Identification and characterization of murine and human Th2/1 hybrid cells in Th2-driven diseases

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List of abbreviations

-	healthy control
+	mild AD
++	moderate - severe AD
3D	three-dimensional
α	anti-
Ab	antibody
AD	atopic dermatitis
Ag	antigen
ANOVA	Analysis of variance
APC	antigen-presenting cell
APC	allophycocyanin
BNI	Bernhard Nocht Institute for Tropical Medicine
BSA	bovine serum albumin
BV510	Brilliant violet 510
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CLA	cutaneous lymphocyte-associated antigen
cRPMI	RPMI complete
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
d	day
DC	dendritic cell
DCE	dead cell exclusion
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRFZ	Deutsches Rheumaforschungszentrum
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EN	endemic normal
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
FLG	filaggrin

Foxp3	forkhead box transcription factor p3
FCS	forward scatter
g	g-force
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-3	GATA3-binding protein
h	hour
HBSS	Hanks balanced salt solution
HD	healthy donor
H&E	hematoxylin and eosin
HTLV-1	human T lymphotrophic virus 1
Hz	Hertz
i.p.	intraperitoneal
iL3	infective third stage larvae
IC	intracellular
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharid
µg	microgram
µL	microliter
M	molar
mAb	monoclonal antibody
mg	milligram
min	minute
mL	milliliter
mM	millimolar
mLN	mesenteric lymph node
NIH-NIRT-ICER	National Institute of Health - National Institute for Research in Tuberculosis - International Centre for Excellence in Research
ng	nanogram
NKT cells	natural killer T cells
NO	nitric oxide
OCLN	occludin
p.i.	post infection
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Phl p 5.01	*Phleum pratense* major allergen group 5
Phl p 1	*Phleum pratense* major allergen group 1

PMA	phorbol-myristate-acetate
r	recombinant
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
s	second
s.c.	subcutaneous
SA	streptavidin
SCORAD	scoring of atopic dermatitis
SEB	*Staphylococcus* Enterotoxin B
sec	seconds
SD	standard deviation
siLP	small intestinal lamina propria
siRNA	small interfering RNA
SSC	side scatter
SIT	allergen-specific immunotherapy
STAT	signal transducer and activator
Tab.	table
T-bet	T-box–containing protein expressed in T cells
TBX21	T-box transcription factor TBX21
TCR	T cell receptor
TCR-iNKT	TCR Vα24-Jα18
Teff	effector T cell
Th	T helper cell
TLR	Toll-like receptor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
U	units
w	with
wRPMI	wash RPMI
w/o	without

Zusammenfassung

CD4$^+$ T-Zellen lassen sich in verschiedene Subpopulationen unterteilen, die aufgrund ihrer produzierten Zytokine unterschiedliche Effektorfunktionen erfüllen. Frühere Studien haben gezeigt, dass einige Populationen von T-Helferzellen (Th) Eigenschaften zweier gegensätzlicher Differenzierungsprogramme aufweisen und damit einen gemischten, so genannten „Hybrid"-Phänotyp darstellen. Mäuse entwickelten als Teil der Immunantwort auf Th2-polarisierende Wurminfektionen Th2/1-Hybridzellen, welche die Th2- und Th1-Linien-spezifischen Transkriptionsfaktoren GATA-3 sowie T-bet co-exprimieren. Dies ermöglicht den Th2/1-Zellen die Co-Produktion von Th2-(IL-4/-5/-13) und Th1-Zytokinen (IFN-γ). Dieser Th2/1-Phänotyp blieb über Monate hinweg stabil und löste im Vergleich zu konventionellen Th2- und Th1-Zellen eine geringere Immunpathologie in murinen Typ-1 und Typ-2-Entzündungsmodellen aus.

Basierend auf diesen Daten war das erste Ziel dieser Arbeit Th2- und Th2/1- Zellen am Beispiel von Zwergfadenwurminfektionen (Strongyloidiasis) zu detektieren und phänotypisch zu charakterisieren. Dafür wurden T-Zellen aus Organen von Mäusen und periphere Blutproben indischer Patienten, die mit Zwergfadenwürmern infiziert waren, mittels durchflußzytometrischer Analysen untersucht. Das zweite Ziel war die Existenz des hybriden Phänotyps bei einer nicht-parasitären Th2-vermittelten entzündlichen Hauterkrankung, der atopischen Dermatitis (AD), nachzuweisen. Hierfür wurden Blutproben europäischer AD-Patienten hinsichtlich Th2/1-Zellen untersucht und überprüft, ob deren Zellfrequenzen mit der Schwere der Erkrankung assoziiert sind. Zusätzlich wurden die pro-inflammatorischen Th2- sowie Th22-Populationen und regulatorische Foxp3$^+$ T-Zellen untersucht. In einem weiteren Teil dieser Arbeit wurden die Effektorfunktionen humaner Th2/1-Zellen in einem 3D-Hautmodell für AD analysiert.

Die vorliegende Arbeit zeigt, dass die Bildung von Th2/1-Zellen in Mäusen ebenfalls durch Fadenwürmer induziert wird. Dabei hatten weder der Untersuchungszeitpunkt noch Unterschiede in der Parasitenlast einen Einfluss auf die Th2/1-Zellfrequenzen. Die untersuchten Organe unterschieden sich jedoch deutlich im Th2/1-Hybridanteil. Darüber hinaus konnten in dieser Arbeit erstmalig humane Th2/1-Zellen nachgewiesen werden, die ähnlich wie murine Hybride Th2- und Th1-Zytokine co-exprimieren. Eine vergleichende Analyse des murinen und humanen Th2/1-Phänotyps zeigte Ähnlichkeiten, wie die gegenüber Th2-Zellen verminderte Th2-Zytokinproduktion. Es wurden aber auch artspezifische Unterschiede, wie die stärkere IFN-γ Produktion durch humane Th2/1-Zellen, festgestellt. Die Analyse von Blutproben atopischer Patienten ergab, dass der Mehrheit der zirkulierenden Th2/1-Zellen der Rezeptor CLA fehlt, der für das Einwandern in die Haut benötigt wird. Somit stellt der Großteil der Th2/1-Zellen in AD-Patienten vermutlich keine hautinfiltrierende Zellpopulation dar. Im Gegensatz dazu waren pro-inflammatorische Th2- und Th22-Zellen vor allem in Patienten mit stark ausgeprägter AD in der hautinfiltrierenden CLA$^+$ Population angereichert. Schließlich legen Co-Kulturen von *in vitro* generierten Th2- und Th2/1-Zelllinien mit AD-Hautmodellen nahe, dass der Th2/1-Phänotyp nur schwache Entzündungsreaktionen in der Haut induziert.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass der murine und humane T-Helferzellpool Th2/1-Hybridzellen umfasst. Diese Zellpopulation unterscheidet sich zwischen Maus und Mensch in bestimmten Merkmalen, scheint jedoch in beiden Spezies die Vermittlung von Th2-Zytokinvermittelten Entzündungsreaktionen zu beeinträchtigen. Zukünftige Untersuchungen werden aufdecken, ob Th2/1-Zellen aufgrund ihrer geringeren Th2-Zytokin-Expression Th2-Antworten passiv abmildern oder aber aktiv durch die Produktion von IFN-γ gegenregulieren und ob Th2/1-Zellen damit potenzielle therapeutische Werkzeuge bei Th2-getriebenen Erkrankungen darstellen.

Summary

CD4$^+$ T cells differentiate into diverse cell lineages with distinct effector functions, based on their produced signature cytokines. Previous studies have reported that T helper (Th) cell subsets can adopt characteristics of two opposing T cell differentiation programs, resulting in a mixed so-called "hybrid" phenotype. It was shown that mice develop Th2/1 hybrid cells, co-expressing the Th2 and Th1 lineage-specifying transcription factors GATA-3$^+$ and T-bet$^+$ and Th2 cytokines together with IFN-γ$^+$ in response to Th2-polarizing worm infections. This Th2/1 phenotype was stably maintained for months and caused lower immunopathology in murine models of type-1 and type-2 inflammation compared to conventional Th1 and Th2 cells. Type-2 immune responses are essential for protective immunity against parasitic worm infections, but also play a pathologic role in promoting acute and chronic inflammation.

Based on these data, the first aim of this work was to detect and phenotypically characterize Th2 and Th2/1 cells in threadworm infections. Therefore, organs of mice and peripheral blood samples of Indian patients infected with threadworms were analyzed using flow cytometry. The second aim was to prove the existence of the hybrid phenotype in the non-parasitic, Th2-mediated inflammatory skin disorder atopic dermatitis (AD). Blood samples of European patients with AD were analyzed for the occurrence of Th2/1 cells alongside pro-inflammatory Th2 and Th22 cell subsets and regulatory Foxp3$^+$ T cells, surveying whether the proportions of Th2/1 hybrid cells were linked to distinct manifestations of the disease. Furthermore, the effector function of human hybrid cells was examined in 3D skin equivalents mimicking the hallmarks of AD.

This work demonstrates that the induction of Th2/1 cells in parasitic worm infections can be generalized to murine threadworm infections. While neither the time point of infection nor the parasite burden had a significant impact on hybrid frequencies, Th2/1 cell levels differed depending on the examined organ. This work shows for the first time that also the human peripheral CD4$^+$ T cell pool comprises Th2/1 hybrid cells co-expressing Th2 and Th1 markers. A comparative analysis between the murine and human Th2/1 phenotype revealed similar (e.g. the lower Th2 cytokine production by Th2/1 compared to Th2 cells) but also species-specific differences (such as the stronger IFN-γ production by human Th2/1 cells). Moreover, the analysis of patients with AD demonstrated that the majority of circulating Th2/1 cells lacked the skin-homing marker CLA. In contrast, especially in severe AD patients, pro-inflammatory Th2 and Th22 subsets were enriched in the skin-homing CLA$^+$ subpopulation. Finally, co-cultures of *in vitro* generated Th2 or Th2/1 cells with skin models mimicking the hallmarks of AD suggest that the Th2/1 hybrid phenotype is a poor inducer of skin inflammation.

In conclusion, the CD4$^+$ T cell pool of mice and humans comprises Th2/1 cells displaying species-specific features, but most likely sharing a poor capacity for supporting Th2-associated inflammatory reactions. Future investigations will show whether Th2/1 cells 'passively' regulate Th2 responses due to their lower Th2 cytokine expression or actively cross-regulate an overt Th2 bias by e.g. the expression of IFN-γ and thus, whether Th2/1 cells can be used as a therapeutic tool in Th2 driven diseases.

1. Introduction

1.1. T lymphocytes and their role in the immune system

Protective and balanced immune responses against pathogens and other antigens are a vital prerequisite for health. Immunity in vertebrates depends on complex interactions between numerous cells and molecules of two axes: the early non-specific reactions of the innate immune system and the later responses of the highly specialized adaptive immune system.

T cells are an important subgroup of lymphocytes playing a pivotal role in adaptive immunity. They participate in a variety of cell-mediated immune responses including alloreactivity, cell-mediated cytotoxicity and delayed type hypersensitivity [1]–[3]. T lymphocytes provide help for the production of antibodies by B cells, induce the enhanced activation of macrophages, and recruit neutrophils, eosinophils and basophils to sites of infection and inflammation [4]–[6]. These diverse functions are realized through the production of cytokines and chemokines by different T cell subtypes, which are distinguished by their combination of cell surface proteins. The majority of T lymphocytes express the surface proteins CD4 (T helper cells) or CD8 (cytotoxic T cells). The current work focuses on $CD4^+$ T helper (Th) cells that orchestrate and regulate immune responses by “helping” other immune cells to conduct their functions, while $CD8^+$ cytotoxic T cells produce and release cytotoxic granules to kill cells infected by viruses or bacteria. To fulfill these roles, but also to coordinate lymphocyte development and the immunological memory, T cells continuously migrate into and out of tissues and the vascular-lymphatic circulation. This migration is required to perform repeated serial encounters with diverse host cells in seeking for cognate peptide antigens presented on their surface to activate T cells [7], [8].

1.1.1. $CD4^+$ T cells differentiate into diverse cell lineages with distinct effector functions

Particular cytokines released by activated $CD4^+$ T cells at the onset and expansion of an immune response are thought to be decisive for pathological or physiological consequences [9]. Immune responses against a wide range of pathogens and the regulation of self-tolerance are achieved through the differentiation of naïve $CD4^+$ T cells into distinct Th cell lineages, each possessing unique phenotypic and functional characteristics. These cell lineages develop upon T cell activation and are characterized by the enhanced production of a key transcription factor that acts as a lineage-specifying ‘‘master regulator’’ coordinating the accessibility and expression of defined subsets of cytokines and other genes [10]–[12].

The Th cell activation is dependent on three signals (see **Fig. 1A**) provided by competent antigen-presenting cells (APC), such as dendritic cells (DC). First, APC present peptide antigens via major histocompatibility complex (MHC) II proteins to naïve $CD4^+$ T cells (Th0), which may recognize the cognate antigen by their T cell receptor (TCR), supported by CD4 as a co-receptor. The second signal

confirms and enhances T cell activation. The co-stimulatory molecules CD80 and CD86 expressed on the APC bind to CD28 on the T cell, providing an important co-stimulatory signal [13]. The third signal is mediated by the particular cytokine milieu that plays a major role in inducing the transcription factors that determine the polarization towards different effector T cell populations [14], [15]. Based on initial experiments by Mosmann & Coffman *et al*., 1986 [16], Th cells were classified into two stable and terminally differentiated cell lineages, Th1 and Th2 cells.

Th1 cell differentiation is induced by interferon (IFN)-γ [17], [18] and interleukin (IL)-12 [19], [20] signaling through signal transducer and activator of transcription (STAT) 1 and STAT4, respectively [18], [21]. These transcription factors in turn induce the expression of the master transcription factor T-box expressed in T cells (T-bet) directing Th1 cell differentiation. Th1 cells selectively produce IFN-γ required for efficient immune responses against intracellular pathogens (**Fig. 1B**) [22]. Th2 cell differentiation is driven by IL-4 [23], [24] signaling via STAT6, which activates the expression of the Th2 key regulator GATA-binding protein 3 (GATA-3) [25]. GATA-3 then directs the Th2 differentiation by regulating the production of the Th2 cell lineage-specifying cytokines IL-4, IL-13, and IL-5 [26]–[28]. These Th2 cytokines are critical for immunity against helminths and other extracellular pathogens, but Th2 cells also mediate various allergic responses including asthma and atopic dermatitis (**Fig. 1B**).

Since the introduction of the concept of the Th1/Th2 paradigm in the mid-1980s, considerable progress has been made in the field of $CD4^+$ T cell line differentiation. Besides Th1 and Th2 cells, other $CD4^+$ T cell subsets have been identified in the past decades; each characterized by a certain master regulatory transcription factor and signature cytokines with distinct effector functions on other cells. These include Th17, Th22 and regulatory T cells (Treg) (**Fig. 1B**). The combination of IL-6 and transforming growth factor (TGF)-ß induce the differentiation of Th17 cells, expressing retinoic acid-related orphan receptors (ROR)γt as their master transcription factor, resulting in IL-17 production [29]. Th17 cells play a critical role in autoimmune diseases, such as rheumatoid arthritis, psoriasis as well as allergic responses [29], [30]. Naïve $CD4^+$ T cells that are exposed to TGF-β alone express forkhead box P3 (Foxp3), the key transcription factor of Treg, which secretes primarily the anti-inflammatory cytokines IL-10 and TGF-β [31]. Tregs are known to suppress inflammation and inhibit autoimmunity [29], [31]. The presence of IL-6 together with TGF-α promotes Th22 lineage commitment. Th22 cells are characterized by their master regulatory transcription factor aryl hydrocarbon receptor (AHR) leading to a strong production of IL-22, whereas it inhibits IL-17 production [32], [33]. IL-22 contributes to skin homeostasis but also play an important role in inflammatory and autoimmune diseases such as atopic dermatitis [32], [34], [35], psoriasis [32], [36] and rheumatoid arthritis [37].

