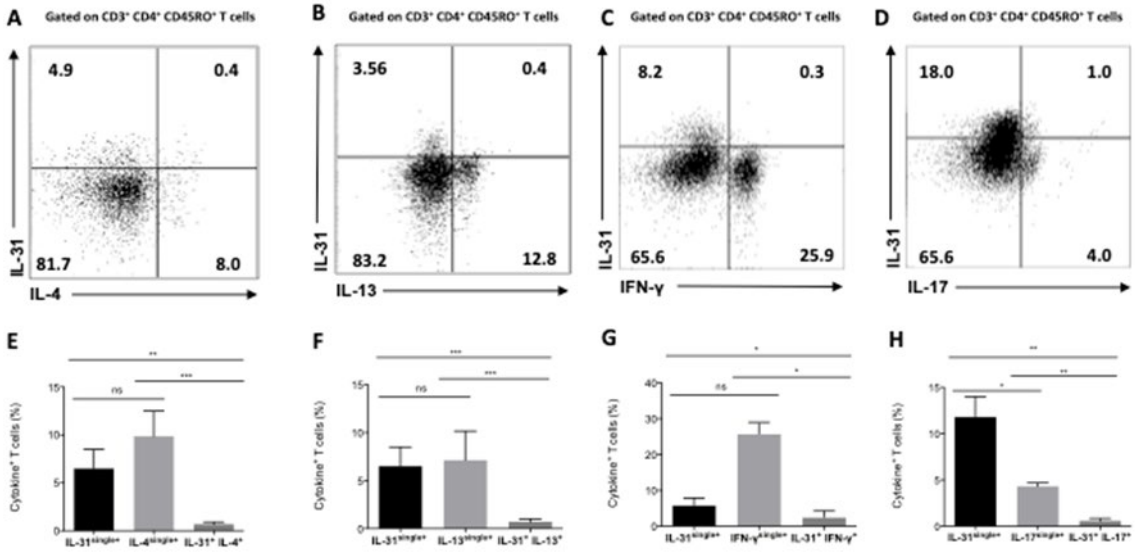

Figure 15: IL-31$^+$ T cells mark a unique single producing cell population within CD4$^+$ T cells. (A-D) Representative dot plots of 6h PMA/Iono stimulated CD3$^+$ CD4$^+$ CD45RO$^+$ cells derived from a healthy donor displaying the expression of IL-31 with IL-4, IL-13, IFN-γ or IL-17 from different experiments. **(E-F)** Frequencies of cell subsets expressing the indicated cytokines reflect predominant single expression of all cytokines when compared to their respective co-producing populations. IL-31 is mainly expressed in the absence of IL-4, IL-13, IFN-γ or IL-17. Gates and quadrants were set according to negative controls. Data are representative of independent experiments with ≥10 healthy donors and presented as mean $\pm$ SD. 1-way ANOVA (Kruskal Wallis Test * P $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$. Abbreviations: ns, not significant.

Instead of the bulk approach performed in the previous experiments, next a clonal approach was chosen, identifying cytokine patterns of individual T cell clones after PMA/Iono stimulation. Therefore, single cells of two healthy donors were sorted into a 96-well plate containing irradiated feeder-cells. The single cells were sorted from a CD4 enriched bulk cell population and gated on the surface markers CD3, CD4 and CD45RO. A primary stimulation of the clones with PHA and consecutive feeding with IL-2 achieved clonal expansion of the cells. On day 28 of the clonal expansion, cells were stimulated with PMA/Iono for 6h and stained for IL-4, IL-13, IL-31, IL-10, IL-17 and IFN-γ, a panel of cytokines differentially expressed by the various distinct functional T cell subsets.

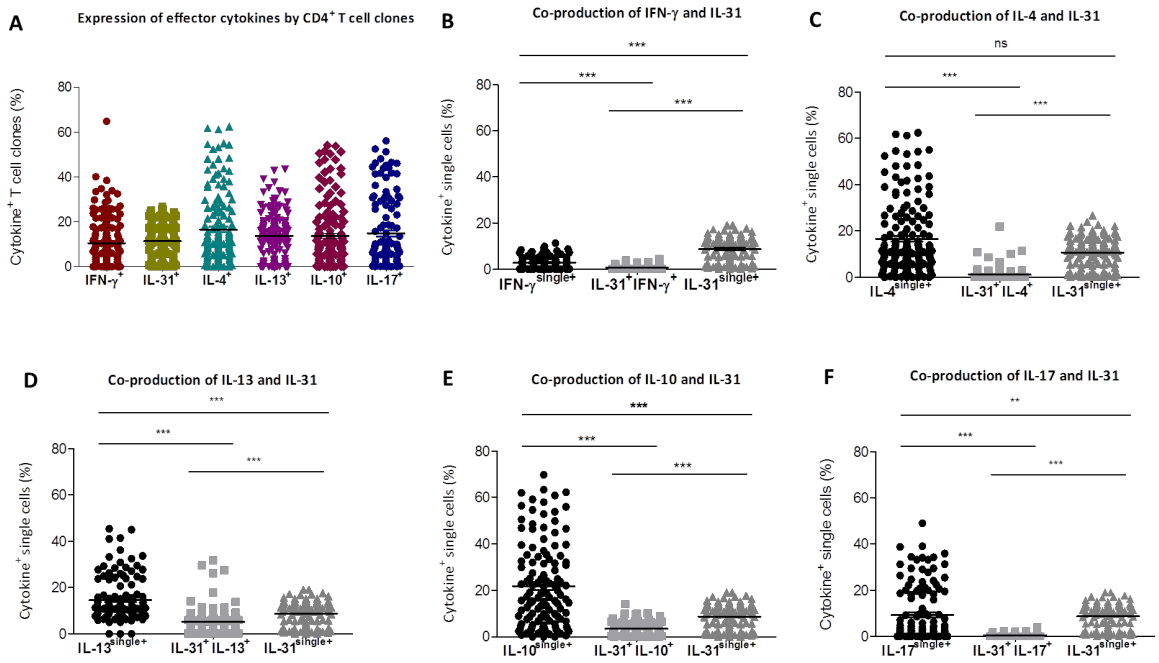

Figure 16: IL-31 producing T cell clones mark a distinct population compared to the double producing cell clones. (A) Single cell cloning of 160 single cells was performed utilizing whole blood derived PBMCs of two healthy donors. PBMCs were enriched for $CD4^+$ T cells and sorted for $CD3^+$ $CD4^+$ $CD45RO^+$ memory T cells, deposited into single wells of a 96-well plate containing feeder cells and clonally expanded with PHA and IL-2. On day 28 of the clonal expansion, clones were analyzed after PMA/Iono activation for the production of a cytokine panel consisting of IFN-γ, IL-17, IL-4, IL-13, IL-31 and IL-10. Cells were gated on living $CD4^+$ T cells. **(B-F)** Flow cytometric analysis of clonal T effector cell populations producing the respective cytokines and/or IL-31 reveals a markedly lower frequency of double-producers compared to single producers of the respective cytokines. Data are representative of independent experiments with 160 clones of 2 healthy donors and each dot represents the result of a single experiment while the mean value is marked by a horizontal line. 1-way ANOVA (Kruskal Wallis Test), $**P \leq 0.001$ $***P \leq 0.0001$. Abbreviations: n.s., not significant.

Figure 16A shows the total expression of the distinct effector cytokines (IFN-γ, IL-31, IL-4, IL-13, IL-10 and IL-17) produced by the 160 characterized T cell clones derived from two

different healthy donors with the highest frequencies of IL-4$^+$ clones (16.5 ± 1.2%), followed by IL-17-producers (14.9 ± 1.4%), IL-13$^+$ clones (13.7 ± 0.8%), IL-10-producers (13.7 ± 1.2%), and lowest for IFN-γ$^+$ clones (10.5 ± 0.9%). On clonal basis, IL-31 is expressed by 11.4% (± 0.7%) of the clones.

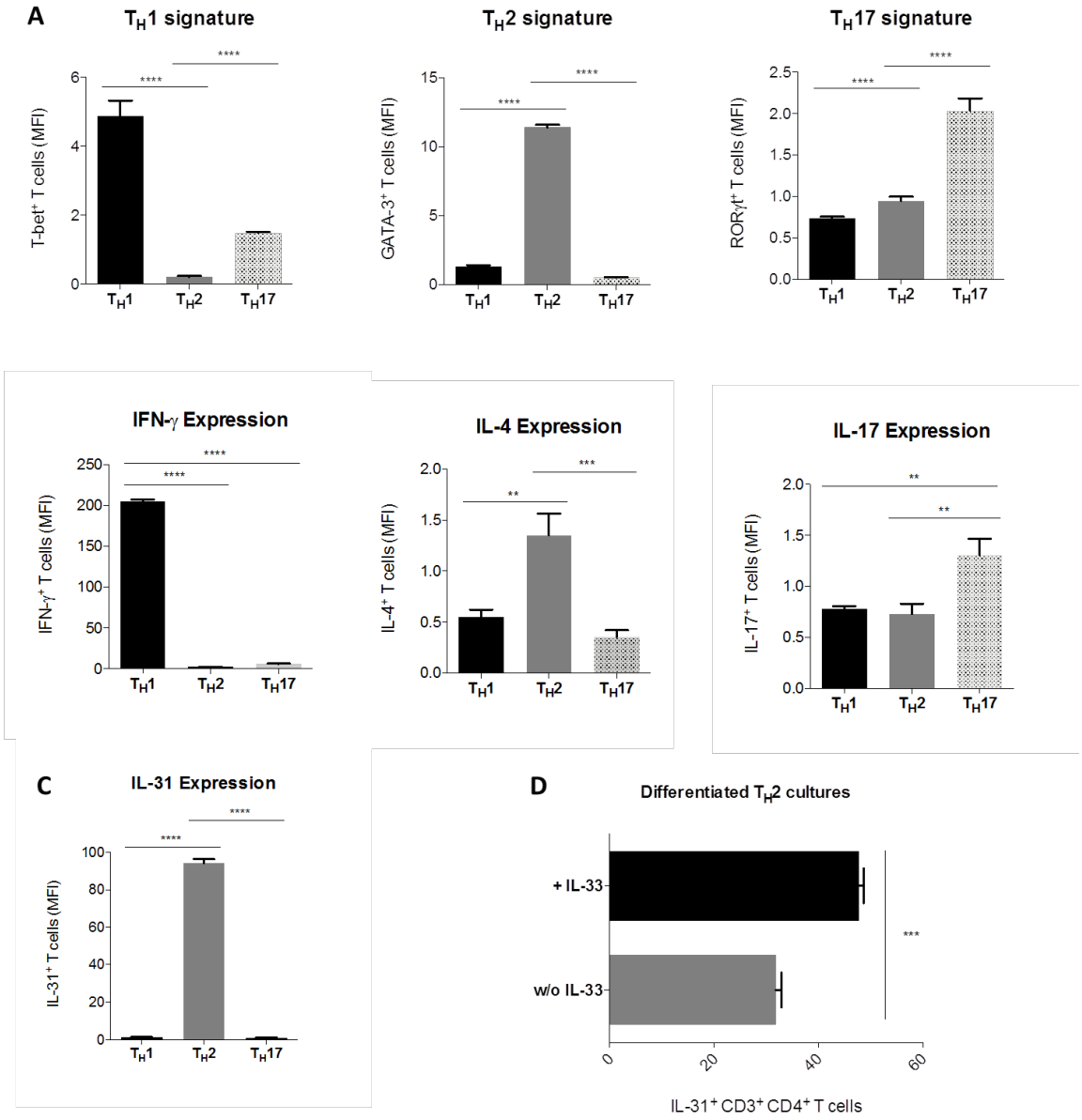

Figure 17: IL-31 is predominantly expressed by in vitro-generated T_H2 cells and increases upon stimulation with the alarmin IL-33. (A) The successful differentiation of the distinct T cell subsets is confirmed by the expression of the transcription factors, where T_H1 cells are governed by T-bet, T_H2 cells by GATA3 and T_H17 by RORγt. **(B)** The three typical T helper cell subsets T_H1, T_H2 and T_H17 are generated *in vitro* from enriched naïve T cells of healthy individuals and after 2 weeks of differentiation stimulated for 6h with PMA/Iono and analyzed for their cytokine profile (IFN-γ, IL-4 and IL-17). **(C)** Additionally to the classical subset characterizing cytokines, the three different cell cultures were analyzed for IL-31. Here, IL-31 is only produced by T_H2 polarized cells. **(D)** The T_H2 cultures are set up either with the alarmin IL-33 (black bar; 100 ng/mL) during the differentiation process or differentiated in the absence of IL-33 (grey bar). After 2 weeks of differentiation intracellular expression of IL-31 was determined. Data are representative of independent experiments of up to 6 healthy individuals and presented as mean ± SD: IL-33: 47.7 ± 1.6%; Ctrl: 31.8 ± 1.8% donors). Unpaired *t*-test, ****$P \leq 0.0001$. Abbreviations: MFI, mean fluorescence intensity.

However, IL-31 single-producers are represented with a frequency 10.6% (± 0.5%), while IFN-γ$^+$ IL-31$^+$-, IL-4$^+$ IL-31$^+$-, IL-13$^+$ IL-31$^+$-, IL-10$^+$ IL-31$^+$- and IL-10$^+$, IL-31$^+$- double-producing clones are almost absent or present with very low frequencies (Fig.16 B-F). The most dominant IL-31-double-producing T cell clones are the IL-13$^+$ IL-31$^+$ clones (5.2 ± 0.4%) as well as the IL-10$^+$ IL-31$^+$ clones (3.5 ± 0.2%).

Hence, findings of Fig. 15 and Fig. 16 might support the speculation of either a T_H31 subset or a unique transient IL-31-producing population within the T_H2 subset of T helper cells. Therefore, naïve T cells were enriched (see chapter 2.3.1) and differentiated towards T_H1, T_H2 and T_H17 cells (see chapter 2.6.1) and after 8 days of polarization and subsequent 6h stimulation with PMA/Iono, they were analyzed for distinct effector cytokine expression.

IL-31 production could only be detected in T_H2 polarized cells, which also expressed the master transcription factor GATA3 (Fig. 17A, B), clearly identifying IL-31-producing T cells as T_H2 polarized cells. There was no or only little IL-31 expression in T_H1 and T_H17 polarized cells expressing the transcription factors T-bet and RORγt, resopectively.

Further, the alarmin IL-33 has been implicated in the pathogenesis of atopic dermatitis. Therefore, it has been assessed whether it is involved in the up-regulation of IL-31 in T cells. T_H2 cultures were set up as described above and either substituted with 100 ng/mL IL-33 or cultured in its absence. Figure 17C demonstrates that the treatment of T_H2 cells with 100 ng/mL IL-33 during the differentiation process indeed increases the frequency of IL-31 producing cells (mean + SD: IL-33: 47.7 ± 1.6%; Ctrl.: 31.8 ± 1.8%).

3.6 Atopic dermatitis patients exhibit elevated levels of IL-31$^+$-expressing T_H2 cells

In humans, elevated levels of IL-31 in serum of patients with atopic dermatitis have been reported previously[44,73,106]. Interestingly, patients suffering from atopic dermatitis also show elevated serum levels of the classical T_H2 cytokines IL-4 and IL-13 in past studies[107]. However, the up to date published studies have utilized different techniques to determine the amount of IL-31 protein in serum or plasma, but the results were not consistent. Therefore, the question was raised if these results can be confirmed on cellular basis. Hence, the next aim was to investigate the frequency of the intracellular cytokine positive cells for IL-31, IL-4 and IL-13 in CD4$^+$ T cell population derived from

PBMCs gated on CD3$^+$ CD4$^+$ CD45RO$^+$ T cells of healthy volunteers (n=10) in comparison to PBMCs derived from AD patients (n=26) after a 6h stimulation with PMA/Iono. The frequency of IL-31 producing CD4$^+$ CD45RO$^+$ T cells was significantly elevated in AD patients (16.0 ± 2.9%) when compared to healthy subjects (6.5 ± 2.0%) (Fig. 18A).

The intracellular frequency of IL-4 was considerably lower in AD patients after one round of a 6h stimulation with PMA/Iono (3.4 ± 0.7%) when compared to healthy individuals (9.9 ± 2.6%) (Fig.17A). The frequencies of IL-13$^+$ T_H2 cells did not show differences between healthy volunteers (4.1 ± 0.6%) and AD patients (5.6 ± 0.9%). With regard to the co-production of IL-31 and either IL-4 or IL-13, frequencies were elevated for both co-producers (IL-4$^+$ IL-31$^+$: 2.4% ± 0.6% and IL-13$^+$ IL-31$^+$: 2.3 ± 0.7%) in patients with atopic dermatitis in comparison to healthy volunteers (IL-4$^+$ IL-31$^+$: 0.8 ± 0.2% and IL-13$^+$ IL-31$^+$: 0.7 ± 0.3%) (Fig. 18B).

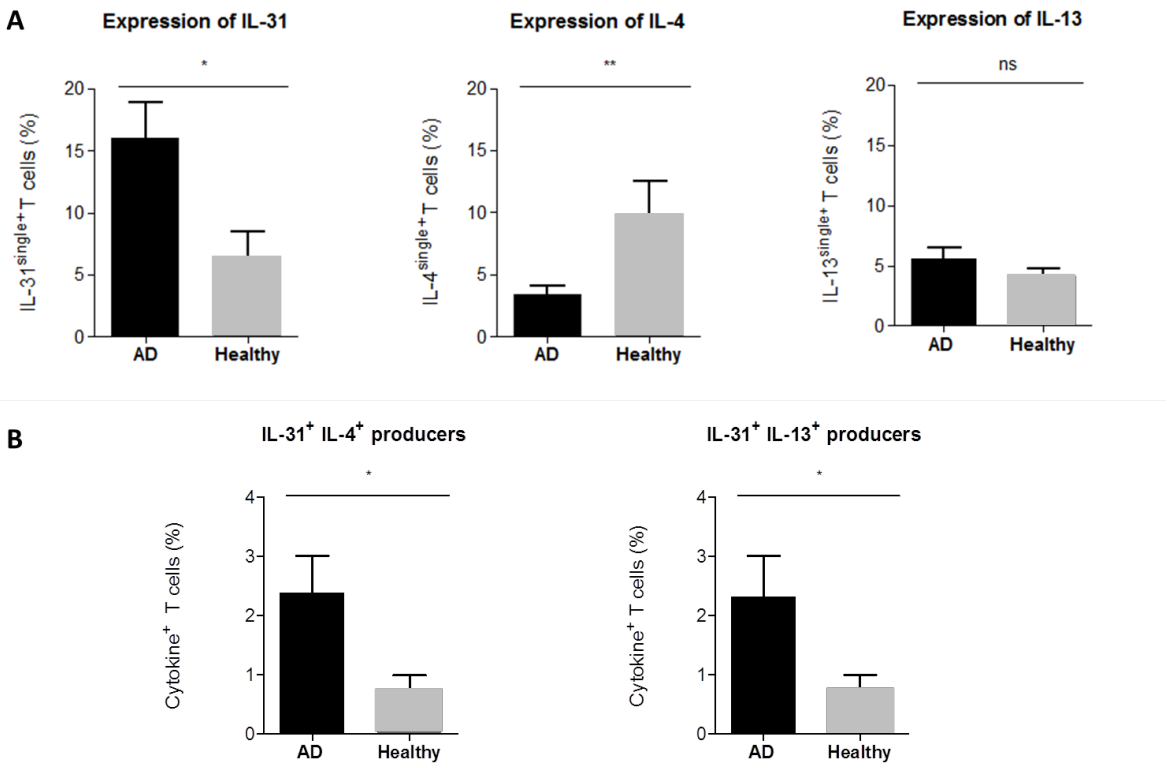

Figure 18: IL-31 frequency is markedly increased in patients with atopic dermatitis. (A) CD3$^+$ CD4$^+$ CD45RO$^+$ T cells from PBMCs of either healthy individuals (n=10) (grey columns) or AD patients (n=26) (black columns) were analyzed for their cytokine production profile (IL-31, IL-4 and IL-13) after stimulation with PMA/Iono for 6h. Populations were gated on live, CD3$^+$ CD4$^+$ CD45RO$^+$ T cells. **(B)** Frequencies of IL-4$^+$, IL-31$^+$ and IL-13$^+$ T cells in healthy individuals versus AD patients assessed as in (A). T cells producing both cytokines simultaneously are referred to as "double-producers". Data are representative of independent experiments with 10 healthy individuals and 26 AD patients and presented as mean ± SD. Unpaired *t*-test with Welch's correction, **P* ≤ 0.05, ***P* ≤ 0.001. Abbreviations: AD, atopic dermatitis; ns, not significant.

3.7 Altered expression of skin-homing associated receptors on IL-31$^+$ T cells in patients with atopic dermatitis

As IL-31$^+$ T cells show a distinct skin-homing phenotype (Fig. 13), and atopic dermatitis is accompanied by an elevation of skin-resident immune cells, the question was raised if AD patients show an altered skin-homing chemokine receptor repertoire within the IL-31 expressing T cells when compared to healthy individuals. To provide proof for this hypothesis, PBMCs of healthy volunteers (n=12) and AD patients (n=26) were assessed for co-expression of intracellular IL-31 and cell surface CLA, CCR4, CCR10 or CCR8.

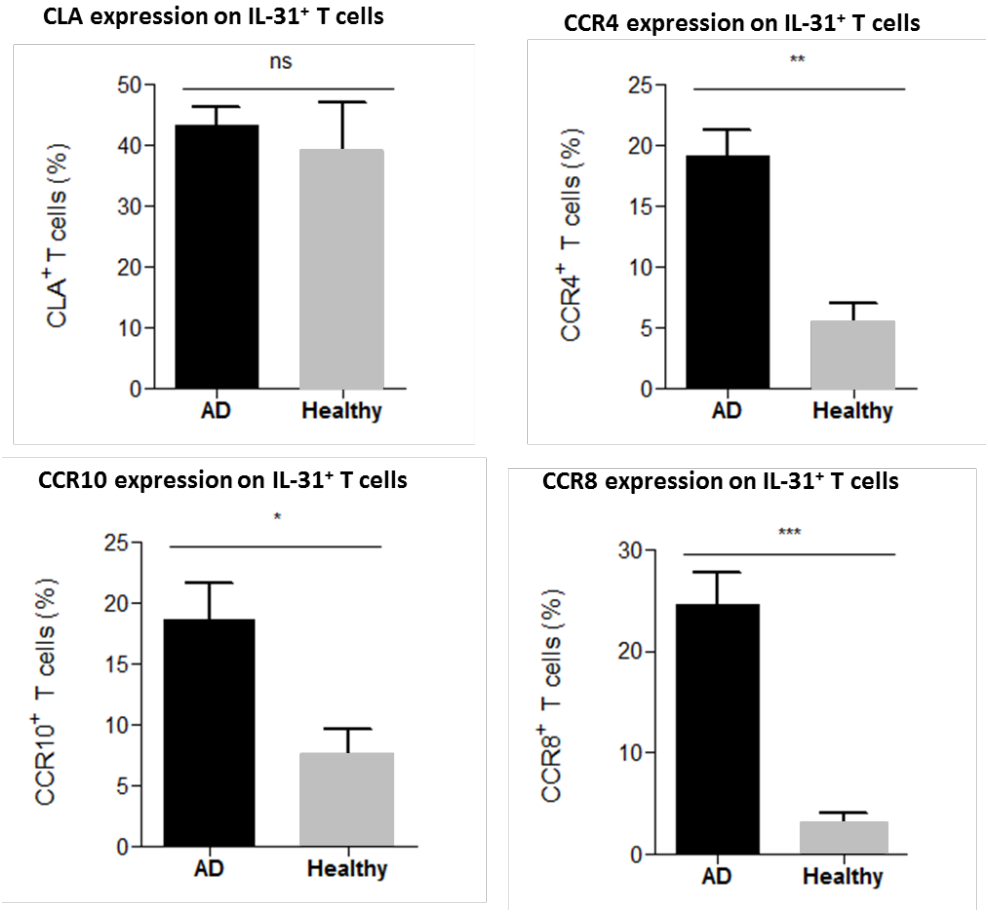

Figure 19: Skin-homing receptors are elevated on IL-31$^+$ CD4$^+$ memory T cells of AD patients. Flow cytometric analysis of PMA/Iono stimulated PBMCs gated on CD3$^+$, CD4$^+$, CD45RO$^+$ and IL-31$^+$ cells of AD patients (black bars) and healthy individuals (grey bars) show expression of the skin-homing associated receptors CLA, CCR4, CCR10 and CCR8 on IL-31$^+$ T cells. The frequencies of CCR4, CCR10 or CCR8 co-expressing cells are significantly higher for AD patients. Data are representative of independent experiments with 10 healthy individuals and 26 AD patients and presented as mean ± SD. The gating strategy is shown for healthy individuals in figure 13 and performed likewise for AD patients. Mann-Whitney-U Test, *$P \leq 0.05$, **$P \leq 0.001$, ***$P \leq 0.0001$. Abbreviations: AD, atopic dermatitis; CCR, CC chemokine receptors; ns, not significant.

Figure 19 shows the co-expression patterns of chemokine receptors CCR4, CCR8, CCR10 and CLA with IL-31 in healthy individuals vs. AD on $CD3^+$ $CD4^+$ $CD45RO^+$ T cells. With regard to CLA/IL-31 co-expression, frequencies were 43.3 ± 2.9% in AD and 39.0 ± 8.1% in healthy subjects indicating no aberrant expression of the skin-homing receptor CLA in IL-31^+ T cells of patients with AD compared to healthy individuals. However, IL-31^+ cells co-expressing CCR4, CCR10 or CCR8 were significantly elevated in AD patients (IL-31^+ $CCR4^+$: 19.1 ± 2.1%; IL-31^+ $CCR8^+$: 18.6 ± 3.0%; IL-31^+ $CCR10^+$: 24.6 ± 3.2%) compared to healthy individuals (IL-31^+ $CCR4^+$: 5.5 ± 1.5%; IL-31^+ $CCR8^+$: 7.7 ± 2.0%; IL-31^+ $CCR10^+$: 3.2 ± 0.9%).

3.8 Basophils express the heterodimeric IL-31 receptor IL-31RA and OSMRβ

While $CD4^+$ T cells, which belong to the adaptive immune system, secrete IL-31, expression of the cognate heterodimeric receptor IL-31RA/OSMRβ by cells of the innate immune system has not been fully elucidated. A basophilic innate immune cell population obtained from urticaria patients has been described to express IL-31RA/OSMRβ[86]. To test whether quiescent basophils express the IL-31RA/OSMRβ heterodimer, basophils from whole blood derived PBMCs of healthy volunteers (n=12) and AD patients (n=34) were analyzed by multicolor flow cytometry utilizing antibodies recognizing a surface marker typical for basophils, $CD123^+$, in combination with antibodies directed against IL-31RA or OSMRβ.

Expression of the OSMRβ and IL-31RA on this subset of basophils was found (Fig. 20). The frequency of IL-$31RA^+$ basophils with 1.8 ± 0.2% was 2-fold higher compared to cells expressing the OSMRβ subunit (0.8 ± 0.2%) in healthy and AD-patients, but no differences in the frequencies of IL-$31RA^+$ and $OSMR\beta^+$ basophils was detected (Fig. 21B).

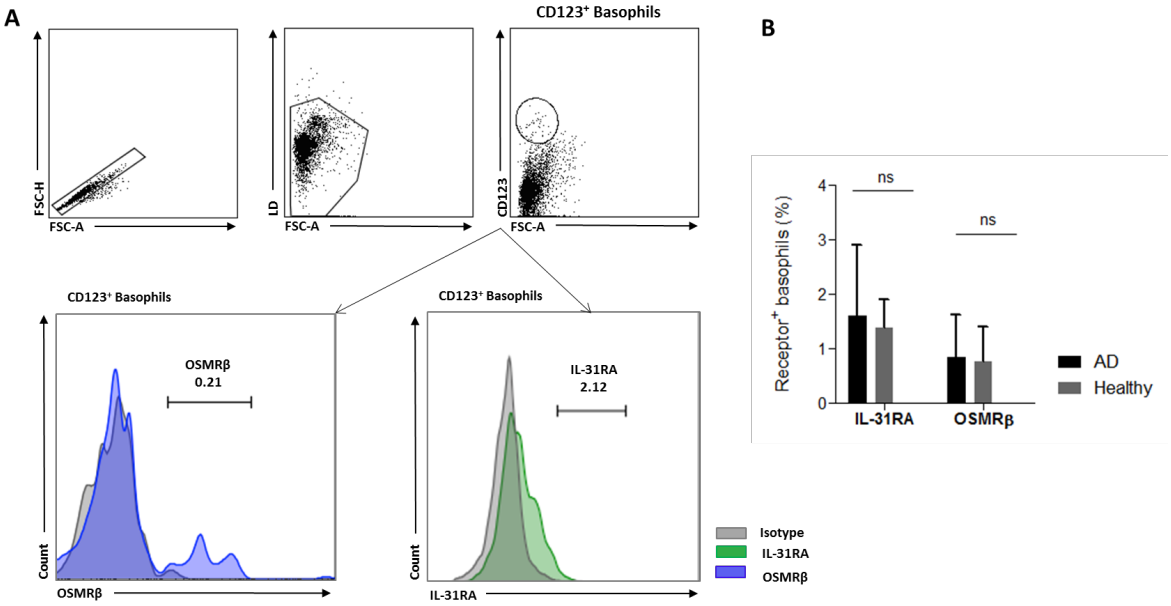

Figure 20: Basophilic innate immune cells express the heterodimeric surface receptors IL-31RA and OSMRβ in comparable frequencies in AD and healthy individuals. (A) Representative dot plots of PBMCs of healthy volunteers (n=12) pre-gated on living CD123$^+$ cells (stained with a cell viability dye). Within the CD123$^+$ cells, expression of IL-31RA (green curve) and OSMRβ (blue curve) was determined in comparison to their respective isotype controls (grey curves). **(B)** Frequencies of IL-31RA/OSMRβ expressing basophils of AD patients are comparable to those of healthy individuals. Gates and quadrants were set according to negative controls. Data are representative of independent experiments of 12 healthy individuals and presented as mean ± SD. Unpaired *t*-test with Welch's correction, Abbreviations: LD, live/dead; ns, not significant.

3.9 Basophils of AD patients show equal frequencies, but are higher activated based on ST2 expression

The role of basophils in the onset or perpetuation of AD in humans has not yet been elucidated in greater detail. However, since the IL-31/IL-31RA/OSMRβ axis has been described in the AD pathophysiology in mice[44,68] and is considered to be involved in the development of AD in humans, the aim was to assess a putative involvement of basophils in human AD, especially as both receptor subunits are expressed on quiescent basophils (Fig. 20).

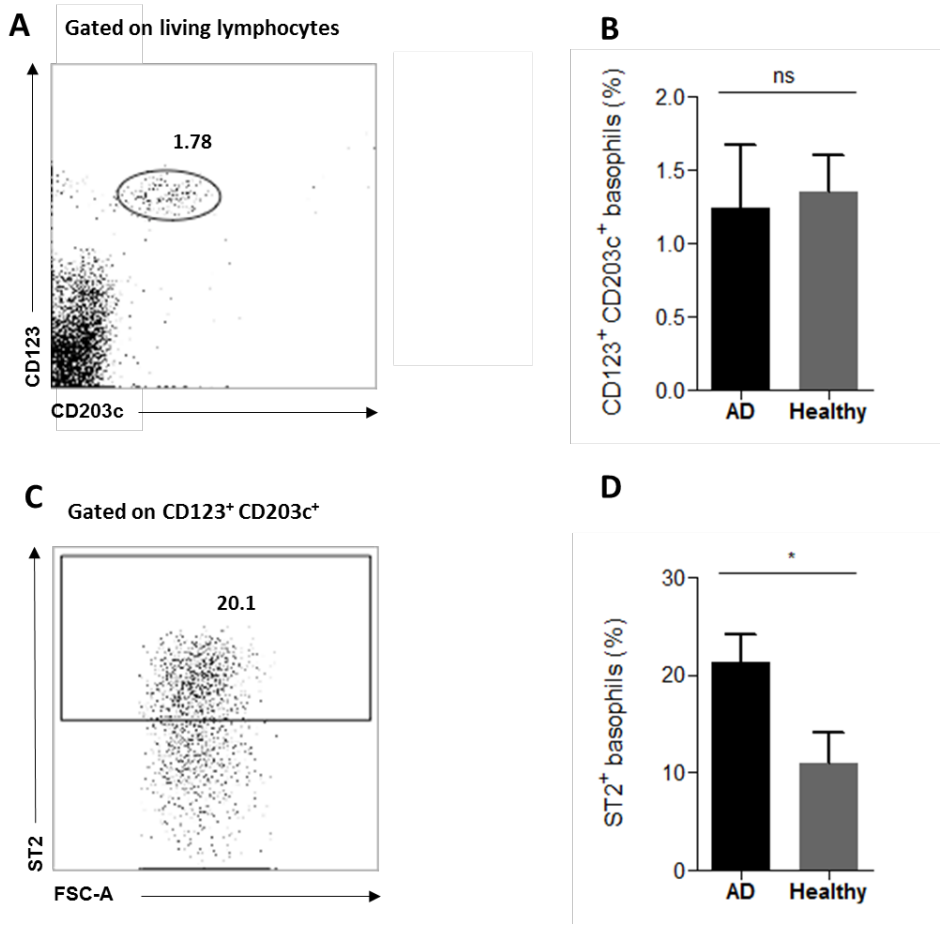

Figure 21: Basophils are equally distributed in AD patients and healthy individuals, but express higher frequencies of ST2. (A) Representative dot plot of CD123$^+$ CD203c$^+$ basophils identified in living PBMCs of AD patients. **(B)** PBMCs of AD patients (n=34) and healthy individuals (n=12) were gated on cells positive for the surface markers CD123 and CD203c. Frequencies of basophils are comparable between healthy individuals and AD patients. **(C)** Representative dot plot for ST2 expression on cells pre-gated on CD123$^+$ CD203c$^+$ basophils derived from peripheral blood of AD patients. **(D)** Expression of the activation marker ST2 (IL-33R) is significantly upregulated on CD123$^+$ CD203c$^+$ basophils of AD patients (n=34) in comparison to healthy individuals (n=12). Data are representative of individual experiments for 34 AD patients and 12 healthy individuals and presented as mean ± SD. Unpaired *t*-test with Welch's correction, $*P \leq 0.05$. Abbreviations: ns, not significant.

Basophil frequencies of AD patients (n=34) and healthy volunteers (n=12) were determined in PBMC by flow cytometry, identifying a subtype of basophils as CD123$^+$ and CD203c$^+$ cells, referred to as "activated basophils" in the subsequent study. The total frequency of activated CD123$^+$ CD203c$^+$ basophils did not differ between AD patients (1.2 ± 0.4%) and healthy individuals (1.4 ± 0.3%) (Fig. 21A, B).

However, assessing the activated subpopulations of basophils derived from these AD patients and healthy individuals pointed to an enhanced activation status of AD-derived basophils, as indicated by the elevated expression of the alarmin IL-33-targeting receptor IL-33R (T1/ST2 receptor) (AD: 21.3 ± 2.9%; Healthy: 11.0 ± 3.1%) (Fig. 21C, D).