Based on the distinct expression profiles of master transcription factors and signature cytokines resulting from highly controlled *in vitro* stimulation conditions and the multiple mechanisms of positive feedback [38], [39] and reciprocal inhibition [40], [41], the differentiation of the Th1 and Th2 lineages were assumed to be mutually exclusive [42]. However, early studies have described that both human and murine T cell clones can co-express cytokines of two Th cell lineages, such as Th1 and Th2 cytokines [43]–[45]. These findings were thought to represent an uncommitted and pluripotent state, but that ongoing stimulation would finally lead to the stable conventional Th1 and Th2 cell lineages [46]–[48].

However, Hegazy *et al.*, 2010 [10] showed that differentiated murine Th2 cells can also express T-bet and IFN-γ when stimulated appropriately. In fact, a Th1-inducing virus infection reprogrammed otherwise stably committed murine GATA-3$^+$ Th2 cells to adopt a GATA-3$^+$T-bet$^+$ and IL-4$^+$IFN-γ$^+$ ‘‘Th2+1’’ phenotype (hereinafter referred to as Th2/1). This phenotype was maintained *in vivo* for months, suggesting ‘‘lineage-like’’ properties [10]. The observation of other hybrid differentiation phenotypes revealed a previously overlooked flexibility of the classic Th cell lineages [49]–[51] leading to intensive discussion on CD4$^+$ T cell plasticity and its functional relevance [31], [52]–[54]. Moreover, Peine *et al*., 2013 [48] described for the first time that Th2/1 hybrid cells develop alongside classical Th2 cells in mice infected by parasitic worms (helminths).

1.1.2. The Th2/1 hybrid phenotype – commitment of naive CD4$^+$ T cells to a simultaneous Th1/Th2 differentiation program

Soon after the first description of Th1 and Th2 cells it was shown that both human and murine T cell clones have the capability to co-produce the Th1 and Th2 cytokines IFN-γ and IL-4, respectiveley [43]–[45]. Moreover, as addressed above, fully differentiated Th2 cells can adopt Th1 features triggered by a Th1-inducing infection, leading to a Th2/1 phenotype co-expressing Th2 and Th1 markers [10]. Remarkably, further studies revealed that Th2/1 cells also arise naturally during parasitic helminth infections, which induce Th2 immune responses. Mice infected with the trematode *Schistosoma mansoni* displayed a substantial cell population co-expressing GATA-3 and T-bet [48] as well as IL-4 and IFN-γ [55] at the single-cell level. Of note, schistosome infections are characterized by an early Th1 response driven by the larval stage and adult worms before egg deposition, followed by the development of a strong Th2 response when eggs are released by the worms dwelling in the venous system and when many of these eggs are trapped in host tissue [56]. This left the possibility that the cells co-expressing Th2 and Th1 markers were reprogrammed Th1 cells induced before egg deposition. However, the bifunctional Th2/1 phenotype also occurred in mice directly immunized and challenged with *S. mansoni* eggs in the lung [48], which induces a strongly Th2-biased response [57], [58]. Furthermore, also a straightly Th2-polarized response induced by the infection with the murine intestinal nematode *Heligmosomoides polygyrus* comprised a considerable fraction of Th2/1 cells in spleen and blood of mice [48]. The simultaneous commitment of naive CD4$^+$ Th cells to the two opposing Th1 and Th2 differentiation programs (**Fig. 1C**), has been characterized as follows: (I) Murine Th2/1 cells co-express the Th1 and Th2 cell lineage-defining transcription factors and cytokines (IFN-γ/IL-4, IFN-γ/IL-5 or IFN-γ/IL-13) at intermediate levels compared to the conventional counterparts. (II) During memory development, Th2/1 cells follow the same kinetics as conventional Th2 cells, are stably maintained for months and resist reprogramming into Th1 or Th2 cells. (III) The combination of IFN-γ, IL-12 and IL-4 induce the differentiation of Th2/1 cells *in vitro* (**Fig. 1C**). IFN-γ signals are essential for Th2/1 cell development, as IL-4 dominates over IL-12 signals. (IV) Importantly, Th2/1 cells support the immune responses in murine models of type-1 and type-2 inflammation, but cause less immunopathology than conventional Th1 and Th2 cells, respectively (**Fig. 1C**).

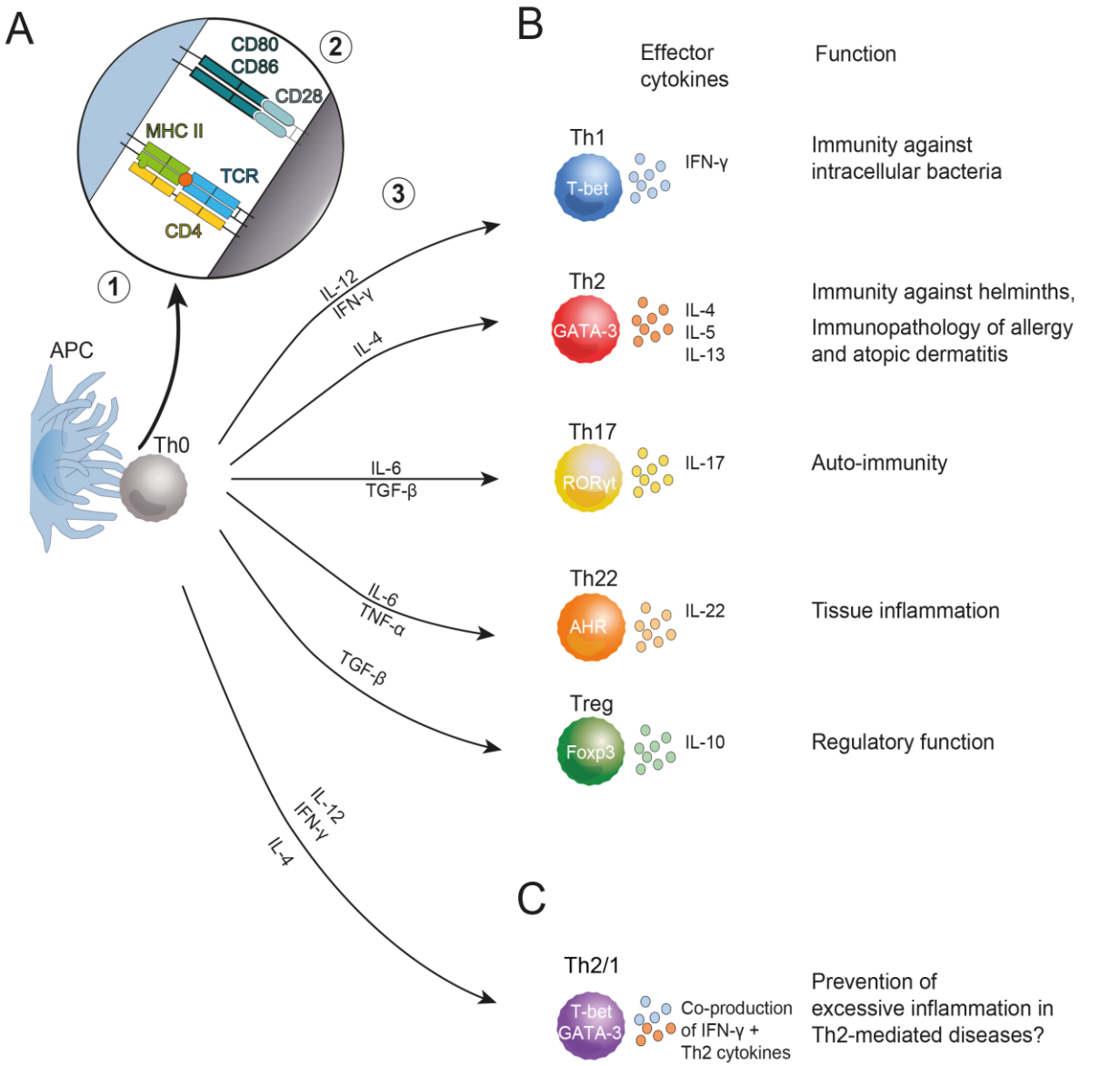

Fig. 1.1: Schematic overview of CD4⁺ T cell activation and differentiation into various T cell lineages. (A) 1. Triggering of the initial activation of CD4⁺ T cells: The T cell receptor (TCR) of a naïve CD4⁺ T cell (Th0) binds to the antigen presented by the major histocompatibility complex II (MHCII) on the surface of an antigen-presenting cell (APC). The T cell co-receptor (CD4) also binds MHCII on the APC, stabilizing the interaction. **2.** Enhancement of T cell activation and initiation of T cell proliferation: T cells require a second signal for full activation and progress to a highly proliferative state, which is provided by the binding of CD80 or CD86 on the APC to CD28 on the T cells. **3.** T cell differentiation: The cytokines present in the microenvironment predominately govern the decision on T cell differentiation. The indicated cytokines induce the corresponding T cell lineages as shown in (B)**. (B)** CD4⁺ T cell lineages with their respective effector cytokines and functions. **(C)** The Th2/1 hybrid phenotype: Simultaneous integration of Th1 and Th2-polarizing signals leading to the development into Th2/1 hybrid cells that stably co-express Th1 and Th2 cell lineage-specifying transcription factors and cytokines.

These findings suggest that the acquisition and maintenance of a stable Th2/1 hybrid phenotype expressing two opposing Th differentiation programs could contribute to the prevention of excessive immunopathology [51]. Based on these interesting and important findings further questions arise: Are Th2/1 cells restricted to mice or also detectable in humans? Is the Th2/1 cell differentiation a common finding in helminth infections? Does the Th2/1 phenotype occur also in other settings of type-2

inflammation? Do Th2/1 cells represent a target for therapeutic approaches in excessive inflammation?

To address these questions, this work assessed Th2/1 responses in threadworm infected mice and humans, but also in patients suffering from atopic dermatitis. An overview on the characteristics of immune responses to threadworm infections and in atopic dermatitis is provided in the following sections.

1.2. Adaptive immune responses in Th2-mediated diseases

Type-2 immunity comprises a spectrum of disorders. While type-2 immune responses are induced by and confer protection against helminths, they can also play pathologic roles, promoting acute and chronic inflammatory responses against a myriad of allergens, resulting in e.g. eczema and asthma [59]. These disorders give rise to a similar course of inflammation and pathology [60]. It is estimated that more than 3 billion people worldwide are affected by diseases resulting from type-2 inflammation [59], [61], [62]. Despite the medical and economic impact of these disorders, it remains elusive how the diverse stimuli trigger type-2 responses and how the cellular and molecular networks orchestrate their protective versus pathologic roles in various infectious or inflammatory settings. As such, there is a great interest in identifying factors that can be therapeutically targeted to reduce disease susceptibility and progression. Hence, further analyses of the bifunctional Th2/1 phenotype, which seems to be associated with attenuated immunopathology in murine inflammation models [51], might add a new piece in unraveling the complex Th2 network in mice and particularly in humans. Therefore, the present work investigated the Th2/1 phenotype in mice and humans infected with threadworms and patients afflicted with atopic dermatitis (AD).

As mentioned above, Th2 responses are characterized by $CD4^+$ T cells, which secrete the signature cytokines IL-4, IL-5 and IL-13. Each of them has a well-defined and relatively specific function. Although IL-4 and IL-13 share receptor components and signaling pathways, differences in the expression of their receptors as well as their affinity for these ligands result in the two cytokines mediating distinct functions [63], [64]. IL-4 induces the differentiation of Th2 cells, is the major driving factor of IgE class switching in B cells, alternative macrophage activation as well as mast cell activation [59], [65], [66]. IL-13 is involved in several stages of B-cell maturation, differentiation and IgE class switching, but it also mediates eosinophilic inflammation, airway hyper-responsiveness, mucus hyper-secretion and worm expulsion in helminth infections [60], [66], [67]. IL-5 is the major eosinophil-supporting cytokine and induces eosinophilopoiesis and eosinophil release from the bone marrow, enhances eosinophil survival, and acts as a co-stimulator for eosinophil activation [67], [68].

1.2.1. Parasitic disease caused by *Strongyloides* spec.

Pathogenic worm infections affect approximately two billion people worldwide, with soil-transmitted helminths being the most prevalent. Infections with the threadworm *Strongyloides stercoralis* are widely distributed, mainly in tropical and subtropical regions, with an estimated 30-100 million infected people worldwide [69]. As the course of disease is usually asymptomatic or leads to only mild symptoms [70], infections with this enteric nematode represent an underestimated health risk with a high number of undetected cases [71]. Moreover, due to its zoonotic transmission capacity [72] and the increase in travels, strongyloidiasis has been rated as an important emerging global infection [73]. This enteric worm differs from other human enteric nematodes in that it may lead to life long infections and can have devastating effects in particular in immunosuppressed individuals. Uncontrolled multiplication of the parasite (hyperinfection) and potentially life-threatening dissemination of larvae to all internal organs in immunocompromised patients result in mortality rates of up to 85 % [72], [74], [75].

Apart from the exceptional impact on health in high-risk patients, *S. stercoralis* has a unique life cycle. An essential feature is that *Strongyloides* is the only helminth parasite of clinical importance that can complete its entire life cycle and hence replicate within the human host [72]. This manifests in two important clinical features: the possibility of autoinfection and the development of persistent infections without further exposure to infective larvae from the environment [76], [77].
Moreover, this nematode can develop in two ways (**Fig.1.2**): The rhabditiform larvae (L1, L2) develop either directly into filariform infectious stages (L3) or into free-living adults, which reproduce sexually in the soil. These infectious L3 larvae penetrate the skin and are transported via the blood stream to the lungs. From there, larvae migrate to the throat, are coughed up and swallowed. In the small intestine where the larvae live threaded in the epithelium, females molt twice and produce eggs via parthenogenesis. The rhabditiform larvae hatch and can be either passed in the stool or autoinfect the host [78], [79].
Strongyloides is the only helminthic parasite that secretes larvae (not eggs) in faeces, appearing approximately one month after skin penetration [73]. However, the low parasitic load in most infected patients and the irregular larval output by intestinal adult females [80], leads to difficulties in diagnosis resulting in low sensitivity in parasitological techniques [72]. Currently a combination of molecular and serological tests are most reliable and the most sensitive diagnostic methods of choice [72], [75]. However, until today no method or biomarker have been identified to assess the severity or allow the prognosis of the course of a *Strongyloides* infection, later potential co-morbidities or co-infections. Chronic helminth infections are known to be associated with modulation of $CD4^+$ T cell responses, but the exact role of $CD4^+$ T cells, their cytokine response patterns or other immunological markers are relatively poorly defined in strongyloidiasis [81]. In order to shed more light on $CD4^+$ T cell responses in infections with *Strongyloides* and based on the findings mentioned above, this work aimed to functionally characterize the bifunctional Th2/1 hybrid phenotype alongside the conventional Th2 response in this parasitic infectious disease. Therefore, the profiles of transcription factor and cytokine expression of T cells were analyzed. Since various larval stages reside in specific locations in the

host, an animal model was used to investigate $CD4^+$ T cell subsets in different organs and to test the suitability of blood samples (the only accessible source for T cells of *Strongyloides*-infected patients in the current work) and to prove the existence of Th2/1 cells in humans.