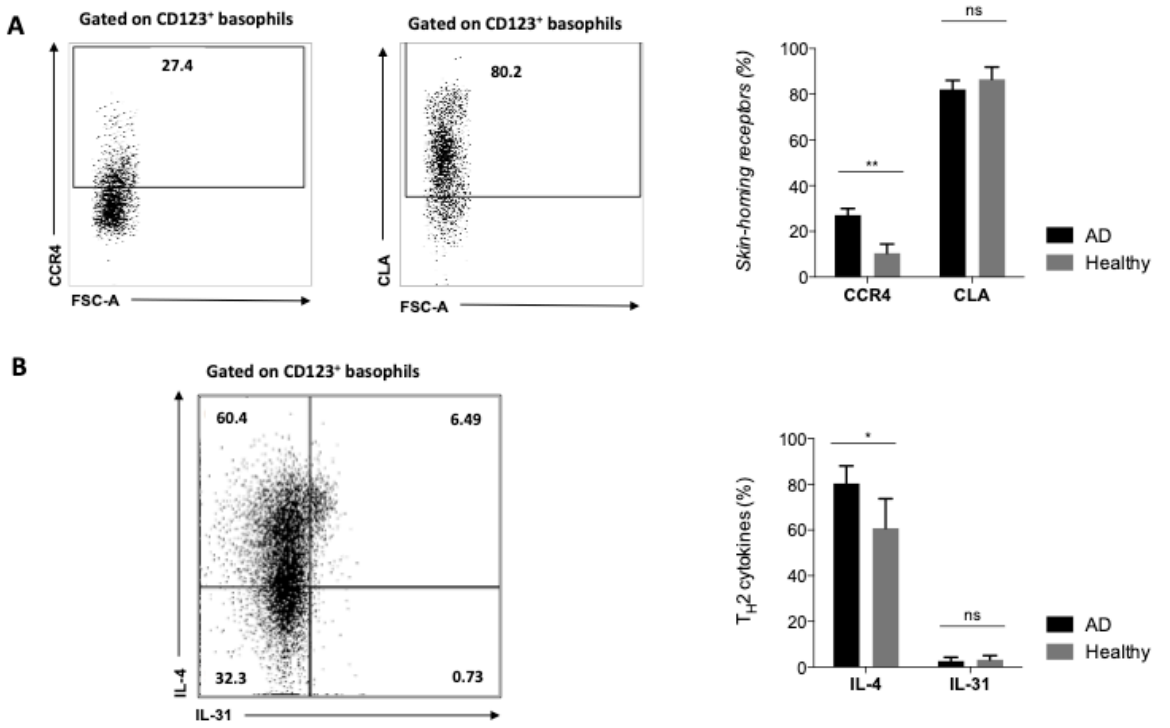

Figure 22: Elevated frequencies of basophils expressing the skin-homing receptor CCR4 and IL-4 in AD. (A) Representative dot plots of pre-gated $CD123^+$ basophils under exclusion of $CD11c^+$, $CD14^+$ and $CD16^+$ cells expressing the skin-homing receptor CCR4 show higher frequencies in AD patients (n=34) when compared to healthy individuals (n=10), but comparable frequencies of CLA^+ basophils. **(B)** Representative dot plot of pre-gated $CD123^+$ basophils expressing IL-4 and IL-31 show a higher expression of IL-4 in AD patients, but no differences in IL-31 expression. Gates and quadrants were set according to negative controls. Data are representative of individual experiments for 34 AD patients and 12 healthy individuals and presented as mean ± SD. Unpaired *t*-test with Welch's correction, $*P \leq 0.05$, $**P \leq 0.001$. Abbreviations: ns, not significant.

Further, basophils from healthy individuals as well as AD patients presented a pronounced, skin-homing potential with high frequencies of CLA^+ basophils (AD: 82.1 ± 4.0%; Healthy: 86.5 ± 5.3%) (Fig. 22A). However, $CCR4^+$ basophils were more abundant in AD patients (AD: 27.1 ± 2.9%) than in healthy individuals (11.8 ± 3.2%) (Fig. 22A).

Further, examination of cytokine expression by basophils from either cohort identified a higher abundance of IL-4 expressing basophils of AD patients (AD: 80.3 ± 4.5%; Healthy: 54.5 ± 2.9%; Fig. 22B). There was no difference in basophil IL-31 expression.

3.10 Stimulation with IL-31 does not affect the skin-homing potential of basophils, but improves survival and alters their activation status, IL-31 responsiveness and cytokine profile

Based on the here presented data indicating the expression of the IL-31 heterodimeric receptor IL-31RA/OSMRβ by resting ($CD203^-$) and activated ($CD203^+$) basophils, the aim

was to investigate the receptor's role in basophil function. Downstream signaling of IL-31RA/OSMRβ initiated by IL-31 has been reported to be involved in multiple cellular programs such as pruritus[45,68,74], inflammation[80], proliferation[79] and survival[108], in particular in tumor cells.

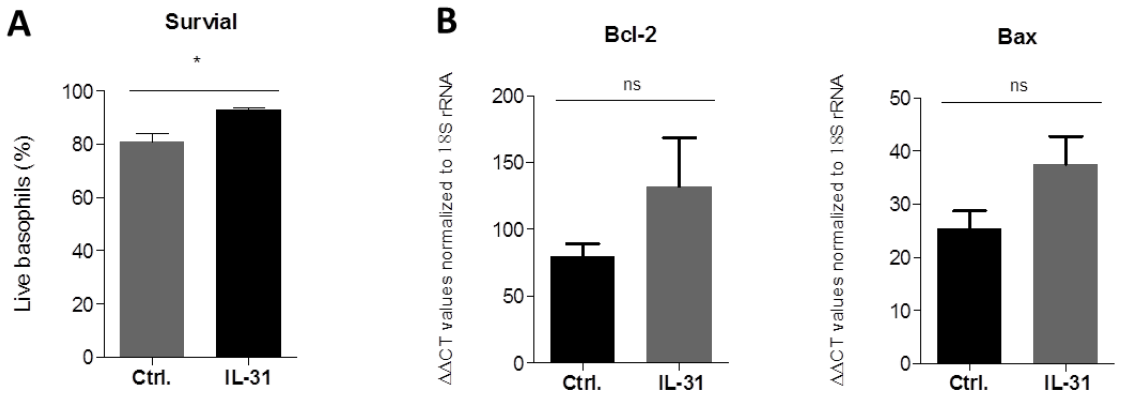

Figure 23: IL-31 treatment increases survival of basophils on protein level, but increased mRNA expression of Bcl-2 and Bax reaches no statistical significance. (A) Flow cytometric analysis of the survival rate of immunomagnetically enriched basophils from PBMCs treated with IL-3 (control; 100 ng/mL, black bars) or IL-31 (100 ng/mL, grey bars) for 24 h (n=6) shows higher frequencies of living basophils after IL-31 treatment (**Ctrl.:** 80.9 ± 1.0%; **IL-31:** 92.8 ± 3.1%). **(B)** Quantitative PCR analysis of gene expression levels of the survival factors Bcl-2 and Bax in human basophils following stimulation with IL-3 (control; 100 ng/mL, black bars) or IL-31 (100 ng/mL, grey bars) for 24 h (n=6). Data are shown as ΔΔCT values normalized to ribosomal 18S rRNA. Data is representative for 3 independent experiments with 2 healthy donors and presented as mean ± SD. Unpaired *t*-test, **P* ≤ 0.05. Abbreviations: ns, not significant.

In this study the impact of IL-31 on the regulation of mediators of survival such as Bcl-2 and Bax was further analyzed for *in vitro* cultured basophils. Basophils were immunomagnetically enriched from whole blood of healthy individuals following a two-step enrichment protocol. In a first step, all non-basophils were depleted and in the following step, $CD123^+$ cells were positively selected via MACS from the negative fraction (see chapter 2.3.2) Subsequently the isolated basophils (94-98% purity) were stimulated with either 100 ng/mL recombinant human (rh-) IL-31 or 100 ng (rh-) IL-3, serving as control.

When survival of basophils was compared after 24 h, this analysis showed a significantly higher survival rate of IL-31- compared to IL-3-treated basophils (Fig. 23A). Quantitative real-time PCR analysis revealed a trend towards increased mRNA levels of the survival markers Bcl-2 and Bax after IL-31 treatment (Fig. 23B).

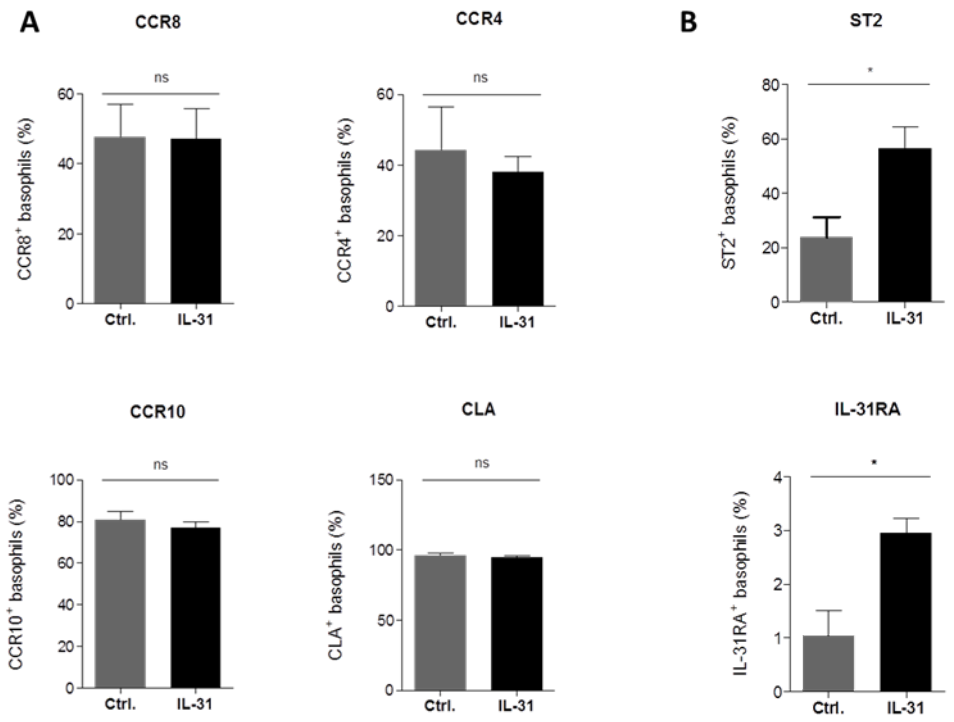

Figure 24: IL-31 signaling does not affect the skin-homing repertoire of basophils, but enhances their activation status and IL-31 responsiveness. (A) Frequencies of CCR8$^+$, CCR10$^+$, CCR4$^+$ or CLA$^+$ basophils were assessed by flow cytometry of CD123 enriched basophils, which were cultured with IL-31 (100 ng/mL) or with IL-3 (control; 100 ng/mL) for 24h. They show no differences in the skin-homing receptor expression**. (B**) Stimulation of basophils, which have been enriched and treated as described in (A), reveals increased frequencies of ST2 receptor$^+$ and IL-31RA$^+$ basophils compared to controls**.** Data are representative for 3 independent experiments with 2 healthy donors each. Unpaired *t*-test and Welch's correction, $*P \leq 0.0001$. Abbreviations: ns, not significant.

Based on the increased survival of IL-31 treated basophils and the tendency towards an upregulation of survival genes, the effect of IL-31 on additional functional relevant surface molecules of basophils (ST2 receptor and IL-31RA) as well as on the expression of skin-homing receptors (CCR4, CCR8, CCR10, CLA) was assessed (Fig. 24). Basophils, enriched from healthy individuals were stimulated with either IL-31 (100 ng/mL) or medium alone (control) for 24h. While the expression frequencies of the skin-homing associated chemokine receptors CCR8 (Ctrl.: 47.5 ± 9.5%; IL-31: 47.1 ± 8.8%), CCR4 (Ctrl.: 44.1 ± 12.5%; IL-31: 37.9 ± 4.6%), CCR10 (Ctrl.: 80.5 ± 4.4%; IL-31: 76.8 ± 3.2%) and the receptor CLA (Ctrl.: 96.2 ± 1.4%; IL-31: 94.8 ± 1.0%) was not altered by IL-31 stimulation (Fig. 24A), the activation marker ST2 (Ctrl.: 23.9 ± 7.4%; IL-31: 56.4 ± 8.1%) and the IL-31 receptor subunit IL-31RA (Ctrl.: 1.0 ± 0.5%; IL-31: 2.9 ± 0.3%) were up-regulated following IL-31 stimulation in basophils (Fig. 24B).

3.11 Basophils initiate T_H2 differentiation of naïve $CD4^+$ T cells independent of dendritic cells

Since basophils are producers of IL-4 and can serve as antigen presenting cells priming T cells towards a T_H2 phenotype[109], the aim was to examine the surface expression of HLA-DR, which is required for the proper function of antigen presenting cells, in untreated human basophils.

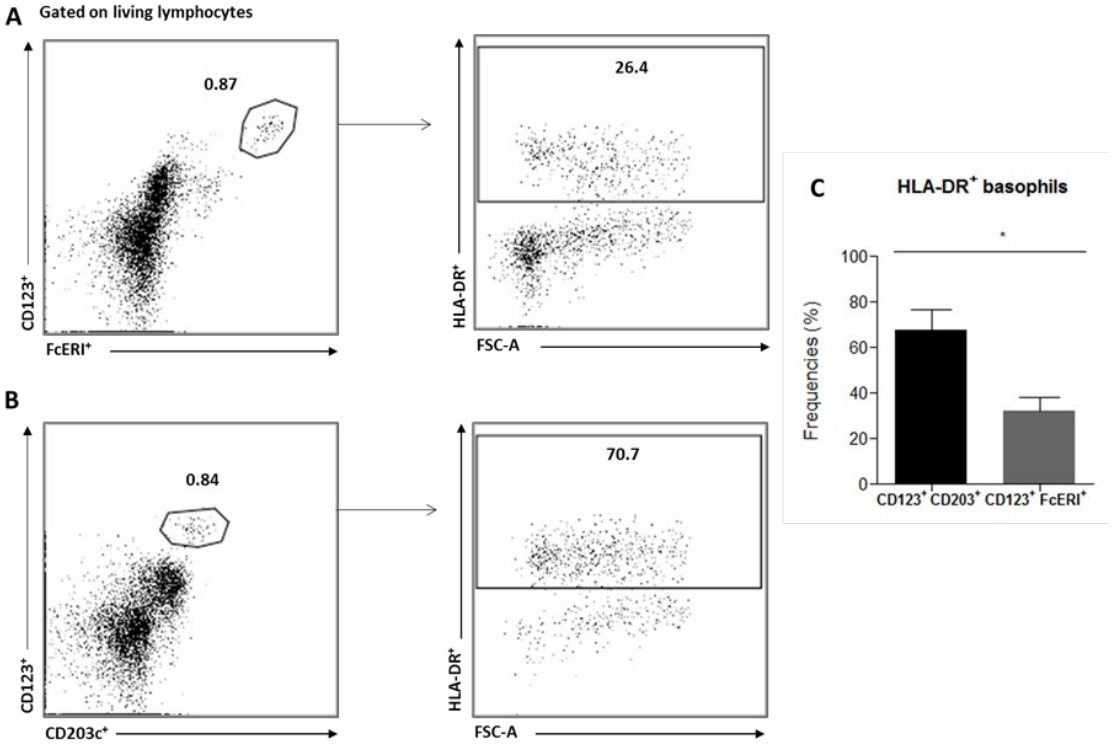

Figure 25: Activated basophils express higher frequencies of HLA-DR. Representative dot plots of HLA-DR expression on **(A)** quiescent ($CD123^+$ $Fc\varepsilon RI^+$) and **(B)** activated basophils ($CD123^+$ CD203c) of peripheral blood from healthy individuals gated on living leukocytes. **(C)** 30.9 ± 6.6% of $CD123^+$ $Fc\varepsilon RI^+$ basophils express HLA-DR, while 53.1 ± 11.8% of $CD123^+$ $CD203c^+$ basophils are $HLA\text{-}DR^+$. Data are representative for 24 healthy individuals. All values are presented as mean ± SD. Unpaired *t*-test and Welch's correction, $*P \leq 0.05$.

Activated $CD123^+$ $CD203c^+$ and quiescent $CD123^+$ $Fc\varepsilon RI^+$ basophils were identified in PBMCs of healthy volunteers (n=24) and expression of HLA-DR was determined by flow cytometry. While both basophil activation stages were identified in comparable frequencies with approx. 0.9 ± 0.2% of $CD123^+/CD203c^+$ and approx. 0.9 ± 0.3% of $CD123^+/Fc\varepsilon RI^+$ in the lymphocyte compartment of living cells within PBMCs, and both expressed HLA-DR molecules, 53.1 ± 11.8% of activated $CD123^+$ $CD203c^+$ basophils expressed HLA-DR molecules, while only 30.9 ± 6.6% of the quiescent ($CD123^+/Fc\varepsilon RI^+$) basophils were positive for HLA-DR (Fig. 25).

The discovery of HLA-DR molecules on the plasma membrane of basophils leads to the determination of the capacity of basophils to prime naïve T cells towards a T_H2 phenotype. Therefore, human $CD123^+$ basophils were enriched from whole blood to a purity of 92-96% (see Fig. 5) and co-cultured with immunomagnetically isolated (see Fig. 4) autologous naive $CD3^+$ $CD4^+$ $CD45RA^+$ T cells (97-99% purity) derived from healthy individuals in the presence of IL-3 for basophil survival, IL-2 and IL-33 (100 ng/mL) or without IL-33 for 8 days. Additionally, naïve T cells of the same donors were incubated with CD3/CD28-coated beads, which are replacing the antigen presenting cells and hence serve as control. Controls without basophils or CD3/CD28-coated beads were not included, as naïve T cells die within the first 2 days in absence of a TCR activating signal. On day 8, the co-cultures were harvested and stimulated with PMA/Iono (5 ng/mL each) for 6h or directly subjected to further analysis.

Analogous to previous observations of this study, the presence of IL-33 resulted in a higher production of IL-31 (6.0 $\pm$ 1.2%) (Fig. 26A, B), when naïve T cells are primed with basophils to initiate their activation compared to T cells primed with basophils in the absence of IL-33 (1.9 $\pm$ 0.1%) or with CD3/CD28 beads (0.8 $\pm$ 0.2%). Further, IL-33 stimulation of co-cultured $CD3^+$ $CD4^+$ $CD45RO^+$ T cells (upon differentiation, naïve T cells downregulate the surface marker CD45RA and upregulate CD45RO instead) presented a slight but robust up-regulation of IL-4 (5.0 $\pm$ 1.5%) expression and a markedly decrease in IL-13 producing T cells (7.2 $\pm$ 1.5%) when compared to co-cultures in the absence of IL-33 (IL-4: 2.1 $\pm$ 0.1; IL-13: 14.5 $\pm$ 0.5) and the CD3/CD28-activated T cells (IL-4: 0.9 $\pm$ 0.1; IL-13: 3.3 $\pm$ 0.2) (Fig. 26B). IL-33 did not alter the production of IFN-γ in co-cultured $CD3^+$ $CD4^+$ $CD45RO^+$ T cells, and none of the conditions resulted in a substantial IL-17A expression (Fig. 26B).

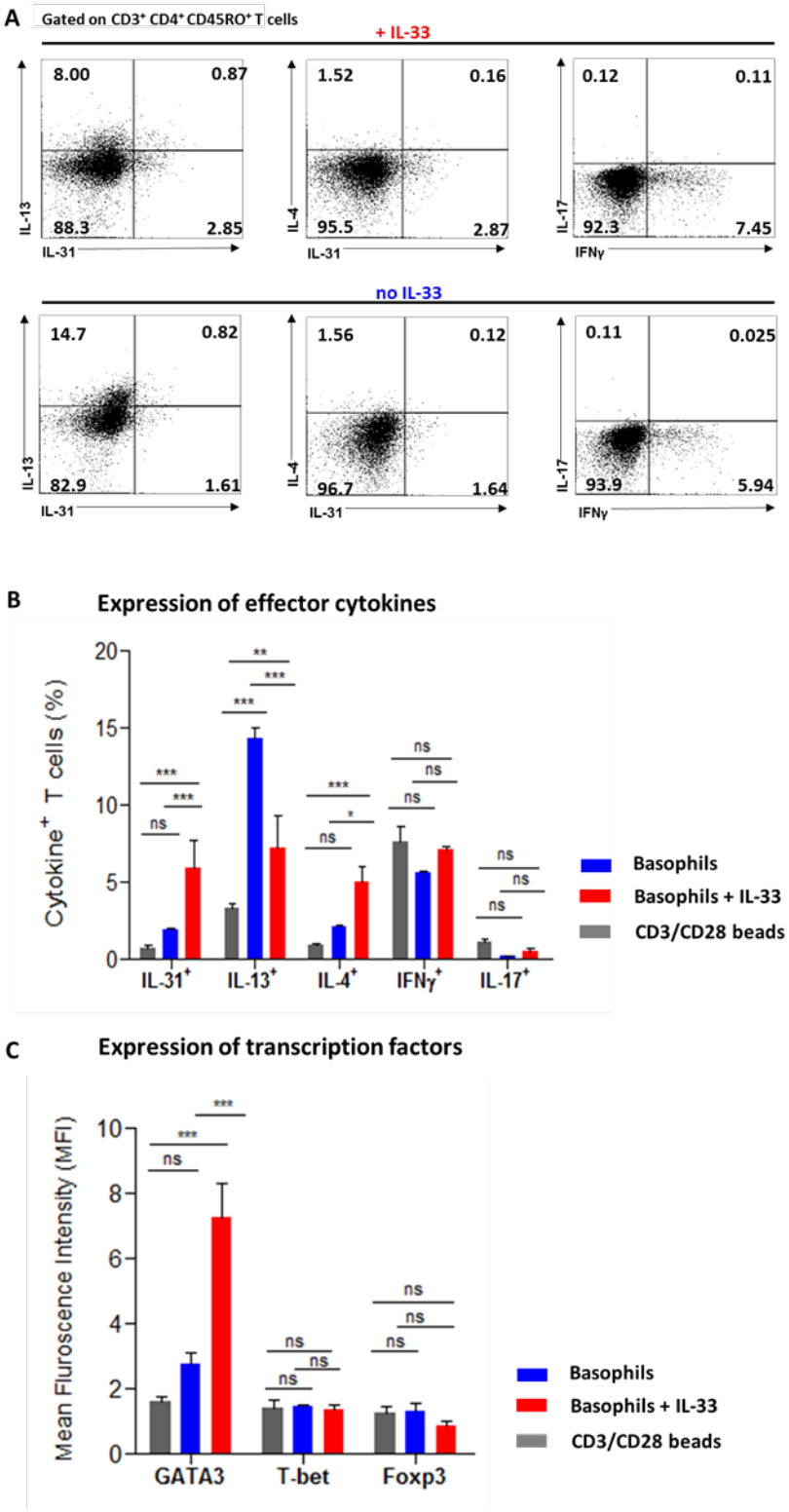

Figure 26: Co-culturing of naïve T cells with basophils drives T cells towards T_H2 differentiation *in vitro*. (A) Representative flow cytometric dot plots of differentiated $CD3^+$ $CD4^+$ $CD45RO^+$ T cells after 8 days of co-culturing with basophils, show production of IL-13 and IL-31 as well as IL-4 and IFN-γ in the presence of 100 ng/mL IL-33 (upper row) or absence of IL-33 (lower row). **(B)** Quantification of IL-31, IL-13, IL-4, IFN-γ and IL-17 production by $CD3^+$ $CD4^+$ $CD45RO^+$ T cells after stimulation by basophils with or without IL-33 or by CD3/CD28 beads (n=6). **(C)** Mean fluorescence intensities (MFI) of the transcription factors GATA3, T-bet and Foxp3 in $CD3^+$ $CD4^+$ $CD45RO^+$ T cells activated by basophils with or without IL-33 or CD3/CD28 beads (n=6). Data are representative for 2 independent experiments with 3 donors each and all values are presented as mean ± SD. 2-way ANOVA, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Abbreviations: ns, not significant.

When the expression of the polarization-associated transcription factors GATA3 (T_H2 subset), T-bet (T_H1 subset) and Foxp3 (T_{reg} cells) in the activated T cell populations was analyzed, the dominating transcription factor present in basophil-co-cultured $CD3^+$ $CD4^+$ CD45RO T cells without IL-33 was GATA3 (2.8 $\pm$ 0.2%), while T-bet (1.5 $\pm$ 0.1%) and Foxp3 (1.3 $\pm$ 0.2%) were almost absent. Further, T-bet and Foxp3 expression was not affected by IL-33 activation of basophil co-cultured $CD3^+$ $CD4^+$ $CD45RO^+$ T cells. However, the T_H2 subset associated transcription factor GATA3 was significantly increased (7.3 $\pm$ 0.8%) in co-cultured T cells following IL-33 stimulation (Fig. 26C).

3.12 Atopic dermatitis mimicking cytokine microenvironment in keratinocytes drives basophils to prime T cells towards a T_H2 phenotype, including IL-31 production

Acute and chronic inflammatory disorders often feature signature expression patterns of distinct cytokines. For instance, atopic dermatitis has been described to be dominated by an influx of T_H2 cells and a cutaneous inflammatory microenvironment predominantly composed of the cytokines IL-4 and IL-13. In psoriasis, the key cytokines are considered to be IL-17 and IL-23, and in a third group of acute as well as chronic inflammatory disorders the signature cytokine expression pattern is composed of TNFα and IL-1β. Up to now, the association of IL-31 to a disease state has been described for AD, urticaria and probably cutaneous T cell lymphoma (CTCL))[45,86,110]. To delineate the influence of the inflammatory cytokine environment on the secretome of normal human epidermal keratinocytes (NHEK) and subsequently on basophils, and basophil-elicited priming of T cells, in a next step the NHEK were stimulated with either IL-4, IL-13 (50 ng/mL, each), IL-17A (100 ng/mL) or a combination of TNFα and IL-1β (30 ng/mL, each) for 6h and 24h. Subsequently, supernatants of cytokine-treated NHEK were added to co-cultures of basophils and naïve T-cells.

Similar to chapter 3.12, human $CD123^+$ basophils were enriched from whole blood to a purity of 93-95% (see Fig. 5) and co-cultured with immunomagnetically isolated (see Fig. 4) autologous naive $CD3^+$ $CD4^+$ $CD45RA^+$ T cells (96-99% purity) derived from healthy individuals in the presence or absence of the NHEK-conditioned culture supernatants for 8 days. Additionally, naïve T cells of the same donors were incubated with CD3/CD28-coated beads, which are replacing the antigen presenting cells and hence serve as

control. Controls without basophils or CD3/CD28-coated beads were not included, as naïve T cells die within the first 2 days in absence of a TCR activating signal. On day 8, the co-cultures were harvested and stimulated with PMA/Iono (5 ng/mL each) for 6h or directly subjected to further analysis.

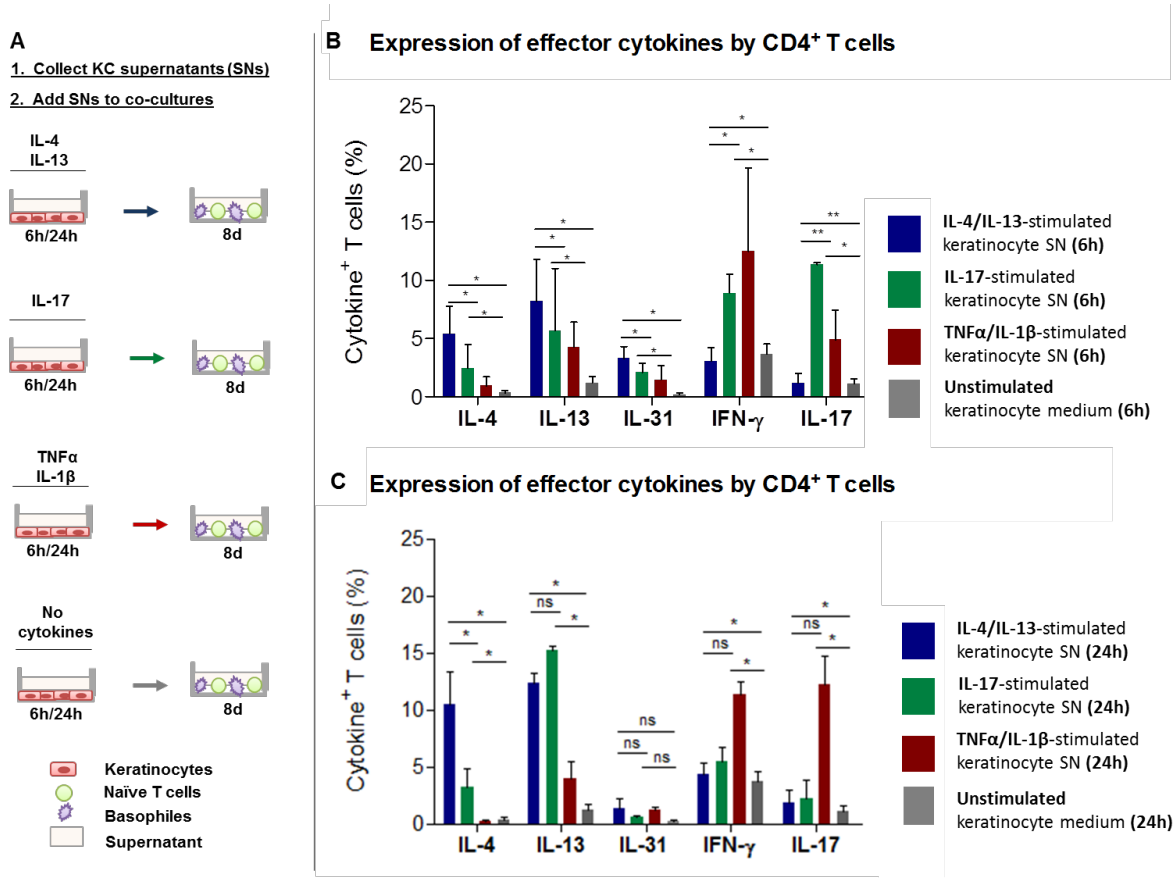

Figure 27: Expression of effector cytokines by basophil-primed CD4⁺ T cells dependent on keratinocyte-conditioned culture supernatants generated under different proinflammatory conditions. (A) Schema of the setup of the co-culture experiment with the stimulated keratinocyte SNs. Kerationcytes were either stimulated with IL-4/IL-13 (50 ng/mL, blue bars), IL-17 (100 ng/mL, green bars),TNFα/IL-1β (30 ng/mL, red bars) or keratinocyte have not been stimulated with cytokines (grey bar) for **(B)** 6 h or **(C)** 24h. **(B, C)** Expression of effector cytokines by basophil-primed CD4⁺ memory T cells in the presence of the different keratinocyte-conditioned supernatants (treated for either 6 or 24h) was determined by flow cytometry after 8 days of T cell stimulation. IL-4/IL-13 conditioning induces T_H2 cytokines (IL-4 and IL-13), whereas IL-17 conditioning initiates production of IL-17 and IFN-γ in basophil-primed T cells. TNFα/IL-1β conditioning induces a rather T_H1 and T_H17 dominated response, while IL-31 and IL-4 are nearly absent. **(**Results summarize data of 3 independent experiments, comprising 2 donors per group (n=6) and values are mean ± SD. 1-way ANOVA Test, **P* < 0.0001, ***P* < 0.0001, ****P* < 0.0001. Abbreviations: ns, not significant; SN: supernatants.

Figure 27 shows the frequencies of CD4⁺ memory T cells (upon differentiation over 8 days, T cells downregulate the surface marker CD45RA and upregulate CD45RO instead) expressing the effector cytokines (IL-4, IL-13, IL-17, IL-31, IFN-γ) associated with the respective T cell subsets T_H1, T_H2 and T_H17 by basophil-primed naïve T cells activated under the influence of supernatants derived from the stimulated keratinocytes. Basophil-

primed T cells presented the highest frequency of T cells expressing the T_H2 cytokines IL-4 (10.4 ± 2.9%), IL-13 (12.3 ± 0.9%), and IL-31 (1.4 ± 0.8%), when priming occurred in presence of IL-4 and IL-13-conditioned NHEK supernatant. However, expression of the T_H2 cytokines in the presence of either IL-17 conditioned NHEK supernatant or TNFα/IL-1β-conditioned NHEK supernatant was almost absent. IL-31 was produced by basophil-primed T cells irrespective of the cytokines used to stimulate the NHEK. The highest frequency of IL-31$^+$ cells was observed after short stimulation for 6h when NHEK had been treated with IL-4/IL-13 (3.3 ± 1.0%). Treatment of the NHEK for 24h showed a much higher frequency for the secretion of all T_H2 cytokines, in particular when the NHEK had been stimulated with IL-4/IL-13. Especially IL-17 secretion of the differentiated T cells was influenced the strongest by the addition of the supernatants, which were treated with TNFα/IL-1β between 6h and 24h (Fig. 27 B, C).

While IL-4 secretion from primed T cells was increased in the AD-like (IL-4/IL-13-treated keratinocyte supernatant) environment, IL-4 production was low in the psoriatic-like microenvironment (IL-17: 2.5 ± 2.0%) or below detection levels under TNFα/IL-1β pro-inflammatory conditions. IL-13 in turn was also highly expressed by basophil-primed T cells in presence of IL-17 conditioned keratinocyte supernatant. Basophil-primed, IL-31 secreting T cells were detected in low frequencies in the range of 1.2 ± 0.2% (TNFα/IL-1β-supernatants) to 1.4 ± 0.8% (IL-4/IL-13-supernatants), while IFN-γ and IL-17 frequencies increase dependent on the presence of the TNFα/IL-1β conditioning (Fig. 26B).

However, primed T cells derived from co-cultured basophils and naïve T cells in the presence of supernatants of TNFα/IL-1β-treated keratinocytes presented with a higher frequency of IFN-γ producing cells, thus a shift towards a T_H1 phenotype. IL-17-conditioned keratinocyte supernatants induced IL-17, thus a T_H17 signature, in primed T cells derived from co-cultured basophils and naïve T cells.

3.13 Basophil numbers are decreased in the absence of Il-31ra signaling in mice and present altered skin-homing potential and might stimulate less T_H2 cells

Although IL-31 signaling has been implicated in the prolonged survival of B cells in humans[108], its function in survival of basophils is not clear. The previous results of this study did show a survival benefit of IL-31 for basophils *in vitro*. To determine whether IL-31 signaling affects in basophil numbers *in vivo*, we compared frequencies of basophils in

whole blood and secondary lymphoid organs (SLOs) of wild type (WT) and *Il-31ra*$^{-/-}$ (knock out) mice. Overall, the frequencies of basophils were lower in the SLOs, including peripheral lymph nodes (pLNs), mesenteric lymph nodes (mLNs) and the spleen, of *Il-31ra*$^{-/-}$ mice as compared to basophil frequencies in WT mice (Fig. 28A). Frequencies of circulating basophils were similar in *Il-31ra*$^{-/-}$ and WT mice. These observations were confirmed, when absolute numbers of basophils were calculated (Fig. 28B).