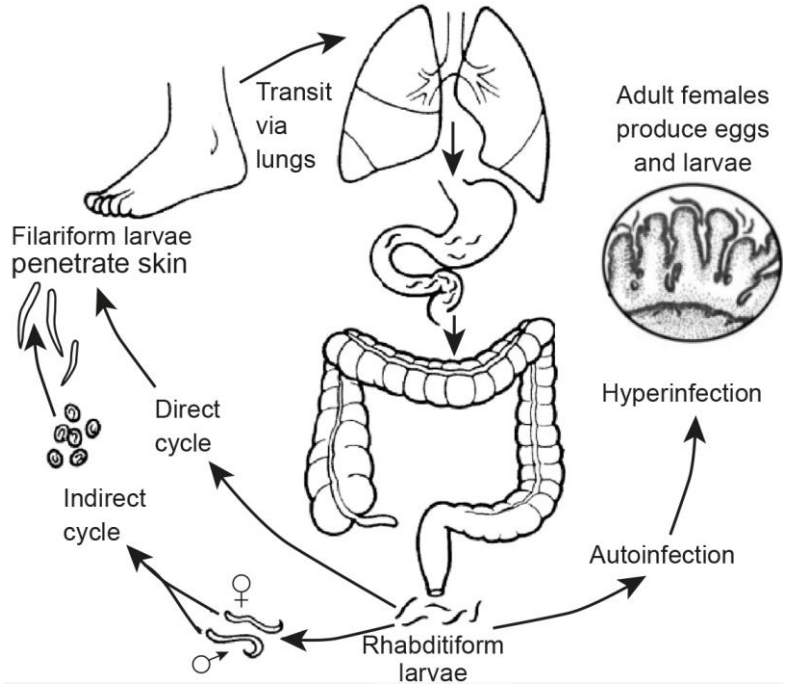

Fig.1.2: Life cycle of Strongyloides stercoralis (Modified from Carvalho & Da Fonseca Porto, 2004 [78]). Rhabditiform larvae transform indirectly, free-living, or directly, inside the human host, to the infectious filariform stage. Larvae migrate via bloodstream to the lungs, are coughed up, swallowed and finally reach the gut. There, adult females produce eggs, larvae hatch, which autoinfect the host and can lead to hyperinfections.

Because *S. stercoralis* infects humans, primates and dogs [72], [82], but does not develop beyond the third larval stage in mice [83], this analysis employed the experimental system of murine *Strongyloides ratti* infections to study the adaptive immune response to strongyloidiasis [82], [84], [85]. *S. ratti* is a rodent-specific threadworm, closely related to *S. stercoralis*, with a rapid life cycle that includes tissue-migrating and intestinal parasitic stages [71]. Infective third-stage larvae (L3i) actively penetrate the skin of their mammalian host and migrate within two days presumably via the lung to the mouth, are swallowed and reach the gut [84], [86]. Even though this and other models provide the opportunity to study the immune response to this type of parasites, it has to be considered that rodent threadworms (*S. ratti* and *S. venezuelensis*) do not lead to hyperinfection and prolonged chronic infections [82], [87], in contrast to *S. stercoralis* infecting humans.

Still, mice are fully susceptible to patent infection with *S. ratti*. Th2 responses essential for the prevention of hyperinfection or severe disease in *S. stercoralis* infections [88], [89] are induced in both host species. In helminth infections, the Th2 response exerts a protective effect in that it leads to recruitment and infiltration of eosinophils, basophils and mast cells, and differentiation of alternatively activated macrophages, which are important players for larvae killing and/or adult worm expulsion [90]–[92] (**Fig. 1.3**). The Th2 cytokine IL-4 is essential for IgE production and mast cell activation,

while IL-13 is critical for mucus production, goblet cell hyperplasia and worm expulsion [60], [93] (**Fig. 1.3**). Moreover, Th2 cytokines regulate tissue repair and inflammatory control [90].

In contrast to helminth-infections, the persistence of Th2 cells and Th2 cytokine production are critical characteristics associated with exacerbation of allergic diseases, such as atopic dermatitis (AD) [10], [94]. To find out whether Th2/1 cells occur alongside pro-inflammatory $CD4^+$ T cell subsets during a helminth-independent Th2-driven disease, blood samples of patients afflicted with AD were analyzed.

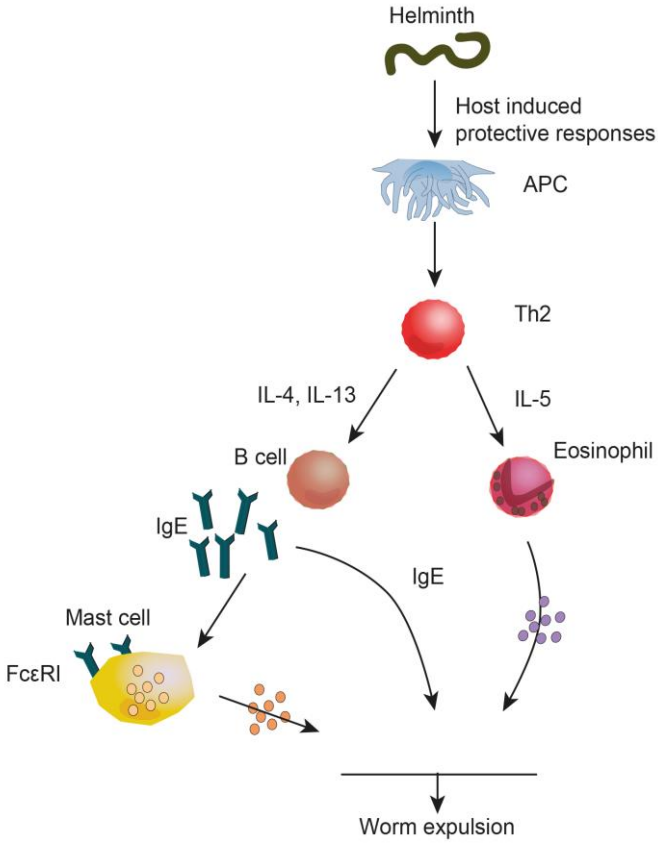

Fig.1.3: Th2 immune response induced by helminth infection. Helminth antigens are processed by APC and presented to $CD4^+$ T cells that differentiate to Th2 cells. Secreted Th2 cytokines activate and attract adaptive and innate immune cells. IL-4 and IL-13 induce the differentiation of antigen-specific B cells, which produce the antibody IgE. Antibodies opsonize helminth antigens and larvae and promote binding of innate immune cells via Fc receptors (R), here the high-affinity IgE receptor (FcεRI) on mast cells is shown. Activated mast cells, as well as eosinophils and neutrophils (not shown) release toxic granule proteins which may damage/kill the parasite larvae in the tissue and other mediators to attract further immune cells, which also permits the expulsion of adult worms from the gut.

1.2.2. Atopic dermatitis

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin disorders. It affects both children and adults but often starts already during early infancy. AD is characterized by acute flare-ups of eczematous pruritic lesions over dry skin and can be the initial step of the so-called “atopic march”, reflecting progression from AD to allergic asthma and allergic rhinitis [94], [95].

Although AD is primarily defined by clinical criteria [96], it is recognized as a complex disease with a wide spectrum of clinical phenotypes based on severity of disease, age of onset, serum level of IgE (extrinsic vs. intrinsic AD that means elevated vs. normal IgE level), ethnic background, acute versus chronic course, mutations in the epidermal protein filaggrin and infectious or allergic/irritant triggers [96]–[99]. Despite the increasing prevalence of AD worldwide [100] and significant psychological and economic burden of the disease due to impaired quality of life (chronic pruritus, sleep disruption and depression [101], [102]), targeted therapies in particular for severely affected patients are insufficient. For an optimal individualized therapeutic concept it might be beneficial to stratify the complex clinical phenotypes into more homogeneous subgroups based on a panel of robust immunological biomarkers [97].

Atopic skin is characterized by enhanced epidermal proliferation, disturbed differentiation, and alterations in skin lipid composition and organization mediated [103], [104] by immunological dysregulation [105]. Excessive immune activation marked by significant skin infiltration by T cells and dendritic cells play a critical role in AD. A predominant Th2 response with increased IgE levels and eosinophilia is widely accepted as central to the pathogenesis of this disease [67], [94], [95], [106]. Allergens are common triggers for eczematous skin lesions in AD. In particular, aeroallergens such as grass pollen can induce a worsening of cutaneous symptoms [107]. About 40 % of allergic patients in Europe are sensitized to the most common grass pollen allergen derived from timothy grass (*Phleum pratense*) [108]. The immunodominant major allergen isoforms Phl p 5.01 and Phl p 1 are responsible for more than 90 % of allergic activity in grass pollen-sensitized patients [109].

Activated Th2 cells lead to two AD hallmarks, including the disruption of barrier integrity proteins such as filaggrin, occludin and others, and a reduction in skin lipids [105]. The Th2 cytokines IL-4, IL-5 and IL-13 are significantly increased in lesional and non-lesional skin in AD [94], [95], [100]. Apart from Th2 cytokines, other markers and pro-inflammatory mediators associated with Th2 responses have been described. Cutaneous lymphocyte-associated antigen-bearing (CLA) T cells are specialized for skin-homing, representing the main T cell population in AD lesions and appear to be an important pathogenic factor [35], [110], [111]. CLA is also expressed on the surface of circulating $CD45RO^+$ memory T cells and may represent a reliable surrogate marker of the inflammatory events occurring in the skin [110], [111]. This allows phenotyping of activated CLA^+ T cell subsets in peripheral blood and may eliminate the need for skin biopsies for studying the impact of T cells in skin diseases. A further surface marker considered as a reliable marker for the detection of circulating Th2 cells is the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [112], [113]. This prostaglandin D2 receptor has been described to be important for allergic skin inflammation after epicutaneous antigen challenge [114]. The tissue alarmin cytokine thymic stromal lymphopoietin (TSLP) is expressed primarily by epithelial cells, including keratinocytes and drives Th2 polarization [95]. This pro-inflammatory cytokine is associated with the activation and migration of dendritic cells within the dermis, which then prime Th cells to produce pro-allergic Th2 cytokines [95], [105].

Further Th cell subsets were described to contribute to skin inflammation are Th22, Th1 and Th17 cells [67], [94], [106], [115]–[117] (**Fig. 1.4**). It has been reported that IL-22 secreted by activated Th22 cells is up-regulated during AD and correlates to disease severity [35], [115], [117]. IL-22 acts on keratinocytes, the predominant cell type in the epidermis. It down-regulates genes for terminal

differentiation of keratinocytes and tight junction products, such as claudins, contributing to barrier defects [118] and leading to epidermal hyperplasia [117], [119], [120].

Historically AD is considered as a biphasic disease with a predominant Th2 response in acute disease and a later contribution of Th1 activity in the chronic phase of disease [121], [122] (**Fig. 1.4**). A recent study of paired non-lesional, acute and chronic skin lesions from AD patients demonstrated that the onset of acute AD is characterized by high levels of Th2 and Th22 cells with intensification of these pathways in chronic disease, rather than a switch to a Th1 polarization [117], [106]. However, this study detected also an increase in Th1 related markers during chronic disease [117]. The Th1 cytokine IFN-γ was postulated to promote skin hypertrophy [123] and to induce epidermal hyperplasia together with IL-22 [124].

Human Th17 cells produce high levels of IL-17A and IL-17F [125]. Their role in patients with AD is still controversial, as several studies reported conflicting data on either increased or decreased levels of Th17 cells in AD [35], [126], [127]. An increase of Th17 cells linked to AD might depend on the ethnic background, as a positive correlation of Th17 cells and disease severity was observed in Asian populations [128], [129], but not in European American populations [35]. Moreover, it has been described that patients with the rare intrinsic AD subtype (normal serum IgE levels) display a higher Th17 immune activation compared to patients with extrinsic AD (elevated IgE levels) [130]. However, in the majority of AD patients Th17 cells seem to play a minor role compared to Th22 and Th2 cells [32], [105]. IL-17 is considered to stimulates epithelial cells and fibroblasts to secrete pro-inflammatory mediators such as IL-8, IL-6 and IL-11 and thereby possibly act in cutaneous remodeling in AD [131], [132].

Besides immunological dysregulation by Th cell subsets, the skin barrier can be disturbed due to loss-of-function mutations in the filaggrin (FLG) gene [133], the most-widely occurring genetic risk factor for AD identified so far [134]. Filaggrin deficiency affects several pathways relevant for epidermal barrier dysfunction, like disturbed keratinocyte differentiation [135], impaired tight-junction formation [136], decreased water retention [137], stratum corneum acidification [138] and enhanced susceptibility to cutaneous infections [96]. In order to investigate the direct interplay between pro-inflammatory processes driven by T cells together with filaggrin deficiency, this work included studies employing filaggrin-deficient skin equivalents mimicking the hallmarks of AD [134].

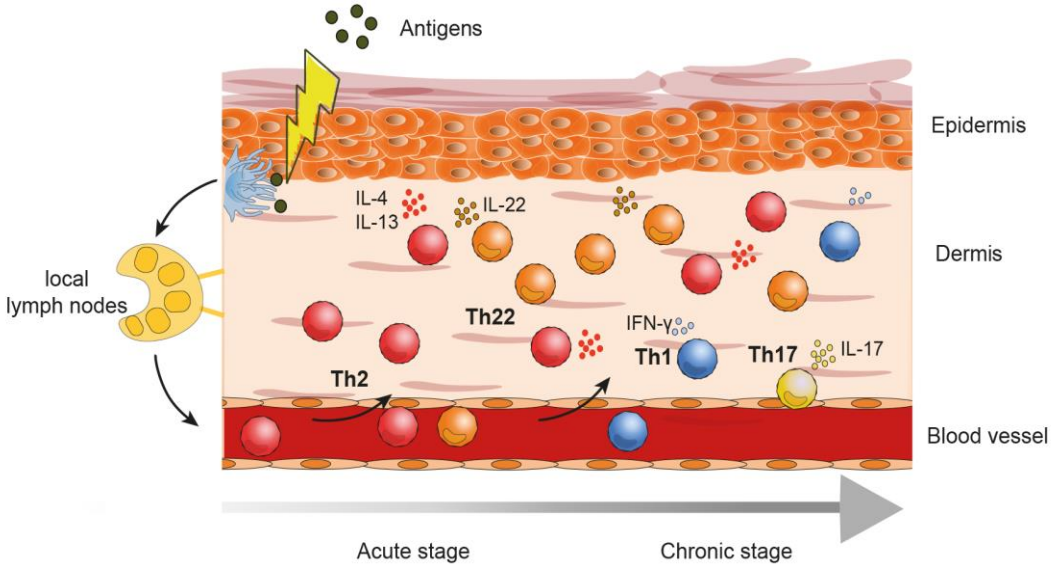

Fig. 1.4. CD4⁺ Th cell subsets that play a role in AD pathogenesis. AD is characterized by the predominant activation of Th2 and Th22 cells, while Th1 and Th17 cells play a minor role. Barrier defects lead to penetration by epicutaneous antigens into the dermis. Activated skin-resident DC migrate to local lymph nodes and polarize naïve Th cells into the Th2 phenotype. Th2 cells are recruited back to the skin. There they induce cutaneous inflammations by effector cytokines accompanied by Th22 cells. A progressive activation of Th2 and Th22 cells are hallmarks of the chronic stage of AD, which is also characterized by a switch to a Th1 cell environment, but not as prominent as the other two Th subsets. Th17 cells may play a role in certain AD-subtypes, such as intrinsic AD.

2. Aims of this work

The induction of a bifunctional $CD4^+$ Th2/1 hybrid subpopulation alongside a strong Th2 response was first described for mice infected with *Heligmosomoides polygyrus* and *Schistosoma mansoni* [48]. Th2/1 hybrid cells with combined characteristics of Th2 and Th1 cells at single cell level demonstrated two prominent characteristics: First, the hybrid phenotype was refractory to reprogramming into classical Th2 or Th1 cells [48]. Second, these cells caused less immunopathology *in vivo* than the respective conventional Th2 subset in experimental models of airway inflammation and delayed type hyperreactivity [48]. These findings suggested the novel concept of the generation of a stable Th2/1 subset leading to the limitation of Th2 responses. This, in turn, might prevent excessive inflammation during chronic helminth infections and leads to the development of an immune response comprising Th2 and Th1-like activity. Based on these assumptions, the present work explored the following hypotheses:

(I) Th2/1 hybrid cell differentiation is a general phenomenon in murine helminth infections.