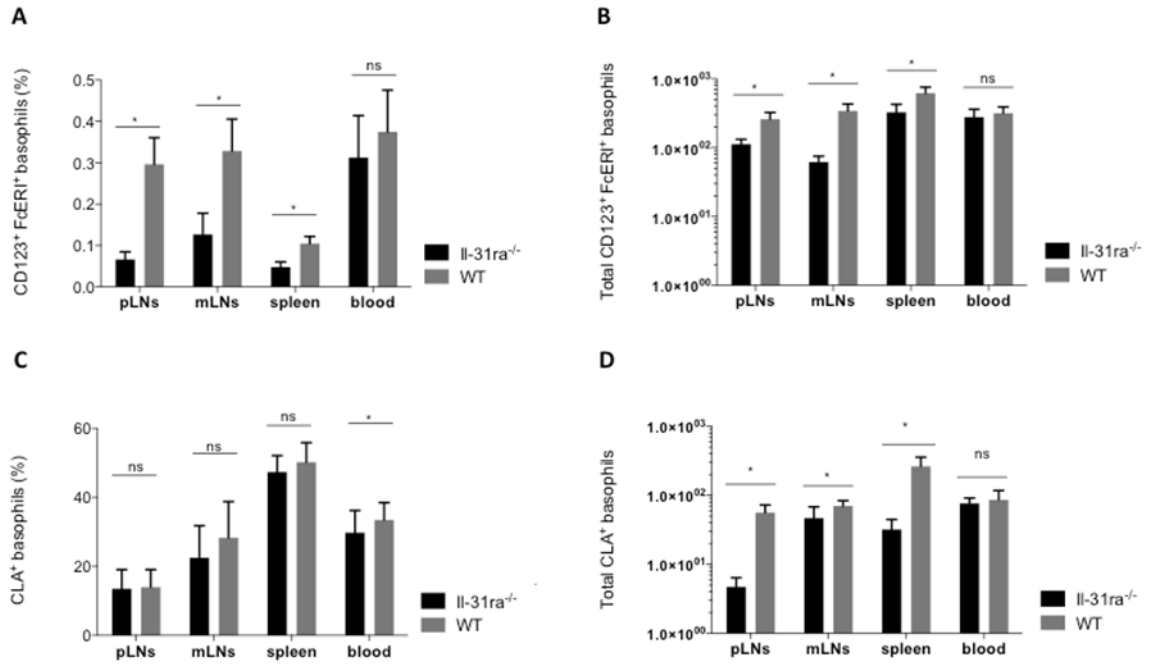

Figure 28: Basophil numbers are decreased in secondary lymphoid organs of *Il-31ra*$^{-/-}$ mice and display an altered skin-homing potential. (A) Frequencies of CD123^{+} FcεRI^{+} basophils in peripheral lymph nodes (pLNs), mesenteric lymph nodes (mLNs) and spleen are markedly reduced in *Il-31ra*$^{-/-}$ (black columns) when compared to WT mice (grey columns), with similar frequencies of basophils in peripheral blood. **(B)** Calculation of total numbers of basophils in SLOs and blood of *Il-31ra*$^{-/-}$ and WT mice shows significantly more basophils in the SLOs of WT mice, but not in peripheral blood. **(C)** Frequencies of CLA^{+} basophils in SLOs and peripheral blood of *Il-31ra*$^{-/-}$ and WT mice. **(D)** Calculation of total numbers of CLA^{+} basophils reveals a decrease of skin-homing basophils in the SLOs of *Il-31ra*$^{-/-}$, while absolute numbers of CLA^{+} basophils are unaffected in peripheral blood. Results are obtained from 2 independent experiments, comprising each 4 mice per genotype (n=8). 1-way ANOVA Test, **P* ≤0.05. Abbreviations: ns, not significant; WT, wildtype; SLOs, secondary lymphoid organs. All values are mean ± SD.

Since previous findings of this study revealed a distinct expression of skin-homing receptors by human basophils, the impact of Il-31 signaling on the skin-homing phenotype of murine basophils was of particular interest. Therefore, basophils from peripheral blood of *Il-31ra*$^{-/-}$ and WT mice were characterized by assessing the basophil's expression of CLA and CCR4. No marked differences in the frequencies and absolute numbers for CCR4^{+} basophils were observed in both mouse strains (data not shown). However, absolute numbers of CLA^{+} basophils (Fig. 28D), but not their frequency (Fig

28C), were decreased in the spleen, pLNs and mLNs of *Il-31ra*$^{-/-}$, whereas there was no difference in the blood of the animals.

Further, the potentially active role of human whole blood-derived basophils in the priming of CD4^{+} T cells towards a T_H2 phenotype, and the observation of decreased basophil frequencies in *Il-31ra*$^{-/-}$ mice suggested that this may cause a lower abundance for T_H2 cells and IL-31 production in the secondary lymphoid organs of *Il-31ra*$^{-/-}$ mice. Hence, in a next step CD4^{+} CD45^{high} effector T cells from pLNs, mLNs as well as spleens of *Il-31ra*$^{-/-}$ and WT mice were isolated and flow cytometrically assessed for their expression of GATA3 (T_H2 cells). Figure 29 shows the mean fluorescence intensities (MFIs) of the CD4^{+} T cell-expressed GATA3 in *Il-31ra*$^{-/-}$ and WT mice (Fig. 29A). The data show a significant decrease of GATA3-expressing T cells for Il-31ra$^{-/-}$ animals in pLNs (*Il-31ra*$^{-/-}$: 9.5 ± 0.2; WT: 12.3 ± 0.6), mLNs (*Il-31ra*$^{-/-}$: 13.1 ± 0.4; WT: 16.6 ± 0.1) as well as spleens (*Il-31ra*$^{-/-}$: 3.8 ± 1.9; WT: 15.0 ± 1.9). Despite of these differences in GATA3 expression, there was no difference detected in other lineage defining transcription factors, such as T-bet, Foxp3 and RORγt (data not shown).

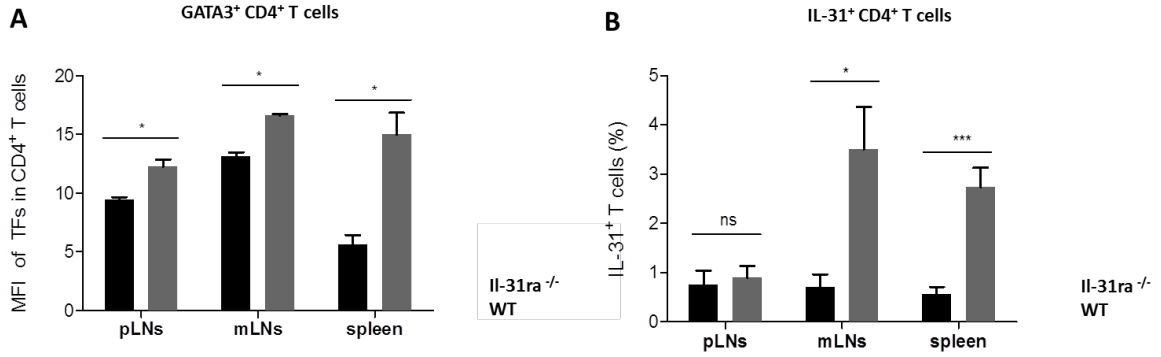

Figure 29: *Il-31ra*$^{-/-}$ mice show decreased GATA3-expressing T cells and diminished IL-31 production. (A) Flow cytometric analysis of the mean fluorescence intensity (MFI) of GATA3 in CD4^{+} CD45^{high} T cells in pLNs, mLNs and spleen of *Il-31ra*$^{-/-}$ compared to WT mice shows a decrease in GATA3 governed T cells in all peripheral organs of the *Il-31ra*$^{-/-}$ mice. **(B)** In *Il-31ra*$^{-/-}$ mice frequencies of IL-31^{+} CD4^{+} CD45^{high} T cells are markedly decreased in mLNs and spleen when compared to WT mice, but are not affected in pLNs. Results are obtained from 2 independent experiments, comprising 4 mice of each genotype (n=8). All values are mean ± SD. 1-way ANOVA Test, *$P \leq 0.05$, *++$P \leq 0.001$. Abbreviations: ns, not significant; WT, wildtype.; MFI, Mean Fluorescence Intensity.

In order to assess potential differences in IL-31 signaling in T cells, the expression of IL-31 by CD4^{+} CD45^{high} T cells in *Il-31ra*$^{-/-}$ and WT mice was analyzed. In mLNs (*Il-31ra*$^{-/-}$: 0.7 ± 0.3; WT: 3.5 ± 0.9) and spleens (*Il-31ra*$^{-/-}$: 0.6 ± 0.2; WT: 2.7 ± 0.4) of *Il-31ra*$^{-/-}$ mice a significant reduction in IL-31-expressing T cells was observed, whereas there was no difference in pLNs (*Il-31ra*$^{-/-}$: 0.8 ± 0.3; WT: 0.9 ± 0.2).

4. Discussion

Atopic dermatitis is a complex skin disorder, which finds its clinical manifestation as a result of an interplay of genetic alterations, barrier dysfunctions[78,111], and a dysregulated immune system[78]. Inflammatory responses in the skin arise from a breakdown of the normal skin barrier, microlesions and a response of diverse immune cell subtypes of the innate and adaptive immune system, resulting in chronic pruritus and skin inflammation. Pruritus, considered as the cardinal symptom of chronic AD, can be caused by a number of pruritogenic signals that originate from immune cells, epidermal cells or even neuronal cells[57,112].

One pruritogenic agent is the newly described IL-6 family member IL-31, which is predominantly produced by $CD4^+$ T cells and reported to serve as a neuro-immune link. This cytokine directly mediates pruritus by signaling through IL-31RA^+ DRGs neurons in mice and probably humans[68].

The importance of IL-31 as an “itchy cytokine” has increased over the past decade. Firstly, Dillon *et al*. described that lymphocyte-specific overexpression of IL-31 in the murine model resulted in a significant formation of an AD-like phenotype accompanied with severe pruritus in mice[44]. Further, subcutaneous injection of IL-31 induced Trpv1 and Trpa1-dependent itch in mice[68]. Thirdly, patients with AD exhibit elevated serum levels of IL-31 when compared to non-pruritic individuals, and IL-31 levels correlate with the patient’s SCORAD score[45] and with the expression of IL-4 and IL-13[113]. Fourthly, the expression of IL-31 and IL-31RA/OSMRβ was found to be up-regulated in the skin of AD patients[106]. Based on these and further findings, including IL-31-associated growth of small diameter neurons[74] and regulation of the release of pruritogens[114], the IL-31/IL-31RA axis is currently investigated for its deployment as a therapeutic target in AD.

To date, several approaches targeting IL-31/IL-31RA have been developed for therapeutic AD intervention, e.g. a humanized monoclonal anti-human IL-31 receptor antibody (Nemolizumab) blocks IL-31RA and is currently considered for phase III clinical trials[71]. Another attempt aims to block IL-31 directly, but so far this has only been successfully tested in dogs[115], while anti-human IL-31 antibody for patients with moderate to severe AD has been applied in a placebo controlled phase I trial, but the results are not published

as of today. Identifying the precise phenotype of IL-31-expressing T cells could help to target the dysbalanced IL-31/IL-31RA axis and AD more precisely and could be used to monitor the therapeutic efficacy. Hence, this study focused on the one hand on the identification and characterization of IL-31-producing T cells and on the other hand on the implications of this T cell subset for basophils that might be involved in AD development.

Several previous publications using different detection methods for IL-31, e.g. qPCR[45], ELISA[107,116], immunofluorescence staining[73] and intracellular IL-31 flow cytometry staining[77] identified $CD4^+$ T cells as major source of IL-31, but results varied. While most of these studies point towards a direct link of stimulated skin-homing $CD4^+$ T cells as main producers of IL-31, there has been no study so far, where CLA expression, CD4 expression and IL-31 expression where shown in one and the same sample. Therefore, an *ex vivo* intracellular staining method for the detection of IL-31 was established and was utilized to co-stain IL-31 with several other cytokines, surface markers, including chemokine receptors, as well as T cell polarization determining transcription factors for the first time. This technique allows the identification and a thorough and comprehensive characterization of IL-31^+ immune cells.

This study shows a predominant expression of IL-31 by $CD4^+$ T cells already after 3h of stimulation with PMA/Iono and a maximum after 5-6h (Fig. 10). IL-31 expression in $CD8^+$ cytotoxic T cells was low and it was absent in other blood leukocytes subsets (dendritic cells, neutrophils, monocytes/macrophages, B cells, NK cells). These results confirm previous reports describing $CD4^+$ T cells as the main source of IL-31[43,46] and a transient time-dependent induction of IL-31[47]. However, it is the first report directly linking IL-31 expression to the $CD4^+$ T cells using one method.

Naïve T cells express the surface marker CD45RA, which is replaced by CD45RO once T cells encounter the appropriate antigen, are activated and start to produce effector cytokines[2]. Further systematic characterization of the human IL-31^+ T cells identifies IL-31 as part of the effector cytokine family as it is produced by antigen-activated $CD45RO^+$ memory effector T cells (Fig. 11). These effector T cells can be further subdivided based on their expression of CD62L into central memory ($CD62L^+$) and effector memory ($CD62L^-$) subsets, which represent antigen experienced T cells that are mainly located as active effector memory cells in the circulation or reside within lymphoid organs as quiescent central memory cells[32]. This study shows, that IL-31-producing T cells are mainly $CD62L^-$

and are therefore of the effector memory T cell subtype (Fig. 12), which has also been implicated in a previous report, in which IL-31 has been associated with circulating allergen-specific, i.e. previously activated, effector T cells[76].

Although circulating in the periphery, effector T cells express receptors, which can guide them to their target tissue. CLA is a skin-homing receptor contributing to effector T cells migrating to the skin[43]. Indeed, it has been reported to be expressed on IL-31$^+$ immune cells suggesting a skin-homing potential[76,82]. The here presented data confirm that IL-31$^+$ T cells strongly express the skin-homing associated receptor CLA (Fig. 13). However, the understanding of the skin-homing process has advanced adding e.g. the chemokine receptor CCR10 to the list of potent skin-homing receptors[117], in addition to CCR4 and CCR8 as recently summarized by Mueller *et al*.[105]. The present study demonstrates for the first time a comprehensive chemokine receptor repertoire for IL-31$^+$ T cells, which do not express only CLA, but all known skin-homing chemokine receptors (Fig. 13), in the absence of other organ-homing receptors, such as the small intestine-homing receptor CCR9 (data not shown).

Interestingly, all skin-homing chemokine receptors are co-expressed with CLA, which marks also the most dominant skin associated surface molecule. In fact, around 16% of the IL-31-producing T cells are CLA$^+$ CCR4$^+$, 10% CLA$^+$ CCR10$^+$ and around 4% co-express CLA with CCR8 (Fig. 13). However, the three distinct chemokine receptors are not necessarily co-expressed, which is also implied by the different frequencies. While CCR4 shows the highest frequency on IL-31$^+$ T cells, only a small proportion of these cells also expresses CCR8 (3.9 ± 0.9%). Hence, there are IL-31$^+$ cells, which might be CLA$^+$ CCR4$^+$ CCR10$^+$ CCR8$^+$, but also IL-31-expressing T cells, which express CLA and CCR4 only. Whether a different combination of these skin-homing receptors has an effect on the function or distribution of these cells needs further research.

Nevertheless, these observations implies that the T cell-expressed IL-31 most probably functions as a cytokine acting mainly on skin, thereby opposing several other reports describing a functional role for T cell-derived IL-31 in all mucosal sites of the body, including the lung and the gut[3,118,119]. However, although IL-31-expressing T cells co-express all skin-homing receptors, this is only true for about 40% (CLA) or 3 - 16% (CCR4, CCR8 and CCR10) of these cells. Therefore, on subsets of IL-31-producing T cells other homing receptors may be expressed, guiding the T cells to different tissues. There is

further evidence that IL-31 participates in the development of atopic diseases besides atopic dermatitis, including asthma[75] and IL-31$^+$ T cells express relatively high levels of CRTH2 (Fig. 14), which has been described as a potent lung-homing factor. Morevoer, previous studies demonstrated that CRTH2 is involved in asthmatic T_H2 reactions[16,25].

Compared to healthy individuals, expression of the skin-homing chemokine receptors CCR4, CCR8 and CCR10 on IL-31$^+$ T cells was elevated in patients with AD (Fig. 19). In-line with a prominent role of IL-31$^+$ T cells in AD, elevated levels of the respective chemokine ligands, such as CCL27 and CCL17, are secreted by activated keratinocytes and have been reported in the skin of AD patients[44]. This leads to the speculation that the secretion of CCL27 and CCL17 initiates the homing of the IL-31$^+$ T cell subset to the skin. Further research is needed to substantiate this hypothesis.

Analyses of the concomitant profiles of the IL-31-producing T cells connects this study with previous reports, classifying IL-31-producing T cells as T_H2 cells, which produce the effector cytokines IL-4 and IL-13[45,46]. Both cytokines have also been detected in elevated levels in patients suffering from atopic dermatitis. Consequently, therapeutic agents for atopic inflammation have been developed targeting these two T_H2-specific cytokines. Dupilumab, a novel monoclonal antibody binding the alpha subunit of the interleukin-4 receptor (IL-4Rα), thereby blocking the IL-4/IL-13 signaling pathways, was successfully tested in phase 3 clinical trials with patients suffering from moderate to severe AD resulting in a rapid and robust improvement of pruritus and skin lesions[120]. Importantly, despite of the efficacy of dupilumab, its targets IL-4 and IL-13 do not serve as direct neuro-immune mediators with regard to pruritus elicitation[70]. However, IL-31, another T_H2 cytokine, and is induced by IL-4 signaling directly[45,46], and acts on IL-31RA-expressing small diameter DRG neurons, thereby evoking pruritus and neuronal sprouting[68,74]. Hence, dubilumab might have an indirect effect on pruritus by subsequently decreasing the IL-31 expression. Therefore, a more precise delineation of the T effector cells producing IL-31 was warranted.

The detection of CRTH2, a chemoattractant receptor expressed exclusively on T_H2 cells[16] on IL-31-producing T cells (Fig. 14) supported the concept of a T_H2-association. However, the comprehensive characterization of human IL-31-producing T cells in bulk cultures (Fig. 15) as well as on a clonal level (Fig. 16) revealed only a low frequency of IL-4/IL-31 or IL-13/IL-31 co-producing CD4$^+$ T cells, whereas the single producers of these cytokines were

more abundant. In contrast, there was no co-expression with the classical T_H1 (IFN-γ) or T_H17 (IL-17) effector cytokines. The co-producers may represent classical T_H2 cells, however, there appears to be as well a unique sub-population of IL-31-expressing T cells, a bona fide T_H31 T cell, in the absence of other lineage-defining cytokines. Only when specific polarization cultures were used, the T_H2 nature of these IL-31$^+$ cells could clearly be documented by their expression of the T_H2 lineage-defining transcription factor GATA3 (Fig. 17). This rather implies that there may be a unique subset of IL-31$^+$ T_H2 cells. Whether this represents a transient phenomenon remains to be determined.

In AD patients, the IL-31$^+$ T cells are more abundant compared to healthy volunteers (Fig. 18) and expression of the skin-homing chemokine receptors CCR4, CCR8 and CCR10 is increased (Fig. 19). Whether there are further differences is currently unclear, but a more detailed analysis of e.g. TCR specificities or on the regulation of cytokine single-positive versus the double-positive states of T_H2 cells, is needed. So far, technical difficulties prevented the flowcytometric sorting of IL-31$^+$ cells for further analysis, particularly molecular analyses, because the intracellular IL-31 staining results in RNA degradation (data not shown). However, the unique chemokine receptor profile of these cells identified in this study, irrespective of whether derived from healthy individuals or patients, provides a tool for flow cytometric tracking as well as enrichment of these cells for further analysis.

Following reports on the association of IL-31 with pruritus, Hawro *et al*. have described a rather delayed induction of itch upon subcutaneous challenge with IL-31 in a skin prick test[55], suggesting an indirect effect of IL-31 in pruritus onset and maybe AD perpetuation. Such an indirect effect of IL-31 argues for an interaction of IL-31-producing T cells with other cells in the epidermal microenvironment, e.g., IL-31RA$^+$ cells of the innate immune system like basophils, or the need for additional co-factors, for it to exert its pruritic function. For instance, basophils are localized in the circulation and may migrate to tissues, secrete the classical T_H2 cytokine IL-4 upon activation and are implicated in atopic and allergic inflammation[121–123]. Recent evidence linked basophils to the onset of pruritus in spontaneous chronic urticaria, and basophils have been found to express the subunits of the heterodimeric IL-31 receptor IL-31RA/OSMRβ[86]. Moreover, IL-31 serum levels, which are elevated in spontaneous chronic urticaria patients, decrease in response to treatment with omalizumab, a monoclonal antibody directed against IgE, thereby

targeting also basophils[55]. Although results on IL-31 serum or plasma levels have to be regarded with caution, as stated before, basophils appear to contribute together with IL-31 to inflammatory disorders such as AD and asthma.

Here, we could confirm that total (quiescents and activated) $CD123^+$ basophils derived from healthy volunteers express IL-31RA and OSMRβ, with IL-31RA expression exceeding OSMRβ expression in healthy as well as AD patients (Fig. 20). Interestingly, patients with AD often present with elevated IL-3 serum levels[124], the cytokine ligand of CD123, which is responsible for peripheral maintenance and replenishment of basophils from bone marrow, supporting the hypothesis of an involvement of basophils in atopic dermatitis. Increased IL-3 serum levels could trigger maturation of basophils in the bone marrow and their release into the circulation[87,125]. However, when analyzing basophils in the circulation of AD patients in comparison to healthy individuals, no differences in frequencies were observed of activated $CD123^+$ $CD203c^+$ basophils (Fig. 21A; B), but AD patient-derived activated basophils showed an enhanced expression of the ST2 receptor (IL-33 receptor), indicating an even more pronounced activation state (Fig. 21C, D). Hence, basophils may not differ in numbers, but due to their more activated state rather in their responsiveness to incoming danger signals such as the alarmin IL-33 during atopic skin inflammation. Indeed, serum-levels of the alarmin IL-33 in atopic dermatitis patients are increased as well, and it has been shown to activate basophils, triggering the release of IL-31[126,127]. This activation loop of basophilic IL-31 secretion further supports the relevance of the IL-31/IL-31RA signaling in the pathophysiology of AD, although differences in the frequencies of basophils expressing IL-31 (Fig. 22B) or its receptor (Fig. 20B) have not been observed in healthy individuals and AD patients.

Basophils of AD patients also show a higher expression of IL-4, another indication of their higher activation state (Fig. 22B). This may be a result of to the increased ST2 expression on basophils of AD patients observed here (Fig. 21D) and the higher level of its ligand IL-33 in AD patients[48,127], because in a previous report it has been shown that IL-33 induces IL-4 in basophils[128]. Moreover, TSLP has been documented to be responsible for the up-regulation of the ST2 receptor[128]. TSLP has been found to be elevated in AD patients and may originate from FcεRI stimulation by binding of IgE-complexes, which are abundant in extrinsic AD, but not in the intrinsic sub-form[53,117]. Thus, there is evidence for a unique regulatory circuitry involving activated basophils and their expression of IL-31, IL-33 and

their receptors as well as IL-4, which supports an important role of basophil function in AD. Nevertheless, the delineation of the precise regulation of this circuitry and its role in AD is in need of future investigations.

In healthy individuals basophils in the skin are scarce, however, the presence of basophil infiltrates during allergic skin reactions have been described, initially based on Giemsa staining and identification of basophils by their characteristic granules[121,129]. Meanwhile, analyzing tools for the detection of tissue-resident basophils have evolved, and basophil infiltrates have been identified by antibody staining, utilizing either anti-2D7, a cytoplasmic protein, or anti-basogranulin (BB1) antibodies in various inflammatory skin diseases, including atopic dermatitis[130,131]. Thus, basophils selectively appear to migrate to the skin in AD, but to date, little is known about how the circulating basophils enter the skin and analyses of their skin-homing chemokine receptors have not been conducted.

Here, the expression of the skin-homing chemokine receptors CLA and CCR4 was shown for basophils (Fig. 22A). Moreover, a higher frequency of CCR4 expressing basophils could be documented for AD patients, suggesting a potential recruitment of circulating basophils to the site of acute skin inflammation, where the respective CCR4 ligands, CCL22 and CCL17, are produced. In line with this hypothesis, elevated levels of CCL22 and CCL17 have been reported in the skin of AD patients[44]. Considering that CCR10 and CCR8 frequencies on basophils are even higher than CCR4, this argues for a pronounced skin-homing potential of basophils in general. However, $CCR8^+$ and $CCR10^+$ basophils are not significantly elevated in AD patients in comparison to healthy volunteers. Despite that, expression of skin-homing receptors on basophils has not been reported before and their role in atopic skin diseases and the connection to their respective ligands (CCL1 for CCR8 and CCL27) needs further elucidation.

The expression of IL-31RA by basophils has previously been reported[86], but its implication for AD pathophysiology has not been investigated. Therefore, human basophils were isolated as $CD123^+$ cells from peripheral blood of healthy volunteers and stimulated with IL-31. Of note, in peripheral blood, besides basophils, plasmacytoid dendritic cells (pDC) express CD123[132,133]. To prevent co-enrichment of pDC and basophils, cells were depleted of all non-basophils (including $CD4^+$ pDC) expressing the surface markers CD1a, CD3, CD4, CD8, CD14, CD15, CD16, CD11c, CD19, CD56, CD25, NKp44 and CD34 first, prior to CD123

positive selection. In the resulting population, CD123 expressing basophils represented about 96% (data not shown).

When the CD123$^+$ basophils were cultured with IL-31, there was no change in skin-homing chemokine receptor expression (Fig. 24A) and there was only a minor effect on basophil survival, which was around 92.8% for treated basophils vs. 80.9% for untreated basophils after 24h of culture, or expression of the survival factors Bcl-2 and Bax (Fig. 23).

The stimulation of basophils with IL-31 in turn increases the sensing of IL-31RA$^+$ basophils by upregulating the IL-31RA (Fig. 24B), itself resulting in potentially prolonged survival of the normally short lived basophils enabling them to persist longer in the periphery and increase priming of IL-31$^+$ T cells.

Also, there was a significant increase in IL-33 receptor ST2 expression (Fig. 24B), again pointing towards the interplay of these two ligand-receptor pairs in the regulation of basophils as described above. Whether the IL-31-induced up-regulation of ST2 *in vitro* on "healthy" basophils is also responsible for the elevated ST2 levels of basophils from atopic dermatitis patients (Fig. 21) and whether IL-31-expressing T cell, which are more abundant in AD patients (Fig. 18) contribute to this phenomenon, needs further investigation, e.g. by utilizing AD-derived basophils or, ideally, basophils from AD patients treated with IL-33- or IL-31-targeting therapies. However, the pronounced state of activation of basophils from AD patients could contribute to both, elevated IL-33 and IL-31 levels, when stimulated with either of these agents.

AD is commonly described as an inflammatory skin disorder showing an influx of T_H2 cells in the acute phase and a switch towards an additional influx of T_H1 cells in its chronic phase[53]. In the past decade, several groups have identified basophils as antigen presenting cells (APC) and aimed to delineate the role of basophils in the priming of T_H2 cells[134,135], with only few studies focusing on human basophils. Findings are still controversial and it has been suggested that basophils solely provide the essential IL-4 for T_H2 priming, but they have also been shown to act as fully functional APC in the priming of T cell responses and e.g. to trigger T cells towards a T_H2 response in allergic reactions and helminth infections[3,101,134,136]. Therefore, the possible involvement of basophils in the priming of T cells towards T_H2 responses was investigated.

Basophils have been shown to express MHC class II molecules as well as the necessary co-stimulatory molecules (CD80, CD86, CD40) to exert competent APC function[136]. Further, basophils actively migrate to sites of infection as well as to secondary lymphoid organs, where priming of T_H2 cells is considered to occur[101,136]. In this study, expression of the human MHC class II molecule HLA-DR on human basophils was confirmed, with higher expression on $CD203c^+$ activated basophils than on $Fc\varepsilon RI^+$ quiescent basophils. Moreover, when co-cultured with autologous naïve $CD4^+$ T cells, basophils induced the production of the classical T_H2-type cytokines IL-4 and IL-13 and additionally the "itchy" cytokine IL-31 (Fig. 26), associated with expression of the master transcription factor for T_H2 polarization, GATA3, and thus showed all characteristics of a *bona fide* T_H2 response induced by human basophils. Nonetheless, basophil-primed T cells also produced marginal amounts of IFN-γ, a T_H1 cytokine, although no expression of the respective master transcription factor for T_H1 responses, T-bet, was observed.

Generally, naïve T cells are capable to differentiate into each functional subtype, depending on the initial instructing signal. Fully differentiated T cells nevertheless display plasticity and may re-differentiate into another subtype[137]. Thus, after initial activation and instruction in the secondary lymphoid organs, the local microenvironment at the target site of T cell responses may further shape and fine-tune the response. The recurrent presence of the instructing signal, however, may reduce plasticity and stabilize the T cell effector phenotype. Therefore, the marginal presence of T_H1 specific IFN-γ after basophil-priming of autologous T cells might be explained by the lack of recurrent instructing signals, preventing development of a pure T_H2 effector phenotype[137]. Unfortunately, basophils are rare cells and short-lived and so far cannot be kept in culture for longer than 60h. Also, the fragility of basophils prevents cryopreservation of this immune cell population. Therefore, recurrent stimulation of T cells with autologous basophils is difficult. Multiple donors for basophils could be used, however, the resulting allogeneic responses may differ from the autologous responses studied here.

Most importantly though, the here presented data show that basophils are capable to induce a T_H2 polarization in naïve $CD4^+$ T cells in a DC-independent manner (Fig. 26). The polarization of T_H2 cells by basophils serving as the initiating APCs might lead to the accumulation of IL-31-producing T_H2 cells in the setup of AD, although it is still unknown whether the basophil-T cell interaction determines the onset or the perpetuation of AD.

However, the data in this study suggest that basophils are capable of skin-homing, secrete high levels of IL-4 and their action as APCs results in efficient priming of T cells (Fig. 26). Whether the priming of the diverse T cell subsets is primarily mediated antigen-specifically by DCs in SLOs, resulting in the migration of the activated T cells towards the skin and a subsequent shift of these skin-homing T cells towards a T_H2 subset by basophils is the primary pathway is not fully understood. Basophils might as well prime T cells directly in AD and be involved in the generation of a transient subtype of T_H2 cells, which is described here as IL31$^+$ T_H2 cells (Fig. 15). Findings of a recent study by Szegedi *et al*. might argue for the DC-dependent primary pathway. This group has linked the expression of IL-31 to the allergen HDM by identifying HDM-reactive T cells producing IL-31 preferentially increased in patients with AD[77]. The priming of antigen-specific T cells needs the presentation of the antigens by DCs and a potential shift of the T cell polarization mediated by either basophilic interaction or the microenvironment. However, Szegedi *et al.* do not describe any functional or phenotypical properties of the "T_H31 cells", like e.g. a master transcription factor expression of this cell type, which is essential for T cells to be classified as a defined T cell subset. Neither have they discussed any potential pathway for their initial priming HDM-specific T cells[77].

Based on the hypothesis that basophils do not exert the initial priming, but subsequent skewing of T cells towards the IL-31-producing T cells subset, elevated numbers of CD123$^+$ basophils may be expected in AD patients, which however, could not be demonstrated in this study, at least not in the blood of patients (Fig. 21). Although frequencies of basophils were not different in patients with AD when compared to healthy individuals, an increase in CD123$^+$ CD203c$^+$ activated ST2$^+$ basophil frequencies was found (Fig. 21), which could be even further activated with IL-31 (Fig. 24), implying an enhanced activation state of IL-31 responsive basophils in AD patients. These activated basophils produce higher levels of IL-4 and might be the cause for the T_H2 character of atopic skin inflammation.

Basophilic skewing of T cell polarization may be further influenced by the local microenvironmental conditions. AD patients present with elevated levels of IL-3, IL-4 and IL-33, which all can interact with immune cells[103]. When mimicking an AD environment by subjecting keratinocytes to IL-4/IL-13, thus the cytokines, which are increased in AD, the resulting keratinocyte-conditioned culture supernatants strengthened the T_H2 phenotype of the primed CD4$^+$ T cells (Fig. 27). The factor within the culture supernatants of IL-4/IL-

13-conditioned keratinocytes that supports T cell priming towards a T_H2 phenotype remains unknown and awaits elucidation. However, keratinocytes are capable to secrete an array of cytokines and chemokines, particularly in the presence of a pro-inflammatory microenvironment, including TSLP and the alarmin IL-33 upon cell damage[69]. Indeed, the presence of IL-33 in basophil-T cell co-cultures resulted in an increased production of IL-31 and IL-4 (Fig. 26). Although there is clear evidence that there is a hereditary component to AD and a subsequent central role of a break-down of the skin barrier, allowing microbes to invade the dermis[138], this study demonstrates that there is a regulatory network linking keratinocytes, basophils and T cells and a dysregulation of the adaptive immune system in AD might arise from a dysregulation of a basophil-dependent priming and T_H2-polarization of T helper cells, associated with an increase of T_H2 cytokines, particularly of IL-31.

Further support for the concept of a central role of the Il-31/Il-31ra axis in the pathophysiology of AD comes from the observation that in the absence of Il-31ra-signaling in mouse models of AD, a relieve from pruritus and rescue from skin lesions has been observed[139]. When Il-31ra$^{-/-}$ mice were studied here, they showed a reduction in the frequency and absolute number of basophils in secondary lymphoid organs of mice (Fig. 28), whereas basophil frequencies and numbers were not affected in the circulation (Fig. 28) or bone marrow (data not shown), supporting an important role of the Il-31/Il-31ra axis in proper basophil distribution and recruitment. This observation might also explain the comparable frequencies of basophils in human blood of AD patients and healthy individuals (Fig. 21), whereas it remains an open question, whether there are differences in basophil numbers within human lymphoid organs. Interestingly, there are fewer basophils in the SLOs of Il-31$^{-/-}$ mice expressing the skin-homing receptor CLA (Fig. 28D), raising the possibility of a reduction of basophils in the skin of Il-31ra$^{-/-}$ mice as well, which could contribute to the reduction of symptoms in the Il-31ra$^{-/-}$ mice.

The consequences of such a re-localization of basophils might be changes of the developing polarization of T cells: As it has been discussed before, basophils may contribute to stabilize the T_H2 effector type, particularly the development of "T_H31" cells, which produce the "itchy" cytokine IL-31. However, absence of Il-31 signaling could vice versa also interfere with accurate priming of T_H2 cells, particularly "T_H31 cells", leading secondarily to diminished frequencies of basophils in the secondary lymphoid organs. In

Il-31ra$^{-/-}$ mice fewer T_H2 polarized T cells expressing the T_H2 master transcription factor GATA3 were observed (Fig. 29A), and at least in mesenteric lymph nodes and spleen, the frequency of IL-31-expressing T cells was significantly reduce (Fig. 29B), both arguing for an improper T_H2 priming in the absence of IL-31 signaling. These observations are in line with previous studies describing a key role for basophils in the priming of T_H2 cells, and particularly of basophil derived IL-4[3,94,109,125,140].

The accumulation of IL-4 through basophils, which survive longer in the circulation of AD-patients, results in a more potent and abundant priming of T_H2 cells in those patients. These T_H2 cells in turn are then also the main producers of IL-31, leading to intensified pruritic symptoms in AD-patients. Understanding the role of IL-31 in balancing T cell differentiation and plasticity might impel insights to how the T cell phenotype is generated in the different stages of AD.

5. Conclusion

The findings presented in this study extend the understanding of the contribution of IL-31 to the pathophysiology of AD and in particular outline a role of basophils in AD[141].

The systematic characterization of human IL-31-producing T cells revealed simultaneous production of IL-31 with the classical T_H2 cytokines IL-4 and IL-13. Despite of this co-expression, the majority of IL-31-expressing T cells expressed IL-31 only, suggesting that they represent a unique sub-population within the T_H2 subset. With the expression of the T_H2 master transcription factor GATA3, IL-31$^+$ T cells are identified as *bona fide* T_H2 cells. Whether these T_H31 cells represent a stable sub-population of T_H2 cells or are transient remains to be determined. Further characterization identified this subset as CD3$^+$ CD4$^+$ CD45RO$^+$ CD62L$^-$ effector memory T cells, which express the skin-homing receptors CLA, CCR4, CCR10 and CCR8. The frequency of IL-31$^+$ T cells was more abundant in atopic dermatitis patients. The accumulation of this T cell subset might result from higher serum levels of IL-33 in AD patients, an alarmin upregulating IL-31 and serving as a key-player in the pruritic symptoms of AD patients.