(II) Helminth-infected humans generate Th2/1 cells with similar characteristics as murine Th2/1 hybrids.

(III) Th2/1 cells are not restricted to helminth infections, but also occur in patients with other Th2-driven diseases.

(IV) The proportion of human hybrid cells correlates with distinct clinical disease phenotypes and is associated with low pathology.

The first aim of this work was to detect and phenotypically characterize Th2 and Th2/1 hybrid cell responses in murine and human helminth infections. For this purpose, different organs of mice and blood samples of South Indian patients infected with threadworms were examined. That enabled comparative analyses of murine and human T cell responses against an intestinal nematode of the same genus, *Strongyloides*. Murine local and systemic responses as well as human peripheral blood cell responses were assessed with particular emphasis on the profiles of transcription factor and cytokine expression of murine and human Th2/1 hybrid cells.

The second aim was to prove the existence of hybrid cells in patients with a helminth-unrelated Th2-driven disease and to survey whether the proportions of Th2/1 hybrid cells could be linked to distinct disease phenotypes. This part was performed based on blood samples of German patients affected by atopic dermatitis with different clinical outcomes. Finally, the effector function of human Th2/1 hybrid cells was examined in an *in vitro* 3D skin equivalent mimicking the hallmarks of atopic dermatitis. Barrier defects and pro-inflammatory mediators were measured to compare the pathological impact of human Th2 and Th2/1 cells.

In summary, the present work addressed whether $CD4^+$ Th2/1 hybrid cells are common players in the immune response against parasitic worms, whether they occur with similar features in mice and men and whether their proportions differed depending on disease severity in patients with atopic dermatitis. The last aspect was of special interest as Th2/1 hybrid proportions might be a valuable diagnostic marker for the progression of clinical manifestations of atopic/allergic disorders.

3. Results

3.1. Characterization of the Th2 and Th2/1 immune response in murine infection with the nematode *Strongyloides ratti*

3.1.1. Murine *S. ratti* infection leads to systemic and local Th2 responses and suppressed intestinal IFN-γ responses

In order to survey Th2 and Th1 activity in murine threadworm infections, C57BL/6 mice were infected with 200 or 2000 infective stage 3 larvae (iL3) of *Strongyloides ratti*, which were kindly provided by Dr. Minka Breloer, Bernhard Nocht Institute (BNI), Hamburg, Germany. Mice infected with low and high parasite burden were dissected at day 10 or day 20 post infection (p.i.) and organs were analyzed (**Fig.3.1A**). Two infective doses were chosen to see whether a presumably low load of *Strongyloides* worms expected for human threadworm infected patients [72] would hamper the analyses of the immune response in peripheral blood samples (the only material accessible for human patients). The two time points of infection reflected the early versus late stage of infection of the murine experimental model. Worm burdens were determined at day 6 p.i. by quantification of *S. ratti* 28S ribosomal RNA coding DNA in faeces (performed by the team of Dr. Minka Breloer, BNI). Expectedly, mice inoculated with 2000 larvae showed a significantly higher parasite burden than mice infected with 200 larvae (**Fig. 3.1B**) [84]. Histological stains of proximal small intestinal tissue samples (performed by Dr. Anja Kühl, Research Center ImmunoSciences, Charité Berlin, Germany) displayed mild signs of cellular infiltration in some mice at day 20 p.i., whereas no immunopathological changes were detected after 10 days p.i. (**Fig. 3.1C**). The numbers of Th2-driven mucus-producing goblet cells tended to increase in animals infected for 10 days but returned to baseline levels by day 20 p.i. (**Fig.3.1D**). No significant pathological changes were observed in mice with a 10-fold higher parasite burden of 2000 iL3 compared to animals infected with only 200 iL3 or naïve mice (**Fig. 3.1C-D**). These results suggested rather low immunopathological changes during murine threadworm infection.

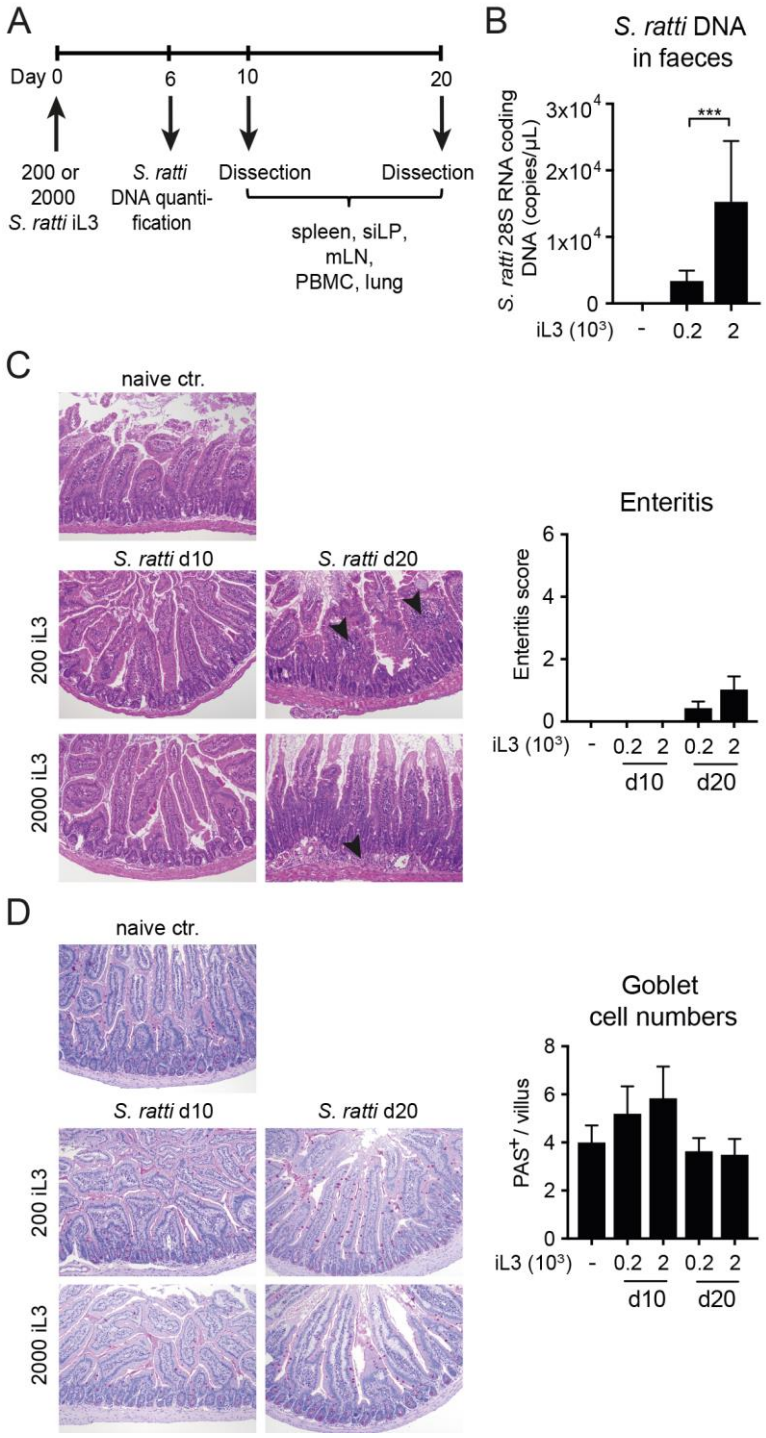

Fig. 3.1: *S. ratti* infection leads to mild immunopathology in mice. (A) Scheme of experimental setup. C57BL/6 mice were infected with 200 or 2000 *S. ratti* infective third-stage larvae (iL3) and dissected 10 or 20 days post infection (p.i.). (B) Quantification of *Strongyloides* DNA at day 6 p.i. (C) Exemplary pictures of proximal small intestinal cross sections stained with hematoxylin and eosin (HE). Magnification x 100. Arrow heads mark mild cellular infiltrates in the lamina propria at day 20 p.i. Bar graph depicts enteritis scores of experimental groups. (D) Exemplary pictures of proximal small intestinal cross sections stained with Periodic acid-Schiff (PAS). Magnification x 100. Mucus filled goblet cells appear magenta stained. Bar graph displays quantification of goblet cells per villus in small intestinal cross sections. Mean + SD of n = 6 (naïve ctr.) and 5 (infected) mice is shown. Data originate from one out of two experiments with similar results. ***$p < 0.005$ comparing infection dose in (B), when analyzed with Student's *t*-test. No significant differences between infected to naïve mice for (C) and (D) when calculated with one-way ANOVA. (Modified from Bock *et al*., 2017)

In contrast to the histological data, flow cytometric analyses of conventional Th2 and Th1 cells as well as Th2/1 hybrid cells revealed significant differences between naïve and infected mice. Cells from spleen, peripheral blood mononuclear cells (PBMC), mesenteric lymph nodes (mLN), small intestinal lamina propria (siLP) and lungs were stimulated with phorbol myristate acetate (PMA) and ionomycin and examined by eight-color flow cytometric antibody panels (details see “Materials and methods” Tab. 6.1, 6.2.3). Following the gating strategy shown in **Fig. 3.2.** $CD4^+$ T cells were further assessed for the frequencies of the T helper cell subsets of interest.

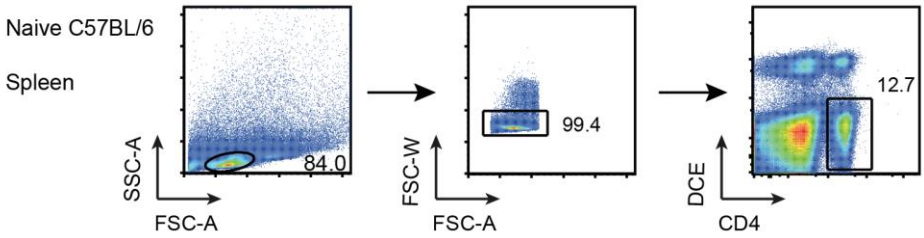

Fig. 3.2: Gating strategy for live $CD4^+$ T cells for further analyses of T cell subsets. Exemplary pseudocolor plots displaying cells isolated from spleen of a naïve control mouse. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed by flow cytometry. Gating on lymphocytes (left), single cells (middle), and live $CD4^+$ T cells (right) is shown. FSC: forward scatter; SSC: side scatter; A: area; W: width; DCE: dead cell exclusion dye.

Infected mice displayed an elevated systemic Th2 response compared to naïve animals, represented by higher levels of $CD4^+$ T cells expressing GATA-3 and IL-4 isolated from spleen (**Fig. 3.3A**, **B**). Similarly, PBMC of infected mice comprised significantly elevated frequencies of Th2 cells based on GATA-3 expression, while the frequencies of IL-4^+ cells tended to increase (**Fig. 3.3C**). Of note, significantly increased frequencies of splenic GATA-3^+ cells were detected in mice infected for 10 and 20 days with a low and high worm burden, whereas the levels of GATA-3^+ cells in blood did not reach statistical significance at day 20 compared to naive controls. Local Th2 responses in mLN followed a similar pattern as spleen responses with significantly more GATA-3^+ cells at day 10 and 20 and for both infection doses, whereas significant IL-4 responses were restricted to the early time point (**Fig. 3.3A**, **D**). Significantly elevated Th2 responses in tissues affected by the adult stage (small intestine), but also by migrating larvae (lung) were more robustly detectable based on GATA-3 expression as compared to IL-4 expression at both time points and for both infection doses (**Fig. 3.3A**, **E** or **Fig. 3.3F**, respectively).

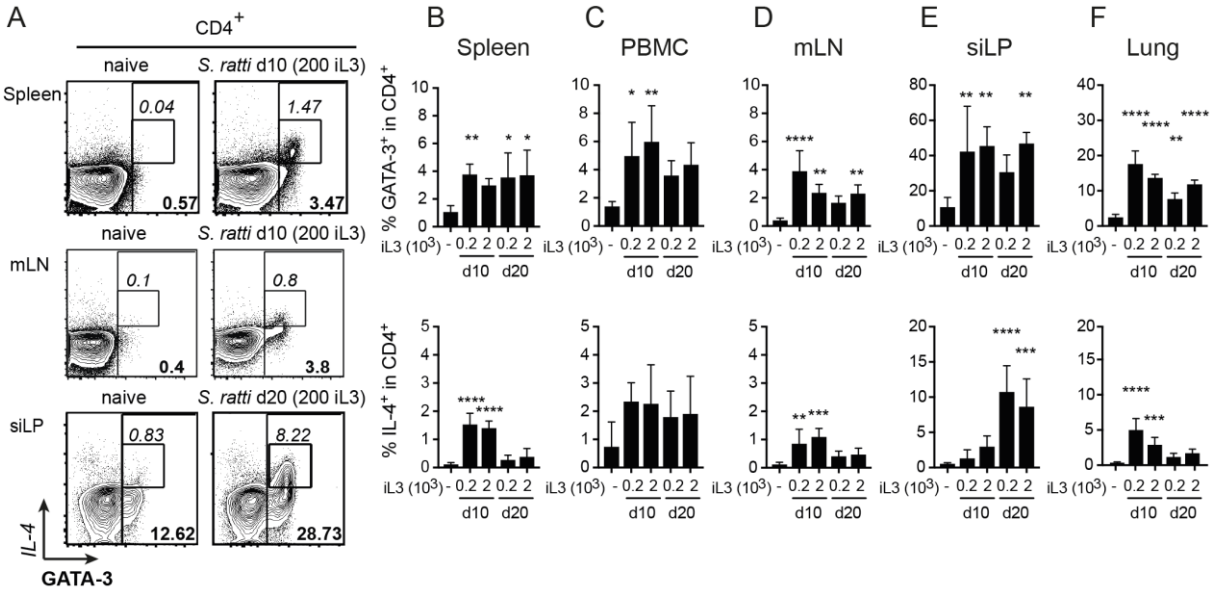

Fig. 3.3: *S. ratti* infection results in an elevated systemic and local Th2 response. CD4⁺ Th2 cells isolated from different organs of infected C57BL/6 mice (200 or 2000 iL3 of *S. ratti*) and uninfected naïve animals. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry. (A) Representative contour plots of live CD4⁺ T cells derived from spleen, mesenteric lymph nodes (mLN) and small intestinal lamina propria (siLP) of an uninfected naïve (left) and a *S. ratti*-infected (200 iL3, right) mouse at the depicted days p.i. Bold numbers indicate frequencies of GATA-3⁺ cells while italic numbers report frequencies of GATA-3⁺IL-4⁺ cells. (B-F) Frequencies of GATA-3⁺ cells (top) and IL-4⁺ cells (bottom) within live CD4⁺ T cells isolated from spleen (B), PBMC (C), mLN (D), siLP (E) and lung (F). Mean + SD of n = 5-6 (naïve control) and n = 4-5 (infected) mice. Data from one out of two experiments with similar results are shown. **p* < 0.05, ***p* < 0.01, ****p* < 0.005, *****p* < 0.001 comparing infected to naïve mice calculated with one-way ANOVA. (Modified from Bock *et al*., 2017)

In contrast to Th2 cells, conventional Th1 cells are characterized by the expression of T-bet and the production of IFN-γ. In spleens of infected mice a strong Th1 response was detected, represented by a significant increase of T-bet⁺ T cells at day 20 p.i., while IFN-γ expression was transiently elevated at day 10 p.i. (**Fig. 3.4A**, **B**). PBMC of infected mice displayed slightly increased levels of both Th1 markers (**Fig. 3.4C**), whereas frequencies of T-bet⁺ and IFN-γ⁺ cells isolated from mLN and lung were similar in all groups (**Fig. 3.4A**, **D, F**). Strikingly, mLN of all groups reflected very low levels of T-bet and IFN-γ. Remarkably, siLP of mice infected with threadworms presented similar frequencies of T-bet⁺ cells, but significantly reduced levels of IFN-γ⁺ cells (**Fig. 3.4E**).

Taken together, the relatively asymptomatic course of murine strongyloidiasis was accompanied by local and systemic Th2 responses confirming previous studies [84], [139]. A transiently increased IFN-γ production and expansion of T-bet expressing Th1 cells, however, was restricted to spleen and PBMC, whereas IFN-γ activity in the infected small intestine decreased significantly in infected mice.

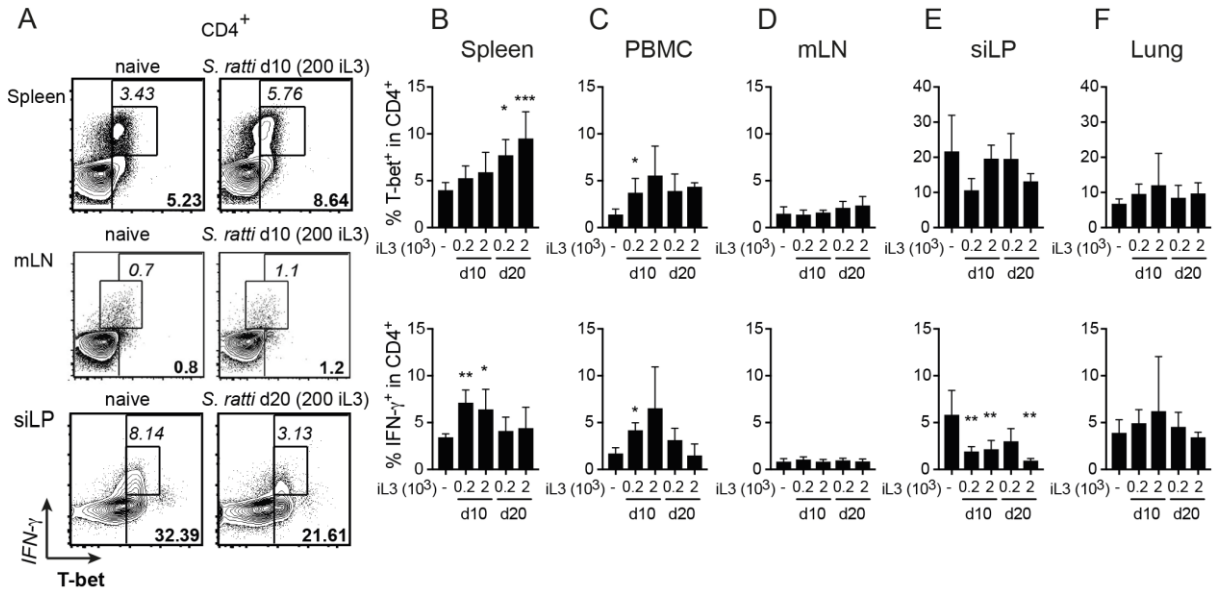

Fig. 3.4: *S. ratti* infection leads to transiently elevated systemic Th1 cell levels, but represses intestinal IFN-γ responses. CD4+ Th1 cells were assessed in C57BL/6 mice infected with 200 or 2000 iL3 of *S. ratti* and uninfected controls. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry. (A) Exemplary contour plots of live CD4+ T cells derived from spleen, mesenteric lymph nodes (mLN) and small intestinal lamina propria (siLP) of a naïve and an infected mouse (200 iL3) at the depicted days p.i. Bold numbers indicate frequencies of T-bet+ cells while italic numbers report frequencies of T-bet+IFN-γ + cells. (B-F) Frequencies of T-bet+ cells (top) and IFN-γ + cells (bottom) within live CD4+ T cells isolated from spleen (B), PBMC (C), mLN (D), siLP (E) and lung (F). Mean + SD of n = 5-6 (naïve control) and n = 4-5 (infected) mice. Displayed data represent one out of two experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ comparing infected to naïve mice using one-way ANOVA. (Modified from Bock *et al*., 2017)

3.1.2. Th2/1 hybrid cells with combined Th2 and Th1 characteristics are induced in mice infected with *S. ratti*

The next analysis addressed whether threadworm infections led to Th2/1 cell differentiation as seen previously in infections with the unrelated intestinal nematode *Heligmosomoides polygyrus* and the blood fluke *Schistosoma mansoni* [48] and if the time point of infection or worm burden affected the proportions of Th2/1 cells within the T cell pool. As shown in **Fig. 3.5**, mice infected with the threadworm *S. ratti* generated Th2/1 hybrid cells. All groups of infected mice displayed significantly increased levels of GATA-3+T-bet+ cells compared to naïve controls (**Fig. 3.5A**, **B-F**). In spleens from mice infected with 2000 iL3 for 10 days, significant increased frequencies of cells co-producing IL-4 and IFN-γ were detected (**Fig. 3.5A**, **B**). Apart from spleens, PBMC, siLP and lung displayed IL-4+IFN-γ+ Th2/1 cells (**Fig. 3.5 C, E, F**), whereas these Th2/1 hybrids were almost absent in mLN (**Fig. 3.5D**).

Methodologically, the detection of Th2/1 hybrids worked most robustly based on GATA-3/T-bet co-expression, whereas their detection based on Th2 cytokine/IFN-γ co-expression was limited by their relatively lower cell numbers compared to Th2 cells and by the commonly lower expression of effector cytokines compared to lineage-defining transcription factors by cells re-stimulated *ex vivo*.

These results confirm the occurrence of the Th2/1 hybrid phenotype characterized by the

co-expression of lineage-defining Th2 and Th1 transcription factors and cytokines at single-cell level during murine infections with *S. ratti*. In general, Th2/1 cells occurred at much lower levels compared to conventional Th1 and Th2 subsets.

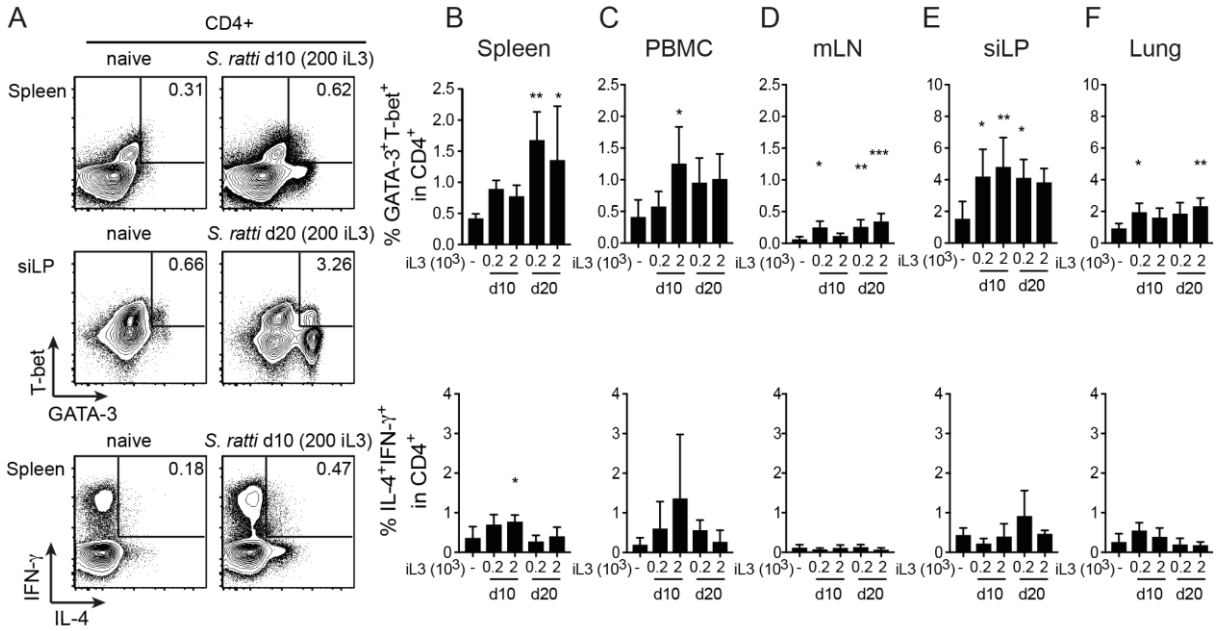

Fig. 3.5: Th2/1 cells co-expressing lineage-defining transcription factors and cytokines of Th2 and Th1 cells are induced during *S. ratti* infection. CD4$^+$ Th2/1 cells isolated from various organs of infected C57BL/6 mice (200 or 2000 iL3) and uninfected naïve animals. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry. (A) Representative contour plots of live CD4$^+$ T cells derived from spleen (top and bottom) and small intestinal lamina propria (siLP) (middle plot) of a naïve and an infected mouse (200 iL3) at the depicted days p.i. Numbers indicate frequencies of GATA-3$^+$T-bet$^+$ and IL-4$^+$IFN-γ$^+$ cells, respectively. (B-F) Frequencies of GATA-3$^+$T-bet$^+$ cells (top) and IL-4$^+$IFN-γ$^+$ cells (bottom) within live CD4$^+$ T cells isolated from spleen (B), PBMC (C), mLN (D), siLP (E) and lung (F). Mean + SD of n = 5-6 (naïve control) and 4-5 (infected) mice. Data from one out of two experiments with similar results are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ comparing infected to naïve mice (one-way ANOVA). (Modified from Bock *et al*., 2017)

For a more comprehensive overview on Th2/1 responses in murine threadworm infections, the proportions of the Th2/1 hybrid subset were examined within the total GATA-3$^+$ CD4$^+$ T cell population in different organs. The highest proportions of T-bet Th2/1 cells were detected within the GATA-3$^+$CD4$^+$ populations isolated from spleen and siLP (**Fig. 3.6A**). As Th2 cells acquire the capacity for the expression of the lineage defining cytokines IL-4, IL-13 and IL-5 with different kinetics (first IL-4 and IL-13, later IL-5 [66]) we next asked if T-bet co-expression and IFN-γ co-production (and hence the Th2/1 hybrid phenotype) was restricted to GATA-3$^+$ T cells at early stages of effector cell differentiation (IL-4$^+$, IL-13$^+$) or also detectable in more thoroughly imprinted, late stage GATA-3$^+$IL-5$^+$ T effector cells. IL-4$^+$ and IL-13$^+$ cells isolated from the spleens of mice infected for 10 days comprised similar frequencies of T-bet expressing Th2/1 hybrid cells (**Fig. 3.6B**). The proportions of T-bet$^+$IFN-γ$^+$ cells within IL-4 and IL-13 producing population were also similar (**Fig. 3.6B**). All Th2 cytokine-producing subsets comprised similar levels of IFN-γ co-producing cells at day 10 and 20 p.i. (**Fig. 3.6C**).

Taken together, Th2/1 hybrid cells co-expressing GATA-3 and T-bet were most prominent in spleen

and siLP, compared to lower levels in peripheral blood and local lymph nodes. Importantly, Th2/1 cells defined as IFN-γ/T-bet co-expressing cells were detected in similar frequencies in cells expressing IL-4, IL-13 and IL-5 during early and later stages of threadworm infection.

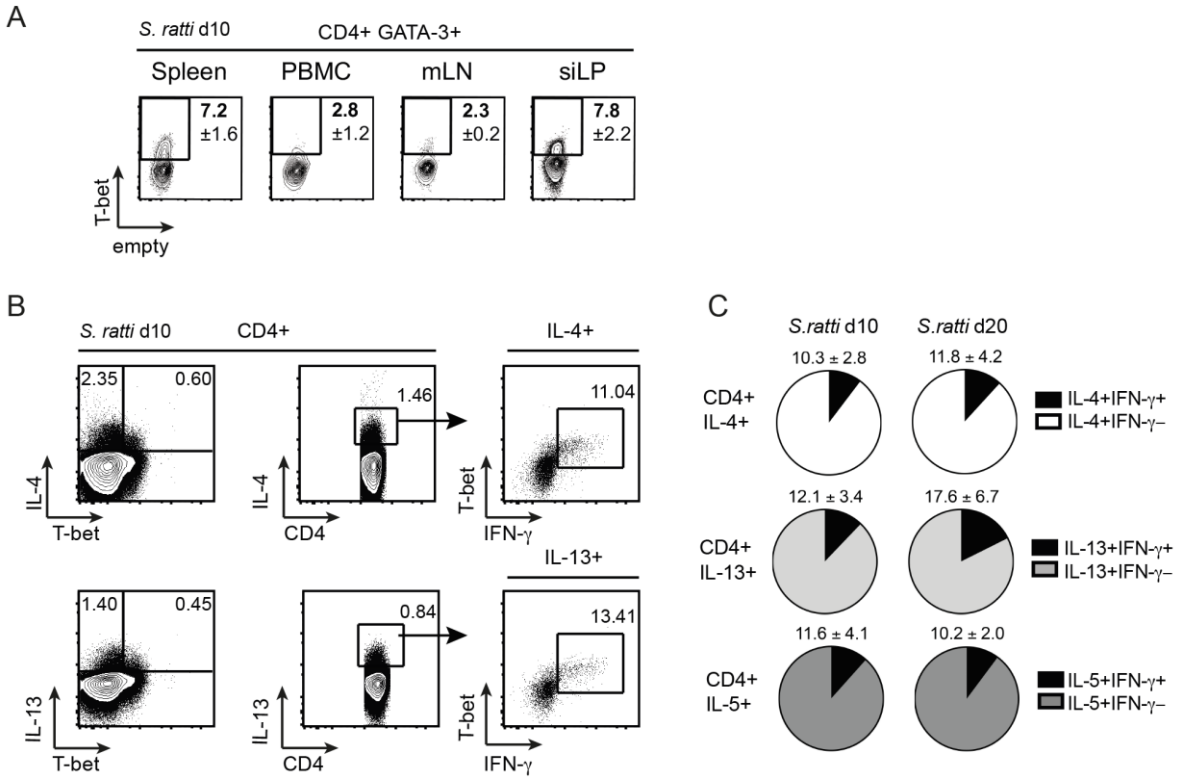

Fig. 3.6: Various cytokine Th2/1 subsets exist and occur in similar proportions within the respective Th2 response. Mice infected with 200 iL3 *S. ratti* were analyzed for Th2 and Th1 features at the depicted days p.i. (A) Exemplary contour plots representing frequencies of T-bet expressing cells within the $CD4^+GATA\text{-}3^+$ subset. T cells were isolated from spleen, peripheral blood (PBMC), mLN and siLP of mice infected for 10 days, stained directly *ex vivo* and acquired by flow cytometry. Numbers indicate mean + SD of 4-5 mice. (B-C) Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed by flow cytometry. (B) Concatenated plots of IL-4 single and IL-4/T-bet co-expressing cells (top left) as well as IL-13 single and IL-13/T-bet co-expressing cells (bottom left) within the $CD4^+$ subpopulation are shown. Further, $IL\text{-}4^+$ or $IL\text{-}13^+$ subsets (center top or bottom, respectively) expressing $T\text{-}bet^+IFN\text{-}\gamma^+$ cells are displayed (right). T cells were isolated from spleens of 5 mice infected with *S. ratti* (200 iL3) for 10 days. (C) Pie charts representing proportions of $IFN\text{-}\gamma^+$ Th2/1 cells (black) within the total IL-4 (white), IL-13 (light gray) and IL-5 (dark gray) producing $CD4^+$ Th2 subset. T cells derived from spleens of mice infected with *S. ratti* (200 iL3) for 10 (left) and 20 days (right). Mean ± SD of n = 5 mice is shown. (Modified from Bock *et al*., 2017)

Next, it was examined if Th2/1 cells induced in murine threadworm infections differed qualitatively from Th2 and Th1 cells in the expression levels of transcription factors and effector cytokines, as shown previously for Th2/1 cells induced in *H. polygyrus* infection [48]. Exemplary FACS plots shown in **Fig. 3.7A** illustrate the cytokine and transcription factor profile of the conventional and Th2/1 hybrid subpopulations. Expectedly, IFN-γ single producing cells expressed the corresponding Th1 transcription factor T-bet, while IL-4 single producers expressed GATA-3 on high levels. A considerable proportion of $IL\text{-}4^+IFN\text{-}\gamma^+$ cells expressed both transcription factors. To quantify the cellular protein expression levels of the Th2 and Th1 markers, the geometric mean fluorescence intensities (geom. MFI) were assessed in Th1, Th2 and Th2/1 cells. Lower mean fluorescence

intensities of GATA-3 and IL-4 signals were detected in Th2/1 cells compared to conventional Th2 cells (**Fig. 3.7B**). Similarly, the expression levels of IFN-γ were significantly reduced in Th2/1 cells compared to conventional Th1 cells (**Fig. 3.7C**). Interestingly, the T-bet expression by Th2/1 cells was significantly elevated compared to Th1 cells (**Fig. 3.7C**), which might reflect the acute activation status of Th2/1 cells compared to mostly quiescent Th1 (memory) cells generated before infection with the parasite.