This study also suggests a strong impact of basophils on the pathophysiology of AD in humans. First, basophils initiated the differentiation of naïve CD4$^+$ T cells to T_H2 cells, including T_H31 cells, thus, the type of response associated with AD. This activity was enhanced by keratinocyte, which had been subjected to an AD-like cytokine microenvironment. Second, basophils express the heterodimeric IL-31 receptor IL-31RA/OSMRβ and therefore are targets of IL-31, which is elevated in AD. Third, basophils of AD patients show a pronounced activation state based on an elevated ST2 expression, a distinct skin-homing receptor repertoire with elevated frequencies of CCR4$^+$ basophils and an elevated expression of IL-4.

Results from *Il-31ra*$^{-/-}$ mice provided direct evidence of a link between IL-31 signaling, basophils and T_H2 responses: Absence of IL-31 receptor signaling resulted in reduced numbers of basophils in the knock-out mice and diminished numbers of T_H2 cells when compared to wild-type mice.

Taken together, activated basophils secrete high levels of IL-4 and support the differentiation of T cells towards T_H2 cells, including a unique subset of T_H31 cells

producing IL-31. The stimulation of the basophils with IL-31 in turn increases their responsiveness to IL-31 through of an upregulation of the IL-31 receptor, resulting potentially in a prolonged survival of the normally short-lived basophils. This enables them to persist longer in the periphery and increase priming of IL-31-expressing T cells. In this regulatory circuitry of activation and re-activation, basophils would be capable to perpetuate their own function in an autocrine manner based on their own IL-31 production, thereby not only persisting longer, but also inducing the migration of basophils to the skin due to an upregulation of the skin-homing receptor CCR4. Using these mechanisms basophils will contribute to the pathophysiology of AD.

Together, understanding the phenotype of IL-31 producing T cells and its interaction with other immune cells may lead to the deciphering of IL-31-induced mechanisms. This in turn might improve the monitoring of the new clinical trials using antibodies against IL-31RA and IL-31 directly, resulting in a higher success rate of the treatment of skin diseases or itch.

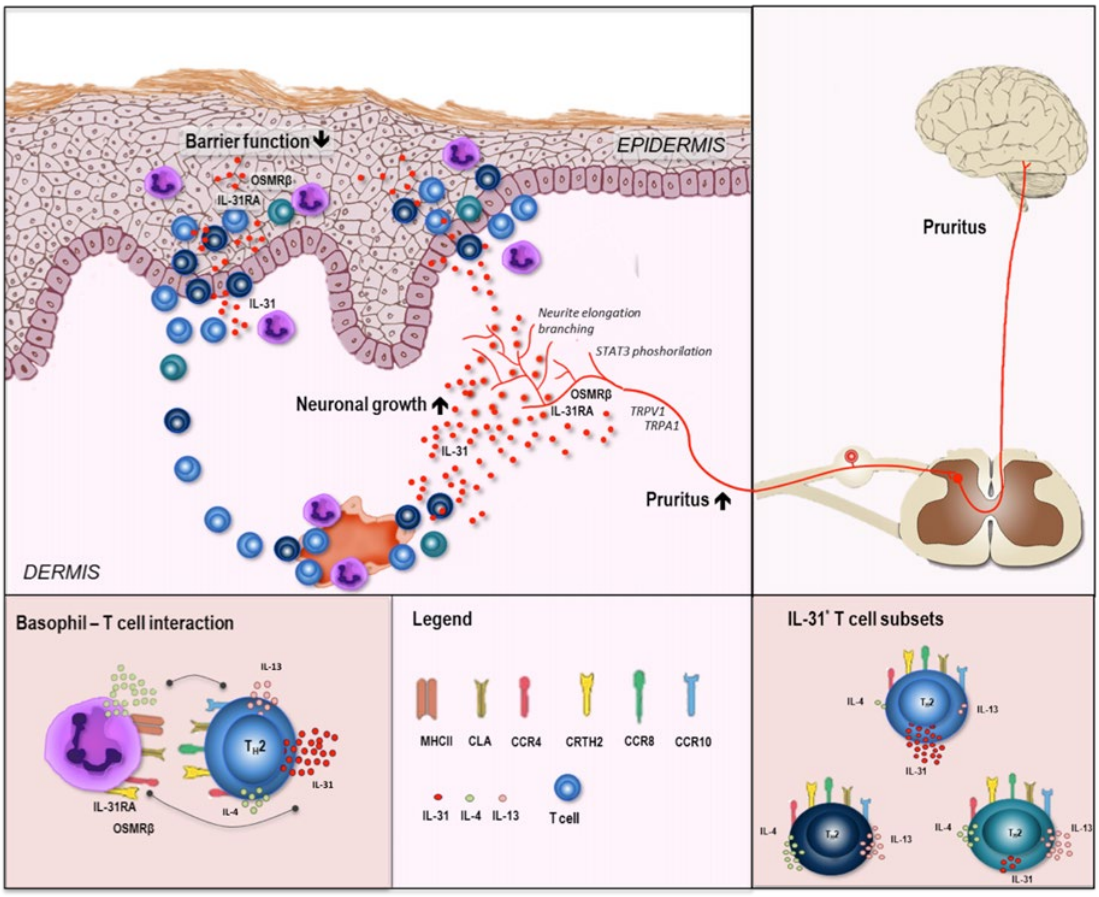

Figure 30: Keratinocyte mediated signaling to basophils results in the subsequent polarization and activation of IL-31-secreting T_H2 cells, which in turn act as neuro-immune linkers. IL-31-secreting T_H2 cells activate IL-31RA$^+$ DRGs provoking the symptoms of barrier dysfunction, pruritus and inflammation in atopic dermatitis patients.

6. References

1. Parkin, J. and Cohen, B. Overview of the Immune System. *Lancet* **357,** 1777–1789 (2001).
2. Banchereau, J. *et al.* Immunobiology of Dendritic Cells. *Nat. Immunol.* **18,** 767–811 (2000).
3. Perrigoue, J. G. *et al.* MHC class II–dependent basophil–CD4+ T cell interactions promote TH2 cytokine–dependent immunity. *Nat. Immunol.* **10,** 697–705 (2009).
4. Smith-Garvin, J. & Koretzky, G. T cell activation. *Annu. Rev. Immunol.* **27,** 591–619 (2009).
5. Zhu, J., Yamane, H. & Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu. Rev. Immunol.* **28,** 445–489 (2010).
6. Moser, B. CXCR5, the defining marker for follicular B helper T (TFH) cells. *Front. Immunol.* **6,** 6–8 (2015).
7. Nurieva, R. I. *et al.* Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. *Immunity* **29,** 138–149 (2008).
8. Zhou, D. *et al.* Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. *Cell. Signal.* **26,** 192–197 (2014).
9. Coomes, S. M., Pelly, V. S., Wilson, M. S. & Wilson, M. S. Plasticity within the ab þ CD4 þ T-cell lineage : when , how and what for ? *Open Biol* **3,** (2013).
10. David Farrar, J., Asnagli, H. & Murphy, K. M. T helper subset development: Roles of instruction, selection, and transcription. *J. Clin. Invest.* **109,** 431–435 (2002).
11. Lantelme, E., Mantovani, S., Palermo R Campanelli, B., Sallusto, F. & Giachino, C. Kinetics of GATA-3 gene expression in early polarizing and committed human T cells. *Immunology* **102,** 123–130 (2001).
12. Szabo, S. J., Kim, S. T., Costa, G. L., Fathman, C. G. & Glimcher, L. H. Pillars Article : A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. *Cell* **100,** 655–669 (2000).
13. Chen, W. Regulatory T cells use " Itch " to control asthma. *JCI* **123,** 2013–2015 (2013).
14. Zheng, W. & Flavell, R. A. The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. *Cell Press* **62,** 117 (1997).
15. Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L. & Paul, W. E. GATA-3 promotes Th2 responses through three different mechanisms: Induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res.* **16,** 3–10 (2006).
16. Mutalithas, K., Guillen, C., Day, C., Brightling, C. E. & Wardlaw, I. D. P. and A. J. CRTH 2 expression on T cells in asthma. *Clin. Exp. Immunol.* **2,** 34–40 (2010).
17. Schlapbach, C. *et al.* Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci. Transl. Med.* **6,** (2014).
18. Licona-limón, P. *et al.* Th9 cells drive host immunity against gastrointestinal worm infection. *Immunity* **39,** (2013).
19. Chen, Z. & O'Shea, J. J. Th17 cells: A new fate for differentiating helper T cells. *Immunol. Res.* **41,** 87–102 (2008).
20. Wei, G. *et al.* Global Mapping of H3K4me3 and H3K27me3 Reveals Specificity and Plasticity in Lineage Fate Determination of Differentiating CD4+ T Cells. *Immunity* **30,** 155–167 (2009).
21. Korn, T., Oukka, M. & Kuchroo, V. Th17 cells: effector T cells with inflammatory properties. *Semin. Immunol.* **19,** 362–371 (2007).
22. Stockinger, B., Veldhoen, M. & Martin, B. Th17 T cells: Linking innate and adaptive immunity. *Semin. Immunol.* **19,** 353–361 (2007).
23. Eyerich, S. *et al.* Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J. Clin. Invest.* **119,** 3573–3585 (2009).
24. Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat. Immunol.* **10,** 857–863 (2009).
25. Lai, T. *et al.* Interleukin-31 expression and relation to disease severity in human asthma.

Sci. Rep. **6,** 1–9 (2016).
26. Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* **6,** 1142–1151 (2005).
27. Josefowicz, S. Z., Lu, L.-F. & Rudensky, A. Y. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu. Rev. Immunol.* **30,** 531–564 (2012).
28. Fairweather, D., Satoskar, A. R. & Garcia, K. C. The compsition and signaling of the IL-35 receptor are unconventional. *Nat. Immunol.* **13,** 290–299 (2013).
29. Huang, A. *et al.* Interleukin-35 on B cell and T cell induction and regulation. *J. Inflamm. (United Kingdom)* **14,** 10–16 (2017).
30. Gagliani, N. *et al.* Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. *Am. J. Transplant.* **13,** 1963–1975 (2013).
31. Farber, D. L., Yudanin, N. A. & Restifo, N. P. Human memory T cells: Generation, compartmentalization and homeostasis. *Nat. Rev. Immunol.* **14,** 24–35 (2014).
32. Sallusto, F., Geginat, J. & Lanzavecchia, A. Central Memory and Effector Memory T cell Subsets : Function, Generation, and Maintenance. *Annu. Rev. Immunol.* **22,** 745–763 (2004).
33. Sallusto, F. & MacKay, C. R. Chemoattractants and their receptors in homeostasis and inflammation. *Curr. Opin. Immunol.* **16,** 724–731 (2004).
34. Cayrol, C. & Girard, J. ScienceDirect IL-33 : an alarmin cytokine with crucial roles in innate immunity , inflammation and allergy. *Curr. Opin. Immunol.* **31,** 31–37 (2014).
35. Dinarello, C. A. Historical Review of Cytokines. *Eur. J. Immunol.* **37,** 34–45 (2011).
36. Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim. Biophys. Acta - Mol. Cell Res.* **1813,** 878–888 (2011).
37. Diveu, C. *et al.* Predominant expression of the long isoform of GP130-like (GPL) receptor is required for interleukin-31 signaling. *Eur. Cytokine Netw.* **15,** 291–302 (2004).
38. Chattopadhyay, S. *et al.* Interleukin-31 and oncostatin-M mediate distinct signaling reactions and response patterns in lung epithelial cells. *J. Biol. Chem.* **282,** 3014–3026 (2007).
39. Dreuw, A. *et al.* Characterization of the Signaling Capacities of the Novel gp130-like Cytokine Receptor *. **279,** 36112–36120 (2004).
40. Fukada, T. *et al.* STAT3 orchestrates contradictory signals in cytokine-induced G1 to S cell-cycle transition. *EMBO J.* **17,** 6670–7 (1998).
41. Fukada, T. *et al.* Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: Involvement of STAT3 in anti-apoptosis. *Immunity* **5,** 449–460 (1996).
42. Maier, E., Werner, D., Duschl, A., Bohle, B. & Horejs-Hoeck, J. Human Th2 but Not Th9 Cells Release IL-31 in a STAT6/NF-κB–Dependent Way. *J. Immunol.* **193,** 645–654 (2014).
43. Bilsborough, J. *et al.* IL-31 is associated with cutaneous lymphocyte antigen – positive skin homing T cells in patients with atopic dermatitis. 418–425 (2006). doi:10.1016/j.jaci.2005.10.046
44. Dillon, S. R. *et al.* Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat. Immunol.* **5,** 752–760 (2004).
45. Sonkoly, E. *et al.* IL-31 : A new link between T cells and pruritus in atopic skin inflammation. *J. Allergy Clin. Immunol.* 411–417 (2006).
46. Stott, B. *et al.* Human IL-31 is induced by IL-4 and promotes TH2-driven inflammation. *J. Allergy Clin. Immunol.* **132,** 446–454.e5 (2013).
47. Park, K. *et al.* Transcriptional activation of the IL31 gene by NFAT and STAT6. *J. Leukoc. Biol.* **91,** 245–257 (2012).
48. Salvo, E. Di, Ventura-Spagnolo, E., Casciaro, M., Navarra, M. & Gangemi, S. IL-33/IL-31 Axis: A Potential Inflammatory Pathway. *Mediators Inflamm.* (2018). doi:10.1155/2018/3858032
49. Hwang, J. S. *et al.* NFAT1 and JunB cooperatively regulate IL-31 gene expression in CD4+ T cells in health and disease. *J. Immunol.* **194,** 1963–74 (2015).
50. Maier, E. *et al.* Prerequisites for functional interleukin 31 signaling and its feedback

regulation by suppressor of cytokine signaling 3 (SOCS3). *J. Biol. Chem.* **290,** 24747–24759 (2015).
51. Weidinger, S., Novak, N. & Kiel, C. Atopic dermatitis. *Lancet* **387,** 1109–1122 (2016).
52. Eichenfield, L. F. *et al.* Guidelines of care for the management of atopic dermatitis - Part 2: Management and Treatment of Atopic Dermatitis with Topical Therapies. *J Am Acad Dermatol* **71,** 116–132 (2014).
53. Bieber, T. Atopic dermatitis. *Ann. Dermatol.* **22,** 125–137 (2010).
54. Kuo, I., Yoshida, T., Benedetto, A. De & Beck, L. A. The cutaneous innate immune response in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **131,** 266–278 (2014).
55. Hawro, T. *et al.* Interleukin-31 does not induce immediate itch in atopic dermatitis patients and healthy controls after skin challenge. *Allergy Eur. J. Allergy Clin. Immunol.* **69,** 113–117 (2014).
56. Leung, D. Y. M. Atopic dermatitis: The skin as a window into the pathogenesis of chronic allergic diseases. *J. Allergy Clin. Immunol.* **96,** 302–319 (1995).
57. Steinhoff, M. *et al.* Neurophysiological, neuroimmunological, and neuroendocrine basis of pruritus. *J. Invest. Dermatol.* **126,** 1705–1718 (2006).
58. Mollanazar, N. K., Smith, P. K. & Yosipovitch, G. Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? *Clin. Rev. Allergy Immunol.* **51,** 263–292 (2016).
59. Bautista, D. M., Wilson, S. R. & Hoon, M. A. Why we scratch an itch : the molecules , cells and circuits of itch. *Nat. Publ. Gr.* **17,** 175–182 (2014).
60. Paus, R., Schmelz, M., Bíró, T. & Steinhoff, M. Review series Frontiers in pruritus research : scratching the brain for more effective itch therapy. **116,** (2006).
61. Hoon, M. A. Molecular dissection of itch. *Curr Opin Neurobiol,* **34,** 61–66 (2015).
62. Usoskin, D. *et al.* Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat. Publ. Gr.* **18,** 145–153 (2014).
63. Ikoma, A., Steinhoff, M., Ständer, S., Yosipovitch, G. & Schmelz, M. The neurobiology of itch. *Nat. Rev. Neurosci.* **7,** 535–547 (2006).
64. Imamachi, N. *et al.* TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *PNAS* **106,** 11330–11335 (2009).
65. Kido-nakahara, M. *et al.* Neural peptidase endothelin-converting enzyme 1 regulates endothelin 1 – induced pruritus. *J. Clin. Invest.* **124,** 2683–2695 (2014).
66. Wilson, S. R. *et al.* The Epithelial Cell-derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch. *Cell* **155,** 285–295 (2013).
67. Arai, I., Tsuji, M., Takeda, H., Akiyama, N. & Saito, S. A single dose of interleukin-31 (IL-31) causes continuous itch-associated scratching behaviour in mice. *Exp. Dermatol.* **22,** 656–681 (2013).
68. Cevikbas, F. *et al.* A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. *J. Allergy Clin. Immunol.* **133,** 448–460.e7 (2014).
69. Werfel, T. *et al.* Mechanisms of allergic diseases Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **138,** 336–349 (2016).
70. Oetjen, L. K. *et al.* Sensory Neurons Co-opt Classical Immune Signaling Pathways to Mediate Chronic Itch. *Cell* **171,** 217–228.e13 (2017).
71. Nemoto, O. *et al.* The first trial of CIM331, a humanized anti-human IL-31 receptor A antibody, for healthy volunteers and patients with atopic dermatitis to evaluate safety, tolerability and pharmacokinetics of a single dose in a randomised, double-blind, placebo-controlle. *Br. J. Dermatol.* **174,** 296–304 (2015).
72. Ruzicka, T. *et al.* Anti–Interleukin-31 Receptor A Antibody for Atopic Dermatitis. *N. Engl. J. Med.* **376,** 826–835 (2017).
73. Kato, A. *et al.* Distribution of IL-31 and its receptor expressing cells in skin of atopic dermatitis. *J. Dermatol. Sci.* **74,** 229–235 (2014).
74. Feld, M., Garcia, R., Buddenkotte, J., Katayama, S. & Lewis, K. The pruritus- and T H 2-

associated cytokine IL-31 promotes growth of sensory nerves. *J. Allergy Clin. Immunol.* **138,** (2016).

75. Vocca, L. *et al.* IL-33/ST2 axis controls Th2/IL-31 and Th17 immune response in allergic airway diseases. *Immunobiology* **220,** 954–963 (2015).
76. Santamaria Babi, L. F. *et al.* Circulating allergen-reactive T cells from patients with atopic dermatitis and allergic contact dermatitis express the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen. *J. Exp. Med.* **181,** 1935–40 (1995).
77. Szegedi, K. *et al.* House dust mite allergens Der f and Der p induce IL-31 production by blood-derived T cells from atopic dermatitis patients. *Experimental Dermatology* **27,** 393–395 (2018).
78. Hänel, K. H. *et al.* Control of the Physical and Antimicrobial Skin Barrier by an IL-31–IL-1 Signaling Network. *J. Immunol.* **196,** 3233–3244 (2016).
79. Singh, B. *et al.* IL-31-driven skin remodeling involves epidermal cell proliferation and thickening that lead to impaired skin-barrier function. *PLoS One* (2016).
80. Kasraie, S., Niebuhr, M., Baumert, K. & Werfel, T. Functional effects of interleukin 31 in human primary keratinocytes. *Exp. Allergy Immunol.* **31,** 845–852 (2011).
81. Lin, Y., Wang, C. & Chiang, B. Role of Bacterial Pathogens in Atopic Dermatitis. *Clin. Rev. Allergy Immunol.* **33,** 167–177 (2007).
82. Torres, M. *et al.* Circulating CLA þ lymphocytes from children with atopic dermatitis contain an increased percentage of cells bearing staphylococcal-related T-cell receptor variable segments. *Clin. Exp. Allergy* **28,** 1264–1272 (1998).
83. Kasraie, S., Niebuhr, M. & Werfel, T. Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins. *Allergy Eur. J. Allergy Clin. Immunol.* **65,** 712–721 (2010).
84. Horejs-hoeck, J. *et al.* Europe PMC Funders Group Dendritic cells activated by interferon- γ / STAT1 express interleukin-31 receptor and release pro-inflammatory mediators upon interleukin-31 treatment. *J. Immunol.* **188,** 5319–5326 (2012).
85. Cheung, P. F. Y. *et al.* Activation of human eosinophils and epidermal keratinocytes by T^h2 cytokine IL-31: Implication for the immunopathogenesis of atopic dermatitis. *Int. Immunol.* **22,** 453–467 (2010).
86. Raap, U. *et al.* Human basophils are a source of - and are differentially activated by -IL-31. *Clin. Exp. Allergy* **47,** 499–508 (2016).
87. Hida, S. *et al.* Fc receptor-g-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils. *Nat. Immunol.* **10,** 214–222 (2009).
88. Siracusa, M. C., Perrigoue, J. G., Comeau, M. R. & Artis, D. New paradigms in basophil development, regulation and function. *Immunol. Cell Biol.* **88,** 275–284 (2010).
89. Hausmann, O. V. *et al.* Robust expression of CCR3 as a single basophil selection marker in flow cytometry. *Allergy Eur. J. Allergy Clin. Immunol.* **66,** 85–91 (2011).
90. Van Beek, A. A. *et al.* Recent developments in basophil research: Do basophils initiate and perpetuate type 2 T-helper cell responses? *Int. Arch. Allergy Immunol.* **160,** 7–17 (2012).
91. Karasuyama, H. & Yamanishi, Y. Basophils have emerged as a key player in immunity. *Curr. Opin. Immunol.* **31,** 1–7 (2014).
92. Morita, H. *et al.* An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation By Promoting Regulatory T Cell Numbers. *Immunity* **43,** 175–186 (2015).
93. Steinbrink, K., Mahnke, K., Grabbe, S., Enk, A. H. & Jonuleit, H. Myeloid dendritic cell: From sentinel of immunity to key player of peripheral tolerance? *Hum. Immunol.* **70,** 289–293 (2009).
94. Sokol, C. L. *et al.* Basophils function as Antigen-presenting cells for an Allergen-Induced Th2 response. *Nat. Immunol.* **10,** 713–720 (2009).
95. Sullivan, B. M. *et al.* Genetic analysis of basophil function in vivo. *Nat. Immunol.* **12,** 527–535 (2011).
96. Siracusa, M. C., Kim, B. S., Spergel, J. M. & Artis, D. Basophils and allergic inflammation. *J.*

Allergy Clin. Immunol. **132,** 789–801 (2013).

97. Kashem, S. W., Haniffa, M. & Kaplan, D. H. Antigen-Presenting Cells in the Skin. *Annu. Rev. Immunol.* **35,** 469–499 (2017).
98. Kaplan, D. H., Jenison, M. C., Saeland, S., Shlomchik, W. D. & Shlomchik, M. J. Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* **23,** 611–620 (2005).
99. Auffray, C., Sieweke, M. H. & Geissmann, F. Blood Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells. *Annu. Rev. Immunol.* **27,** 669–692 (2009).
100. Nestle, F. O. *et al.* Plasmacytoid predendritic cells initiate psoriasis through interferon-α production. *J. Exp. Med.* **202,** 135–143 (2005).
101. Maddur, M. S., Kaveri, S. V. & Bayry, J. Basophils as antigen presenting cells. *Trends Immunol.* **31,** 45–48 (2010).
102. Meng, J. *et al.* New mechanism underlying IL-31-induced atopic dermatitis. *J. Allergy Clin. Immunol.* (2018).
103. Nygaard, U. *et al.* TSLP, IL-31, IL-33 and sST2 are new biomarkers in endophenotypic profiling of adult and childhood atopic dermatitis. *J. Eur. Acad. Dermatology Venereol.* **30,** 1930–1938 (2016).
104. Hamann, C. R. & Thyssen, J. P. Monoclonal Antibodies Against IL-13 and IL-31RA in Development for Atopic Dermatitis. *J. Am. Acad. Dermatol.* **78,** S37–S42 (2017).
105. Mueller, S. N., Gebhardt, T., Carbone, F. R. & Heath, W. R. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annu. Rev. Immunol.* **31,** 137–161 (2013).
106. Nobbe, S. *et al.* IL-31 Expression by Inflammatory Cells is Preferentially Elevated in Atopic Dermatitis. *Acta Derm. Venereol.* **92,** 24–28 (2012).
107. Neis, M. M. *et al.* Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J. Allergy Clin. Immunol.* (2006).
108. Ferretti, E. *et al.* The interleukin (IL)-31/IL-31R axis contributes to tumor growth in human follicular lymphoma. *Leukemia* **29,** 958–967 (2015).
109. Nakanishi, K. Basophils as APC in Th2 response in allergic inflammation and parasite infection. *Curr. Opin. Immunol.* **22,** 814–820 (2010).
110. Cedeno-Laurent, F. *et al.* Improved pruritus correlates with lower levels of IL-31 in CTCL patients under different therapeutic modalities. *Clin. Immunol.* **158,** 1–7 (2015).
111. Cornelissen, C. *et al.* IL-31 regulates differentiation and filaggrin expression in human organotypic skin models. *J. Allergy Clin. Immunol.* **129,** (2012).
112. Yosipovitch, G. & Bernhard, J. D. Chronic Pruritus. *N. Engl. J. Med.* **368,** 1625–1634 (2013).
113. Neis, M. M. *et al.* Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J. Biol. Chem.* 930–937 (2006).
114. Meng, J. *et al.* New mechanism underlying IL-31-induced atopic dermatitis. *J. Allergy Clin. Immunol.* (2018).
115. Michels, G. M. *et al.* A blinded, randomized, placebo-controlled, dose determination trial of lokivetmab (ZTS-00103289), a caninized, anti-canine IL-31 monoclonal antibody in client owned dogs with atopic dermatitis. *Vet. Dermatol.* **27,** 478-e129 (2016).
116. Ezzat, M. H. M., Hasan, Z. E. & Shaheen, K. Y. A. Serum measurement of interleukin-31 (IL-31) in paediatric atopic dermatitis: Elevated levels correlate with severity scoring. *J. Eur. Acad. Dermatology Venereol.* **25,** 334–339 (2011).
117. Homey, B., Steinhoff, M., Ruzicka, T. & Leung, D. Y. M. Cytokines and chemokines orchestrate atopic skin inflammation. *J. Allergy Clin. Immunol.* **118,** 178–189 (2006).
118. Dambacher, J. *et al.* Interleukin 31 mediates MAP kinase and STAT1/3 activation in intestinal epithelial cells and its expression is upregulated in inflammatory bowel disease. *Gut* **56,** 1257–1265 (2007).
119. Perrigoue, J. G., Zaph, C., Guild, K., Du, Y. & Artis, D. IL-31-IL-31R Interactions Limit the Magnitude of Th2 Cytokine-Dependent Immunity and Inflammation following Intestinal Helminth Infection. *J. Immunol.* **182,** 6088–6094 (2009).

120. Ruzicka, T. *et al.* Anti–Interleukin-31 Receptor A Antibody for Atopic Dermatitis. *N. Engl. J. Med.* **376,** 826–835 (2017).
121. Mitchell, E. B. & Askenase, P. W. Basophils in human disease. *Clin. Rev. Allergy* **1,** 427–448 (1983).
122. Gibbs, B. F. Human basophils as effectors and immunomodulators of allergic inflammation and innate immunity. *Clinical and Experimental Medicine* **5,** 43–49 (2005).
123. Yoshimoto, T. *et al.* Basophils contribute to TH2-IgE responses in vivo via IL-4 production and presentation of peptide–MHC class II complexes to CD4+ T cells. *Nat. Immunol.* **10,** 706–712 (2009).
124. Oetjen, L. K., Noti, M., Kim, B. S., Sciences, B. & Pathology, E. New Insights into Basophil Heterogeneity. *Semin. Immunopathol.* **38,** 549–561 (2016).
125. Karasuyama, H., Mukai, K., Tsujimura, Y. & Obata, K. Newly discovered roles for basophils: A neglected minority gains new respect. *Nat. Rev. Immunol.* **9,** 9–13 (2009).
126. Tamagawa-Mineoka, R., Okuzawa, Y., Masuda, K. & Katoh, N. Increased serum levels of interleukin 33 in patients with atopic dermatitis. *J. Am. Acad. Dermatol.* **70,** 882–888 (2014).
127. Guarneri, F. *et al.* Il-31 and il-33 circulating levels in allergic contact dermatitis. *Eur. Ann. Allergy Clin. Immunol.* **47,** 156–158 (2015).
128. Smithgall, M. D. *et al.* IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th 2 cells, iNKT and NK Cells. *Int. Immunol.* **20,** 1019–1030 (2008).
129. Dvorak, A. M. A role for vesicles in human basophil secretion. *Cell Tissue Res.* **293,** 1–22 (1998).
130. Irani, A.-M. A. *et al.* Immunohistochemical detection of human basophils in late-phase skin reactions. *J. Allergy Clin. Immunol.* **101,** 354–362 (1998).
131. Satoh, T., Ito, Y., Miyagishi, C. & Yokozeki, H. Basophils infltrate skin lesions of eosinophilic pustular folliculitis (Ofuji's disease). *Acta Derm. Venereol.* **91,** 371–372 (2011).
132. Schroder, K. & Tschopp, J. The Inflammasomes. *Cell* **140,** 821–832 (2010).
133. Dzionek, A. *et al.* BDCA-2, BDCA-3, and BDCA-4: Three Markers for Distinct Subsets of Dendritic Cells in Human Peripheral Blood. *J. Immunol.* **165,** 6037–6046 (2000).
134. Sokol, C. L. *et al.* Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* **10,** 713–720 (2009).
135. Nakanishi, K. Basophils are potent antigen-presenting cells that selectively induce Th2 cells. *Eur. J. Immunol.* **40,** 1836–1842 (2010).
136. Nakanishi, K. Basophils as APC in Th2 response in allergic inflammation and parasite infection. *Current Opinion in Immunology* **22,** 814–820 (2010).
137. Hirahara, K. *et al.* Mechanisms underlying helper T cell plasticity: Implications for immune-mediated disease. *J Allergy Clin Immunol.* **131,** 1276–1287 (2013).
138. Elias, P. M. & Steinhoff, M. 'Outside-to-inside' (and now back 'Outside') Pathogenic Mechanisims in Atopic Dermatitis. *J. Invest. Dermatol.* **128,** 1067–1070 (2009).
139. Kasutani, K. *et al.* Anti-IL-31 receptor antibody is shown to be a potential therapeutic option for treating itch and dermatitis in mice. *Br. J. Pharmacol.* **171,** 5049–5058 (2014).
140. Sokol, C. L. & Medzhitov, R. Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunol.* **3,** 129–137 (2010).
141. Steinhoff, M., Buddenkotte, J. & Lerner, E. A. Role of mast cells and basophils in pruritus. *Immunological Reviews* 282:248-264 (2018).

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8. Declaration of Authorship

I certify that the work presented here is, to the best of my knowledge and belief, original and the results of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other University.

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9. Curriculum Vitae

Personal Information

Name	Angeliki Datsi
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Education

03/2016- heute	Dissertation at the Heinrich-Heine Universität Düsseldorf, Uniklinikum Düsseldorf Hautklinik; **Topic:** • *Bidirectional IL-31-driven communication between T cells and basophils during atopic skin inflammation*
10/2014- 02/2016	Dissertation at the Deutschen Rheumaforschungszentrum, Humboldt-Universität Berlin; **Topic:** • *Reprogramming of T_H1 T-cells in inflammatory disorders in vivo and in vitro*
10/2012- 08/2014	Master Bio-Science, Westfälische Wilhelms-Universität Münster; Degree M.Sc. (1.4) **Master-Thesis:** • *Identification of the orphan receptor GPR15 as a potential gut homing marker on $CD4^+$ T cells*
10/2009- 08/2012	Bachelor Bio-Science, Westfälische Wilhelms-Universität Münster; Degree B.Sc. (Note: 1.7) **Bachelor-Thesis:** • *Characterization of FoxD in the planarium Schmidtea mediterranea.*
8/2006 – 08/2009	Medical Laboratory Technician Studies (MTLA), Universitätsklinkum Münster (Note: 1.6)

Prof. Experience

04/2018 – today	**Postdoctoral Fellow** Institute for Transplantation Diagnostic and Cell Therapeutics
03/2016 – 04/2018	**Scientific Head of the Neurosurgical Research Lab;** University-Hospital Bergmannsheil Bochum

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09/2013 – 08/2014	**Research internship at Oxford University; England** (Group: Prof. Fiona Powrie, Gastroenterology)
03/2012 - 08/2012	**Bachelor-Thesis** at the Max-Planck-Institute for Molecular Biomedicine, Münster (Group: Stemcells and Regeneration)
10/2011 – 02/2012	**Research internship at Yale University, New Haven, USA** (Group: Prof. Richard. A Flavell, Immunobiology)

Scholarships & Awards

09/2013 – 08/2014	Association at the *Studienstiftung;des deutschen Volkes;* Scholarshifor the Master in Oxford
10/2014- 02/2016	Stipend for the PhD at the ZIBI Graduate School
02/2014	Travel Award for the "9th Congress of ECCO", Copenhagen

Certifications

02/2015	Course on Laboratory Animal Sciences (BfR – Bundesinstitut für Risikobewertung), Berlin
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Additional Skills

Language skills:	German – First language Greek – Mother tongue English – Excellent in speech and writing Italian – Basic knowledge
Office applications:	Microsoft Power Point, Excel, Word
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10. Publications

IL-33 Receptor-Expressing Regulatory T Cells Are Highly Activated, Th2 Biased and Suppress CD4 T Cell Proliferation through IL-10 and TGFβ Release; **PLoS One 2016.**
Siede J, Fröhlich A, **Datsi A**, Hegazy AN, Varga D, Holecska V, Saito H, Löhning M.

Circulating and Tissue-Resident $CD4_+$ T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. **Gastroenterology 2017.**
Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, **Datsi A**, This S, Danne C, Campion S, Duncan SH, Owens BMJ, Uhlig HH, McMichael A; Oxford IBD Cohort Investigators, Bergthaler A, Teichmann SA, Keshav S, Powrie F.

Meningioma assessment: Kinetic parameters in dynamic contrast-enhanced MRI appear independent from microvascular anatomy and VEGF expression; **Journal of Neuroradiology 2018.**
Keil VC, Pintea B, Gielen GH, Hittatiya K, **Datsi A,** Simon M, Fimmers R, Schild HH, Hadizadeh DR.

Silencing of the nucleocytoplasmic shuttling protein Karyopherin a2 promotes cell-cycle arrest and apoptosis in glioblastoma multiforme; **Oncotarget September 2018.**
Martínez-Olivera R*, **Datsi A*** , Stallkamp M, Kohtz I, Pintea B, Köller M, Gousias K

Vemurafenib acts as an aryl hydrocarbon receptor antagonist: Implications for inflammatory cutaneous adverse events; **under revision at Allergy July 2018.**
Hawerkamp H, Kislat A, Gerber P, Pollet M, Soshilov A, Denison M, Momin A, Arold SK, **Datsi A,** Braun, S; Oláh, P, Lacouture, M; Krutmann J, Haarmann-Stemmann, T; Homey B, Meller, S.

The IL-31-producing circulating T cell subset represents a unique population of CLA^+ $CRTH2^+$ $CCR4^+$ T_{EM} cells; **Manuscript in preparation to be submitted to Journal of Allergy and clinical Immunology**
Datsi A, Olaf P**,** Raba K, Kellermann S, Sohn D, Sorg R, Schlappbach C, Buddenkotte J, Homey B