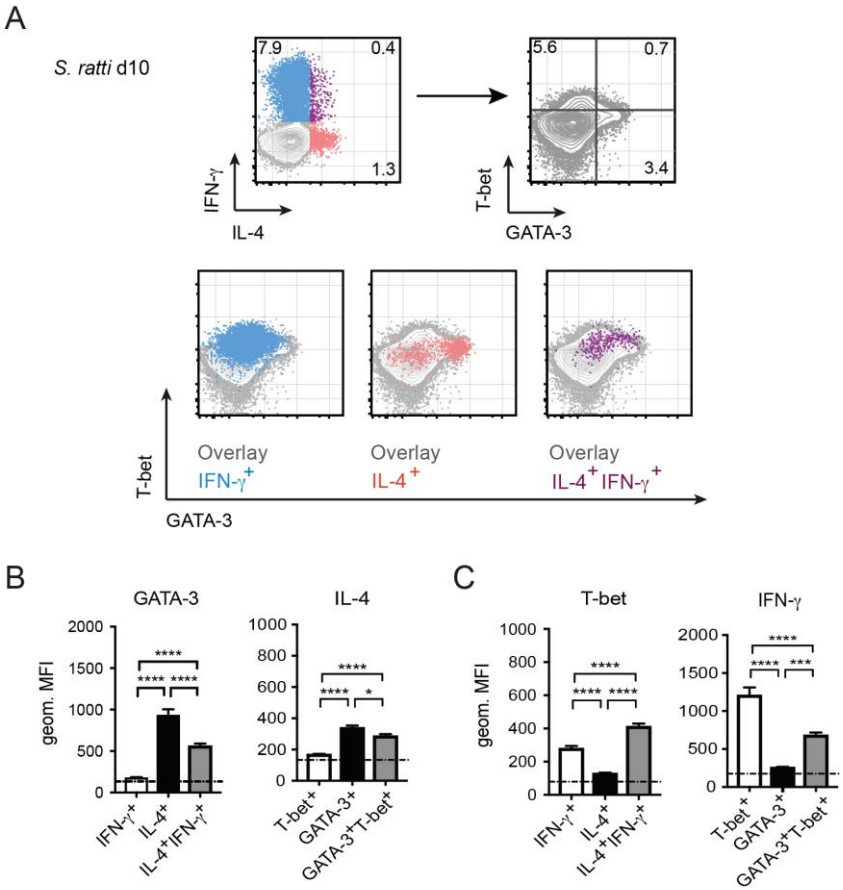

Fig. 3.7: Murine Th2/1 hybrid cells have combined features of conventional Th2 and Th1 cells. Th2/1 cells were screened for characteristics of conventional Th2 and Th1 cells. PBMC were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry. (A) Representative contour plots derived from spleen of an *S. ratti*-infected mouse (d10) displaying the cytokine expression of live $CD4^+$ T cells (top left) indicating single producing IFN-γ (blue) and IL-4 (red) cells and $IL\text{-}4^+IFN\text{-}\gamma^+$ double producers (purple). Each of the depicted cytokine subsets was then analyzed for the GATA-3 and T-bet expression (top right) shown as overlays (lower row) using the aforementioned color code. The total $CD4^+$ population is marked in grey. Numbers indicate frequencies of the respective subset. (B-C) Geometric mean fluorescence intensity (geom. MFI) of GATA-3 or IL-4 (B) and T-bet or IFN-γ signals (C) of the indicated $CD4^+$ subpopulations are shown. Analyzed splenocytes derived from mice infected with *S. ratti* (200 iL3) for 10 days. Dashed lines depict the MFI of $IL\text{-}4^-IFN\text{-}\gamma^-$ and $GATA\text{-}3^-T\text{-}bet^-$ cells. Mean and SD of n = 5 mice is shown. Data originate from one out of two experiments with similar results. $^*p < 0.05$, $^{***}p < 0.005$, $^{****}p < 0.001$ (one-way ANOVA).

In conclusion, murine hybrid cells induced during *S. ratti* infection expressed most markers of Th2 and Th1 cells at intermediate levels compared to the classical T cell subsets, whereas T-bet expression was elevated compared to Th1 cells.

3.1.3. Robust detection of murine Th2 and Th2/1 cells based on transcription factor expression and limitations of blood samples

So far, T cell responses of mice infected with threadworms were compared to naïve controls. To estimate if the analyses of Th2 and Th2/1 responses in patients infected with *S. stercoralis* might be impeded by parameters like low parasite burdens, prolonged duration of infection and the restriction to peripheral blood samples, we next asked if T cell responses of threadworm infected mice differed depending on these parameters.

Cells derived from spleen, PBMC and siLP were chosen to compare Th2 (GATA-3$^+$, IL-4$^+$) and Th2/1 cell responses (GATA-3$^+$T-bet-$^+$, IL-4$^+$IFN-γ$^+$) between mice infected with a low or high parasite dose at the same time point. Organs of mice infected with 200 or 2000 iL3 comprised similar frequencies of Th2 and Th2/1 cells (**Fig. 3.8A-D**). Thus, a 10-fold higher parasite burden did not result in a stronger induction of Th2 and Th2/1 cells. The frequencies of GATA-3$^+$ and GATA-3$^+$T-bet$^+$ cells within a given organ did also not differ between mice infected for 10 or 20 days (**Fig. 3.8A, C**). Compared to the detection of Th2 and Th2/1 cells via their expression profiles of transcription factors, their detection via cytokine expression was more challenging. Frequencies of IL-4$^+$ and IL-4$^+$IFN-γ$^+$ cells isolated from spleens decreased significantly from day 10 to day 20 p.i. (**Fig. 3.8B, D**). By contrast, Th2 cells increased in siLP during a prolonged infection (**Fig. 3.8B**). Similar frequencies of IL-4$^+$ Th2 cells were detected in PBMC for both larval burdens and at both time points (**Fig. 3.8A-D**). However, IL-4$^+$IFN-γ$^+$ cells were only present in low numbers in spleen, PBMC, and siLP samples (**Fig. 3.8D**).

Finally, **Fig. 3.8E-F** summarizes the data on Th2 and Th2/1 frequencies in the different organs of mice infected with a low dose of *S. ratti* for 20 days (thereby mimicking the presumably low parasite load and long lasting infection in asymptomatic humans with strongyloidiasis [72]). Clearly, Th2 and Th2/1 cells were most readily detectable in the infected small intestine, a site not available to sampling in human patients. Both Th2 and Th2/1 cells were present in much lower frequencies in blood of infected mice.

Taken together, these experimental murine data show that Th2 and Th2/1 hybrid responses to threadworm infections are detectable in all organs including blood, but that the latter is suboptimal as cell source compared to e.g. gut tissue. Furthermore, the murine data show that the detection of Th2/1 cells based on transcription factor expression was more robust compared to a cytokine-based approach and that the detection of significant Th2/1 cell responses in helminth infected patients might be complicated by relatively low numbers of these cells in peripheral blood of individuals with low worm burdens and prolonged duration of infection.

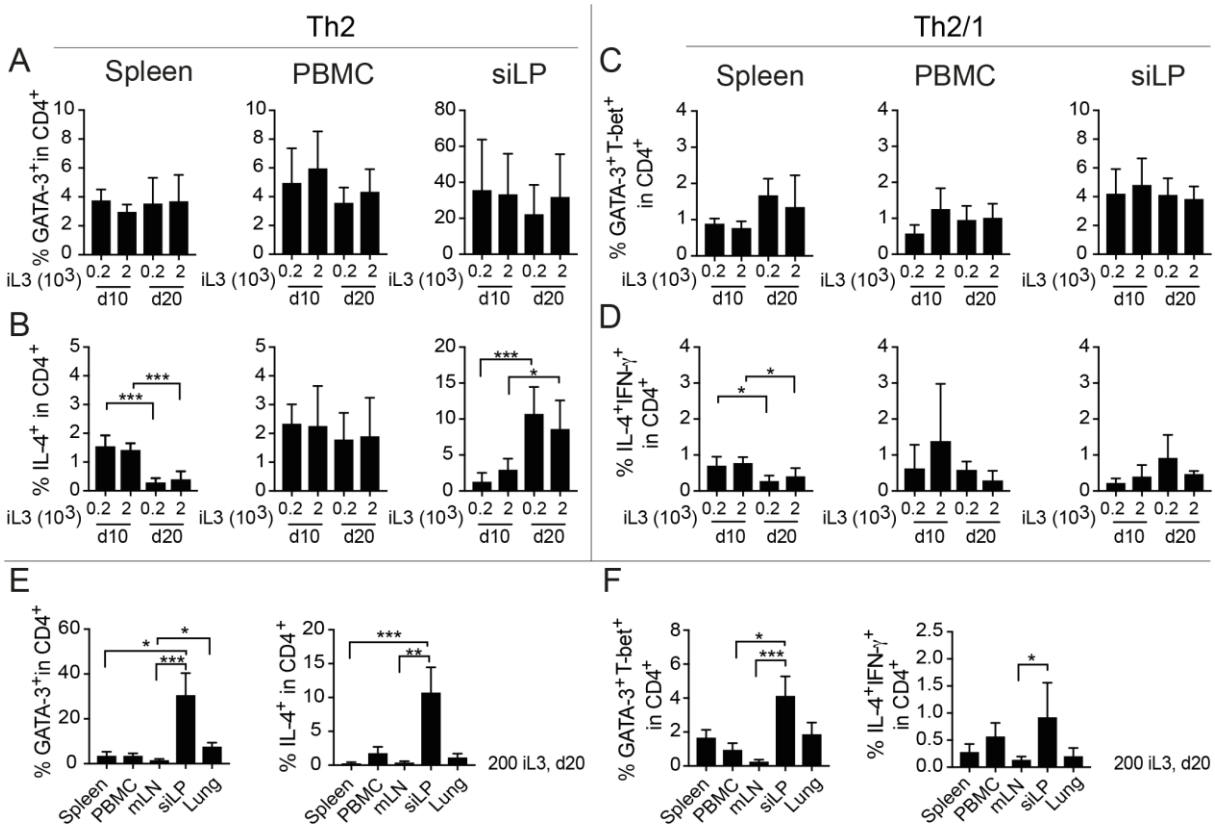

Fig. 3.8: A prominent occurrence of Th2 and Th2/1 cells was not dependent on larval doses or duration of infection, but on the affected organ. CD4⁺ Th2 and Th2/1 cells derived from different organs of C57BL/6 mice infected with 200 or 2000 *S. ratti* iL3 for 10 and 20 days. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry. (A-D) Th2 and Th2/1 responses detected in spleen (left), PBMC (middle) and siLP (right). Frequencies of GATA-3⁺ (A), IL-4⁺ (B), GATA-3⁺T-bet⁺ (C) and IL-4⁺IFN-γ ⁺ (D) within live CD4⁺ T cells. (E-F) Comparison of Th2 and Th2/1 cell levels isolated from spleen, PBMC, mLN, siLP and lung. Organs derived from mice infected with 200 iL3, d20 p.i. Frequencies of GATA-3⁺ (left) and IL-4⁺ (right) Th2 cells (E) as well as GATA-3/T-bet (left) and IL-4/IFN-γ (right) co-expressing Th2/1 cells (F) within the live CD4⁺ subset. Bar graphs display mean + SD of n = 4-5. The *p*-values were calculated by multiple comparison tests using one-way ANOVA, when data were normally distributed, otherwise Kruskal-Wallis test was used. **p* < 0.05, ***p* < 0.01, ****p* < 0.005, *****p* < 0.001 comparing both larval doses at the same time point or one larval dose at d10 vs. d20 (A-D). Mean frequencies of indicated T cell subsets isolated from different organs were compared with each other (E-F).

Summarizing the first part of the present work, murine threadworm infection with its mild course of disease induced Th2/1 cells with combined features of conventional Th2 and Th1 cells. Thus, the induction of the Th2/1 phenotype observed previously in murine nematode and schistosome infection [48] can be generalized to mice infected with *S. ratti*, confirming the first hypothesis of this work. Importantly, the proportion of GATA-3⁺ Th2 and GATA-3⁺T-bet⁺ Th2/1 subsets was neither altered depending on the parasite burden nor the time point of infection. Of note, Th2/1 cells were most robustly detectable based on GATA-3/T-bet co-expression, while their detection based on cytokine co-expression was limited by their relatively low numbers and their reduced per cell cytokine expression levels compared to Th2 and Th1 cells. Furthermore, the Th2/1 phenotype was most prominent in the infected small intestine, but to a lower extent, also detectable in peripheral blood. This indicated that blood as an easy accessible cell source is suitable, but probably not optimal for analyses of Th2/1 cell responses in humans infected with threadworms.

3.2. Characterization of the Th2 immune response and Th2/1 hybrid cells in humans infected with the nematode *Strongyloides stercoralis*

Motivated by the formal proof of $CD4^+$ Th2/1 differentiation in murine threadworm infections the next aim was to proof the existence of Th2/1 hybrid cells in helminth-infected patients. A cooperation with Dr. Subash Babu, NIH-NIRT-ICER at the National Institute for Research in Tuberculosis, Chennai, India, offered the possibility to access blood samples of South Indian patients mono-infected with the threadworm *Strongyloides stercoralis* and endemic uninfected control donors.

First of all, a protocol with appropriate conditions for stimulating and staining human $CD4^+$ Th1 and Th2 cells was established to obtain optimal resolution of the markers of interest (data not shown). Tested parameters included the specimen (PBMC vs. whole blood samples), methods of isolating PBMC, the use of several stimuli (PMA, phorbol 12,13-dibutyrate (PdBU), *Staphylococcus* enterotoxin B (SEB)) and concentrations, different inhibitors of protein transport (Brefeldin A vs. Monensin) as well as different stimulation periods and antibody panels (clones and dyes of markers of interest).

According to the technical setup in place in India, eight-color flow cytometric antibody panels were developed including Th2 and Th1 lineage-defining markers similar to those applied in the experimental mouse studies (details see “Materials and methods” Tab. 6.1, 6.2.3). Participants of the present study were recruited from regions in South India, which are endemic for human strongyloidiasis. Apart from that, efforts have been made to include patients with other helminth infections such as the hookworm *N. americanus* or the filarial nematode *W. bancrofti*. However, the number of samples was too low for a statistical evaluation and therefore the data were omitted here.

Due to the fact that patients were only sporadically available, PBMC were isolated from whole blood and stored in liquid nitrogen (kindly performed by the team of Dr. Subash Babu, NIH-NIRT-ICER) until samples were processed. This procedure ensured the simultaneous analyses of samples of infected and healthy control individuals and therefore allowed a better comparability between the patient cohorts. Before using samples of the study cohort, frozen PBMC were compared with freshly isolated PBMC for Th1 and Th2 cell lineage-specifying features following the protocol mentioned in 6.2.2.5 and 6.2.3. The obtained frequencies were similar (data not shown), confirming that both transcription factor and cytokine profiles were unaffected by the freezing, storage and thawing process. Hence, only cryopreserved PBMC were used for the investigations of samples from patients and healthy controls.

3.2.1. Characteristics of the human study population – *S. stercoralis*

PBMC of 40 uninfected endemic controls and 34 patients mono-infected with *S. stercoralis* were examined during three research stays at the NIH-NIRT-ICER, Chennai in South India. The demographic baseline characteristics of the study population are given in **Tab. 3.1.** No differences in gender, age range and socio-economic status between both cohorts were observed. All individuals were clinically healthy and free of symptoms. The infection status with *S. stercoralis* and the absence of other helminth infections were assessed by the group of Dr. Subash Babu (NIH-NIRT-ICER), as specified in the method section 6.2.2.1.1.

Tab. 3.1: Demographic profile of uninfected healthy donors and individuals infected with *S. stercoralis*

Parameter	Value for the group[a]	
	Uninfected (n = 40)	**Infected (n = 34)**
No. of male subjects	21	16
No. of female subjects	19	18
Mean age (range [year])	33 (18-60)	34 (19-58)
Clinical status	Healthy	Healthy
Symptom(s)	None	None
Socio-economic status	Rural workers	Rural workers
NIE ELISA result	Negative	Positive
Results of stool examination for *S. stercoralis*	Negative	Positive
Presence of other helminth infections	Negative	Negative

[a] Differences in the values for gender and age between infected and uninfected groups were not significant.

Hematological characteristics of both cohorts are depicted in **Tab. 3.2**. No differences in hemoglobin levels, red and white blood cell counts, hematocrit or platelet counts were detected between the groups (Tab. 3.1-3.2 are published in Bock *et al*., 2017).

Tab. 3.2: Hematological profile of uninfected healthy donors and patients infected with *S. stercoralis*

Factor [a]	Mean (range) for the group		
	Uninfected (n = 40)	**Infected (n = 34)**	***P*-value[b]**
Hb (g/dL)	51.77 (8.7 – 174)	13.07 (7.6 –16.9)	NS
RBC (10^6 /µL)	4.80 (3.5 – 6.46)	4.68 (3.7 – 6)	NS
WBC (10^3 /µL)	8.55 (5.3 – 13.3)	8.51 (5.1 – 13.8)	NS
HCT (%)	40.35 (27 – 54)	39.47 (25 – 51)	NS
PLT (10^3 /µL)	287.1 (137 – 446)	267.5 (159 – 413)	NS

[a] Hb, hemoglobin; RBCs, red blood cells; WBCs, white blood cells; HCT, hematocrit; PLTs, platelets.
[b] NS, not significant. (*p*-values were calculated by Unpaired t test)

Differential blood counts (provided by Dr. Subash Babu, NIH-NIRT-ICER) were analyzed to characterize alterations of immune parameters in patients as a consequence of an infection. Cell numbers of lymphocytes and eosinophils were significantly increased in the infected cohort (**Fig. 3.9A, C**), while the count of neutrophils was significantly reduced in *S. stercoralis* infected patients (**Fig. 3.9B**). Numbers of blood monocytes and basophils did not differ between the groups (**Fig. 3.9D, E**).

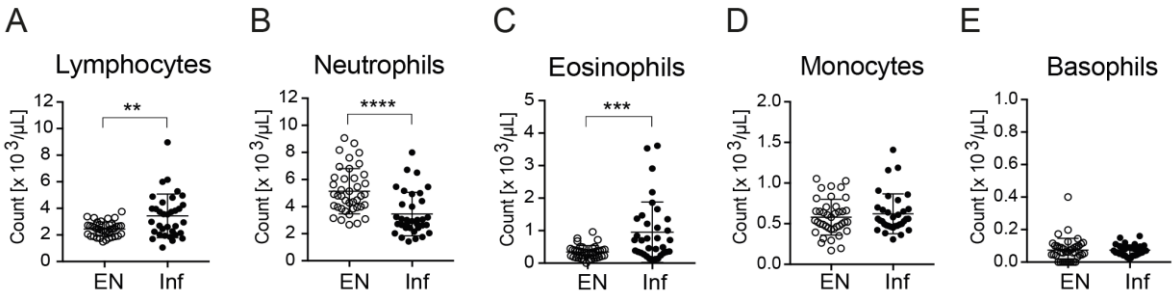

Fig. 3.9: Patients infected with *S. stercoralis* have increased numbers of lymphocytes and eosinophils while neutrophils are decreased. Counts of lymphocytes (A), neutrophils (B), eosinophils (C), monocytes (D) and basophils (E) derived from blood of endemic uninfected controls (EN) and *S. stercoralis*-infected patients (Inf). Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 40 (EN) and n = 34 (Inf). The *p*-values were calculated by Mann-Whitney U test. ***p* < 0.01, ****p* < 0.005, *****p* < 0.001.

Taken together, the study cohorts differed regarding the infection status, but were otherwise healthy and asymptomatic as well as similar in demographic characteristics. Hence, the study population provided a good comparability of the infected with the uninfected control group. The parasitic infection in the patient group was reflected by increased numbers of lymphocytes and eosinophils. Next, the antibody profiles were analyzed, followed by a detailed comparison of the immune status of nematode-infected with uninfected healthy donors in respect of their $CD4^+$ T helper cell responses.

3.2.2. *S. stercoralis* infection is associated with a distinct plasma antibody profile

The plasma antibody profile was assessed in the study population. Patients infected with *S. stercoralis* showed increased levels of Th2-associated IgE and IgG4 (**Fig. 3.10A-B**) compared to the uninfected cohort. The levels of Th1-associated IgG3 were also significantly increased during threadworm infection (**Fig. 3.10C**), while the levels of IgG1 were similar in both groups (**Fig. 3.10D**). Thus, patients with *Strongyloides* infection displayed a distinct plasma antibody profile characterized by elevated levels of Th2-associated antibodies and IgG3.

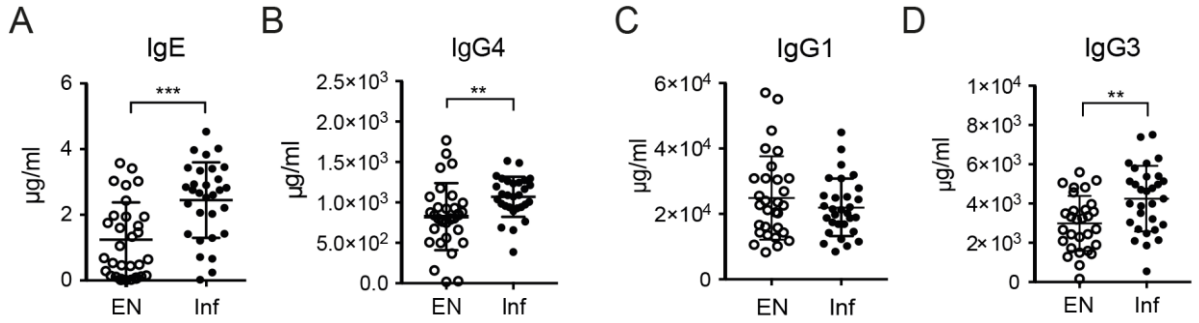

Fig. 3.10: Antibody levels in plasma samples of endemic controls and *S. stercoralis* infected patients. Plasma levels of total IgE (A), total IgG4 (B), total IgG1 (C) and total IgG3 (D) are displayed. The results are shown as scatter plots with each circle representing a single individual. Depicted bars represent mean + SD of n = 30 (EN) and n = 30 (Inf). *P*-values were calculated by Mann-Whitney U test. ***p* < 0.01, ****p* < 0.005. (Bock *et al*., 2017).

3.2.3. Human *S. stercoralis* infection is associated with increased Th2 responses, while Th2/1 cells are detectable in helminth-infected patients and healthy control subjects

PBMC stimulated with PMA/ionomycin were investigated for markers of Th2 and Th1 differentiation by flow cytometry following a similar gating strategy as depicted for mice in Fig. 3.2. Within $CD3^+CD4^+$ T cell population, the *S. stercoralis*-infected group displayed significantly elevated frequencies of GATA-3^+ Th2 cells compared to the uninfected group (**Fig. 3.11A, B**). By contrast, the levels of T-bet^+ Th1 cells did not differ between the cohorts (**Fig. 3.11C**). Consequently, the elevated Th2 response observed in infected donors was also reflected by a higher ratio of Th2 : Th1 cells (**Fig. 3.11D**).

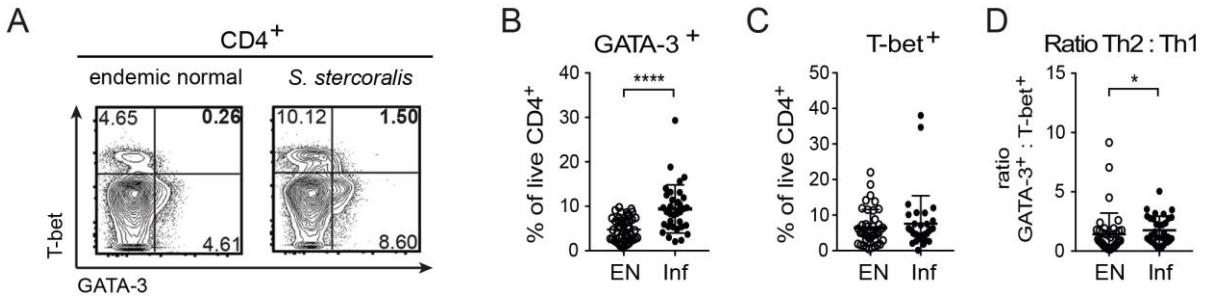

Fig. 3.11: Increased levels of GATA-3^+ Th2 cells in *S. stercoralis*-infected patients. PBMC were stimulated with PMA/ionomycin for 4 h. The expression of transcription factors was detected through intracellular staining via flow cytometry. (A) Representative plots of GATA-3 and T-bet in live $CD4^+$ T cells from an endemic control and a *S. stercoralis* infected donor. Regular numbers indicate frequencies of T-bet or GATA-3 single producers, respectively; bold numbers indicate frequencies of GATA-3/T-bet co-expressing cells. (B) Frequencies of GATA-3 expressing Th2 cells as detected in the endemic normal control group (EN) and *S. stercoralis*-infected group (Inf). The results are shown as scatter plots with each circle representing a single individual. (C) Frequencies of T-bet expressing Th1 cells. (D) Ratio of GATA-3 single producing cells to T-bet single producers. Circles/dots represent individuals, horizontal lines and error bars represent mean ± SD of n = 40 EN and n = 34 Inf. The p-values were calculated by Mann-Whitney U test, $*p < 0.05$, $****p < 0.001$. (Figures are partly published in Bock *et al*., 2017)

Patients with threadworm infection exhibited only slightly increased frequencies of IL-4 and IL-5 expressing T cells (**Fig. 3.12A, B**), but a highly significant elevation of IL-13^+ cells compared to uninfected donors (**Fig. 3.12A, B)**. As observed for T-bet^+ cells, the levels for IFN-γ^+ Th1 cells were similar in both groups (**Fig. 3.12C**). Consequently, the ratios of IL-13^+ to IFN-γ^+ cells were significantly increased in threadworm-infected patients (**Fig. 3.12D**), confirming the Th2 bias induced by the infection.

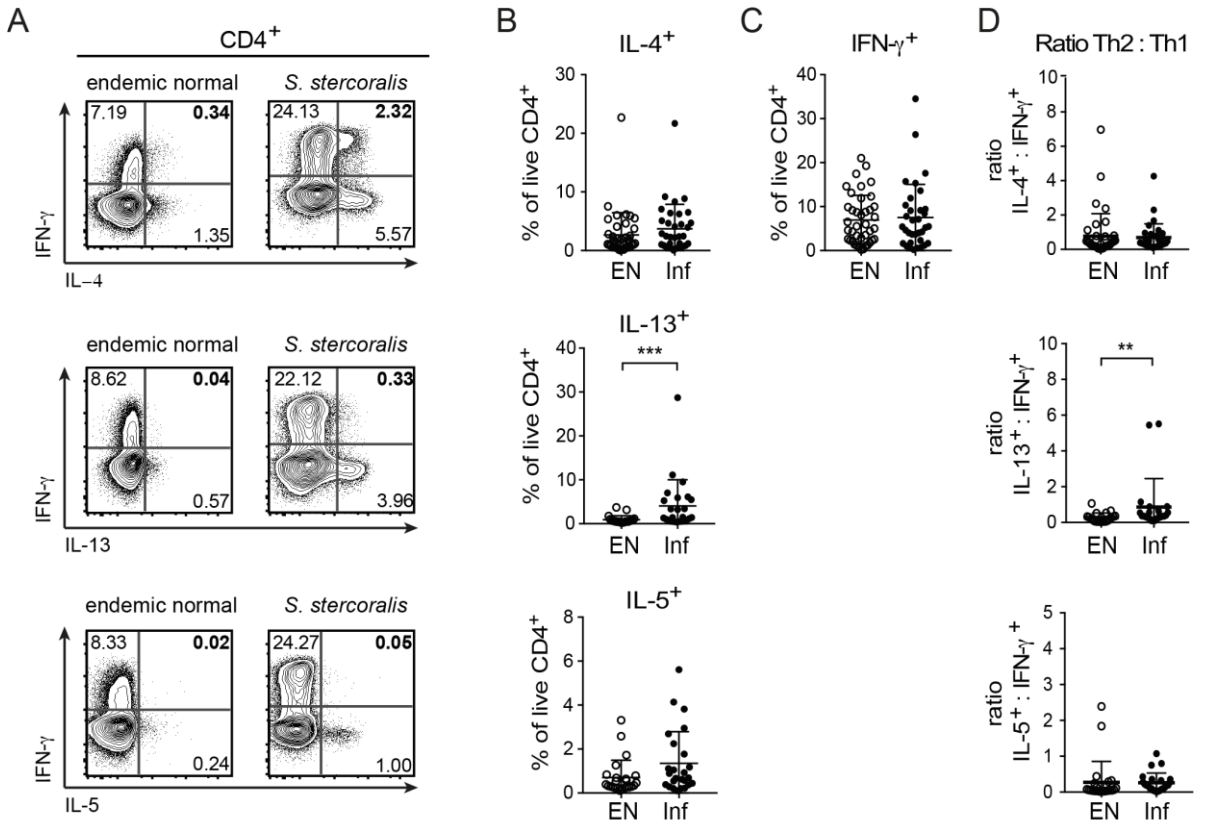

Fig. 3.12: *S. stercoralis* infection leads to increased IL-13 responses. PBMC where stimulated with PMA/ionomycin for 4 h. The production of Th2 and Th1 cytokines was detected through intracellular staining via flow cytometry (A) Exemplary contour plots of live CD4$^+$ T cells expressing IL-4 and IFN-γ (top), IL-13 and IFN-γ (middle), IL-5 and IFN-γ (bottom) from an uninfected endemic control and a *S. stercoralis*-infected donor. Regular numbers indicate frequencies of single producing cells as depicted; bold numbers indicate the respective co-producing subsets. (B) Frequencies of IL-4, IL-13 and IL-5 producing Th2 subsets as detected in the endemic normal control group (EN) and *S. stercoralis*-infected cohort (Inf). (C) Frequencies of IFN-γ producing Th1 cells. (D) Ratios of single producers of Th2 to Th1 cytokines; IL-4 : IFN-γ (top), IL-13 : IFN-γ (middle), IL-5 : IFN-γ (bottom). Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 40 (EN) and n = 34 (Inf) for IL-4 and n = 24 (EN) and n = 25 (Inf) for IL-13 and IL-5. The *p*-values were calculated by Mann-Whitney U test. ** $p < 0.01$, *** $p < 0.005$. (Figures are partly published in Bock *et al*., 2017)

The main objective of the present study was to investigate whether CD4$^+$ T cells of humans comprise a bifunctional Th2/1 hybrid phenotype alongside conventional Th2 cells. Indeed, CD3$^+$CD4$^+$ T cells derived from human PBMC co-expressed Th2 and Th1 transcription factors and cytokines. The *S. stercoralis*-infected group displayed significantly increased frequencies of CD3$^+$CD4$^+$ T cells co-expressing GATA-3 and T-bet compared to healthy donors (**Fig. 3.11A, 3.13A)**. To exclude the possibility that GATA-3$^+$T-bet$^+$ cells were an artifact resulting from strong *in vitro* stimulation via PAM/ionomycin, unstimulated samples were assessed in a paired manner. GATA-3$^+$T-bet$^+$ cells were also detectable in unstimulated cells (data not shown).

Importantly, a distinct subset of IL-4$^+$ cells co-expressed high levels of IFN-γ in nematode-infected patients (**Fig. 3.12A**, bold number). Mean frequencies of IL-4$^+$IFN-γ$^+$ cells, however, were similar in both cohorts (**Fig. 3.13A**). Similarly, a small proportion of IL-13$^+$ cells co-produced IFN-γ, while IL-5$^+$IFN-γ$^+$ cells were barely present (**Fig. 3.12A, 3.13A**). Most patients displayed relatively low levels of Th2/1 cells, however, some individuals in both cohorts had high levels of Th2/1 hybrid cells. Ratios of Th2 : Th2/1 cells were similar in the healthy and infected groups (**Fig. 3.13B**).

Next, the proportions of IFN-γ$^+$ cells within the total CD4$^+$IL-4$^+$ population of infected and healthy blood donors were analyzed and found to be similar in *S. stercoralis* infected patients and uninfected subjects (**Fig. 3.13C**). The proportions of cells co-producing Th1 and Th2 markers within the total Th2 subpopulations were lower for IL-13 and IL-5 producing cells compared to GATA-3 and IL-4 producing T cell subsets (**Fig. 3.13D**).

Finally, human CD3$^+$CD4$^+$ Th2/1 cytokine producers were surveyed for their transcription factor profile (**Fig. 3.14A**), as presented earlier for the *Strongyloides* mouse model (see Fig. 3.7A).
As expected, the IFN-γ single producing cells clustered within the T-bet$^+$ population (**Fig. 3.14A**), while IL-4 single producers expressed high levels of GATA-3 (**Fig. 3.14A, B**). Strikingly, IL-4$^+$IFN-γ$^+$ Th2/1 hybrid cells displayed a GATA-3^{low}T-bet^{high} phenotype (**Fig. 3.14A**), clustered with the population of IFN-γ$^+$ cells and expressed the highest levels of T-bet (**Fig. 3.14A**, **C**). Similar to murine Th2/1 hybrids, human IL-4$^+$IFN-γ$^+$ Th2/1 hybrid cells expressed significantly lower levels of IL-4 than IL-4 single producers (**Fig. 3.14A, B**). By contrast, the mean expression levels of IFN-γ were similar for IFN-γ single and IL-4$^+$IFN-γ$^+$ double producing cells (**Fig. 3.14C**).

As mentioned previously, only few samples of patients with filarial or hookworm infection could be analyzed. However, also these samples comprised Th2/1 cells and the hybrids displayed a similar profile of transcription factors and cytokines (data not shown) as described for the data in Fig. 3.14.

In addition, the attempt was made to detect nematode-specific Th2 and Th2/1 effector cells in infected donors in contrast to uninfected individuals. Cell cultures were stimulated for 24 h with crude extract of the murine *S. ratti* adult worms in combination with co-stimulatory reagents CD49d/CD28. Here, CD40L (CD154) the early activation marker of antigen-reactive CD4$^+$ T cells was included in the analysis. While the detection of CD40L worked readily in PBMC stimulated with PMA/ionomycin, no CD40L and cytokine signals were detected in response to antigen stimulation (data not shown). Thus, it was not possible to assess frequencies of nematode-specific Th2/1 cells.

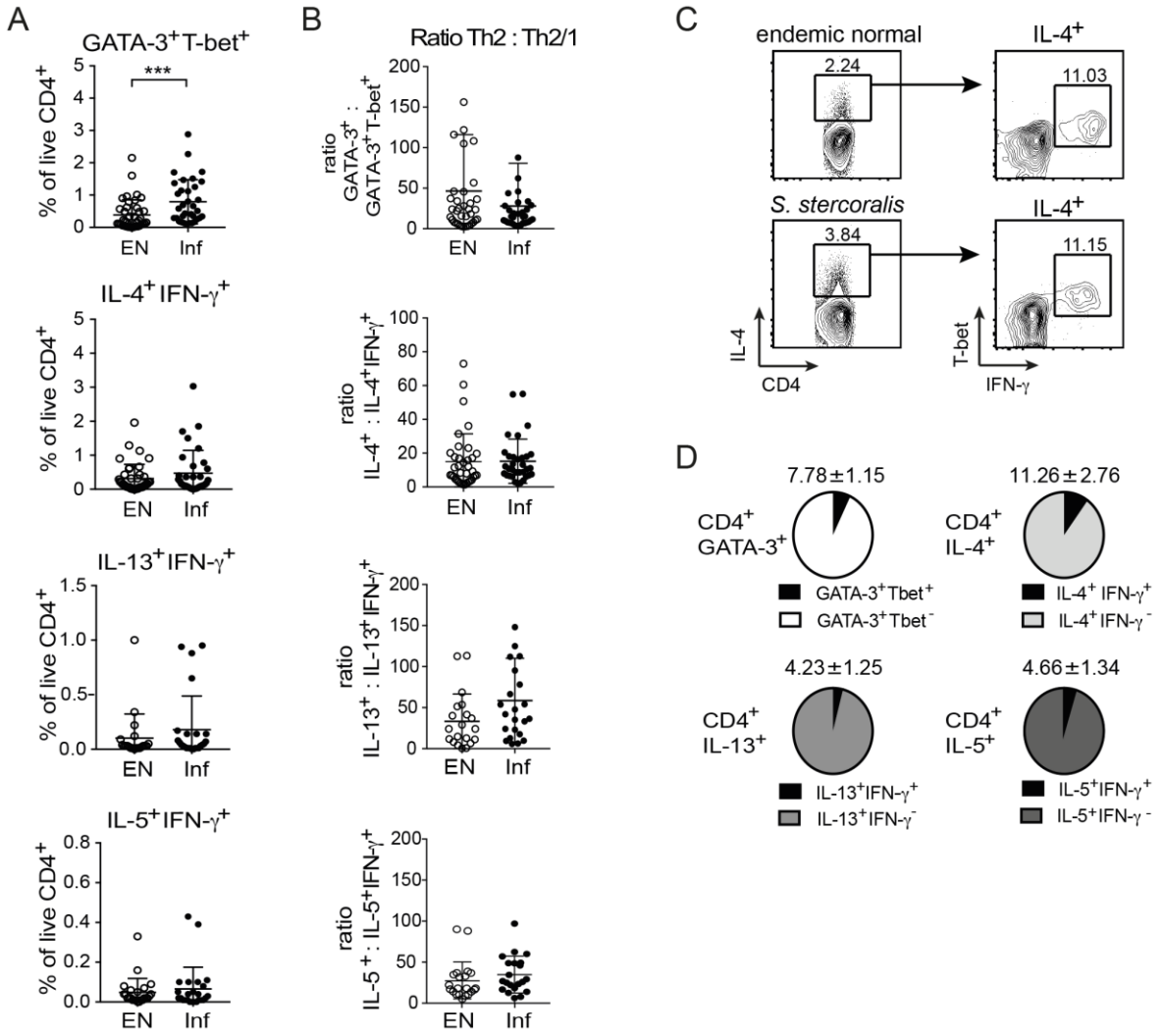

Fig. 3.13: Detection of human Th2/1 cells derived from blood samples. PBMC where stimulated with PMA/ionomycin for 4 h. The expression of transcription factors and production of cytokines were detected through intracellular staining via flow cytometry. (A) Frequencies of Th2/1 hybrid cells on transcription factor level (top) and cytokine level (2nd - 4th row) within the live CD4+ subset from endemic normal controls (EN) and patients infected with *S. stercoralis* (Inf). Circles represent individuals, horizontal lines and error bars represent mean + SD. (B) Ratios of Th2 single producing : Th2/1 co-producing cells for the indicated transcription factors (top) and Th2 cytokines (2nd - 4th row). (C) Exemplary contour plots of IFN-γ co-producing cells within the total CD4+IL-4+ population of a healthy control and *S. stercoralis*-infected patient. (D) Pie charts representing proportions of T-bet+ Th2/1 cells (black) within the total GATA-3 (white) subset or IFN-γ+ Th2/1 cells (black) within the total IL-4 (light gray), IL-13 (gray) or IL-5 (dark gray) producing CD4+ Th2 population of the infected cohort. Mean + SD of n = 40 (EN) and n = 34 (Inf) for GATA-3+T-bet+ and IL-4+IFN-γ+. N = 24 (EN) and n = 25 (Inf) for IL-13+IFN-γ+ and IL-5+IFN-γ+. The *p*-values were calculated by Mann-Whitney U test. ***p* < 0.01, ****p* < 0.005. (Figures are partly published in Bock *et al*., 2017)

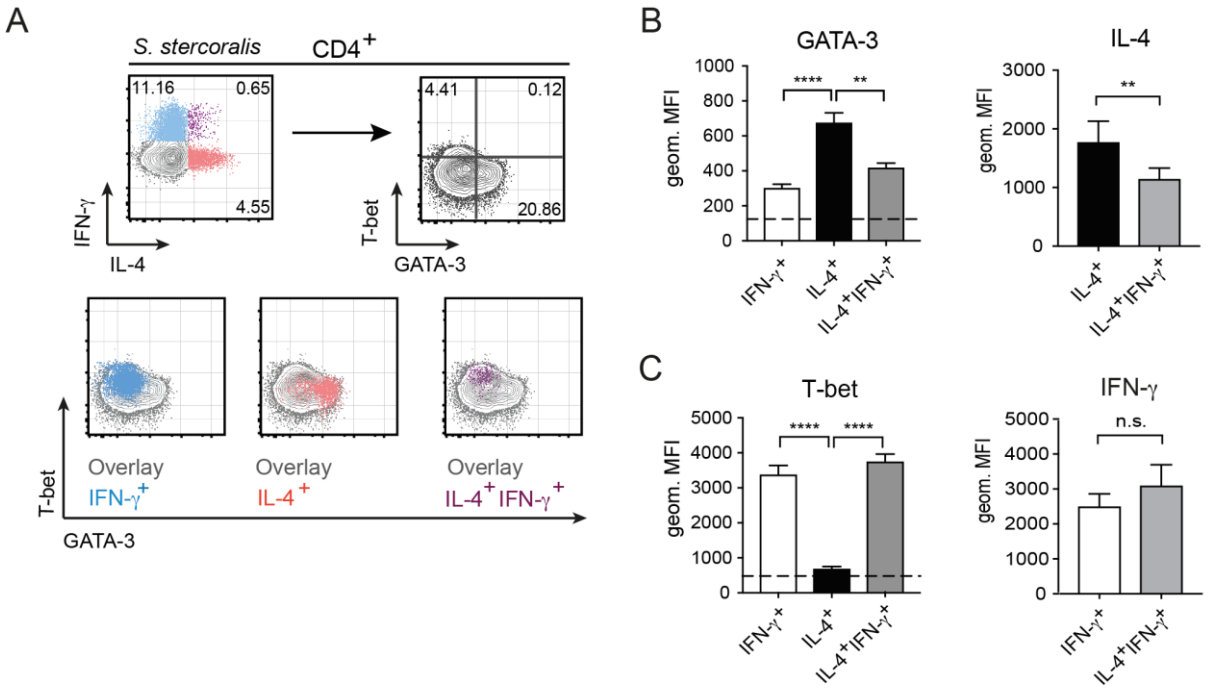

Fig. 3.14: Human Th2/1 hybrid cells express high T-bet and IFN-γ levels, but display poor GATA-3 expression. Th1, Th2 and Th2/1 cells were screened for their transcription factor and cytokine expression levels. PBMC were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed by flow cytometry. (A) Representative contour plots derived from PBMC of a patient infected with *S. stercoralis* displaying the cytokine expression of live CD4$^+$ T cells (top left) indicating single producing IFN-γ (blue) and IL-4 (red) cells and IL-4$^+$IFN-γ$^+$ double producers (purple). The depicted cytokine subsets were then analyzed for their respective GATA-3 and T-bet expression (top right) shown as overlays (lower row). The total CD4$^+$ population is marked in grey. Numbers indicate frequencies of the respective subset. (B-C) Geometric mean fluorescence intensity (geom. MFI) of GATA-3/IL-4 (B) and T-bet/IFN-γ (C) are shown. Dashed lines depict the MFI of IL-4^{neg}IFN-γ^{neg} cells. Mean + SD of n = 5 *S. stercoralis*-infected donors comprising >100 IL-4$^+$IFN-γ$^+$ cells assessed in two independent experiments. The *p*-values were calculated by multiple comparison tests using one-way ANOVA (B, C left) or Student's *t*-test for comparisons of two parameters (B, C right). $^{**}p < 0.01$, $^{****}p < 0.001$. (Figures are partly published in Bock *et al*., 2017)

3.2.4. Characteristics of human versus murine Th2/1 hybrid cells

Comparing the Th2/1 cells isolated from humans and mice, several differences were apparent. Murine IL-4$^+$ hybrids mostly co-produced IFN-γ at intermediate levels, while the human hybrid equivalent co-produced IFN-γ mainly at high levels (**Fig. 3.15A**). Furthermore, the transcription factor profiles of murine and human cytokine hybrids differed. Murine Th2/1 cells expressed both GATA-3 and T-bet, whereas human hybrid cells expressed high levels of T-bet, but low levels of GATA-3 (**Fig. 3.15A**). Besides IL-4$^+$IFN-γ$^+$ cells, also cells co-producing IL-13/IFN-γ and IL-5/IFN-γ were detected in both species. Analyzing the proportions of IL-4$^+$, IL-13$^+$ and IL-5$^+$ cells in spleens of mice infected with *S. ratti* showed that IL-4$^+$ cells dominated the Th2 population, followed by IL-13$^+$ and IL-5$^+$ cells (**Fig. 3.15B**). The proportions of Th2/1 cells expressing the different Th2 cytokines were, however, more evenly distributed in mice (**Fig. 3.15B**). The same analysis in human blood cells revealed that IL-4$^+$ and IL-13$^+$ cells were present in similar proportions within the Th2 cell pool, whereas IL-5$^+$ Th2 cells were present in lower numbers. The human Th2/1 cell pool was dominated by IL-4$^+$IFN-γ$^+$ cells,

followed by IL-13$^+$IFN-γ$^+$, while IL-5$^+$IFN-γ$^+$ cells were detected at very low levels in some donors, but were often absent (**Fig. 3.15B**).

Taken together, cells co-expressing Th2 and Th1 markers were detected in mice and humans, but displayed different features with respect to their transcription factor expression profiles, preferences for Th2 cytokine expression and IFN-γ expression levels.

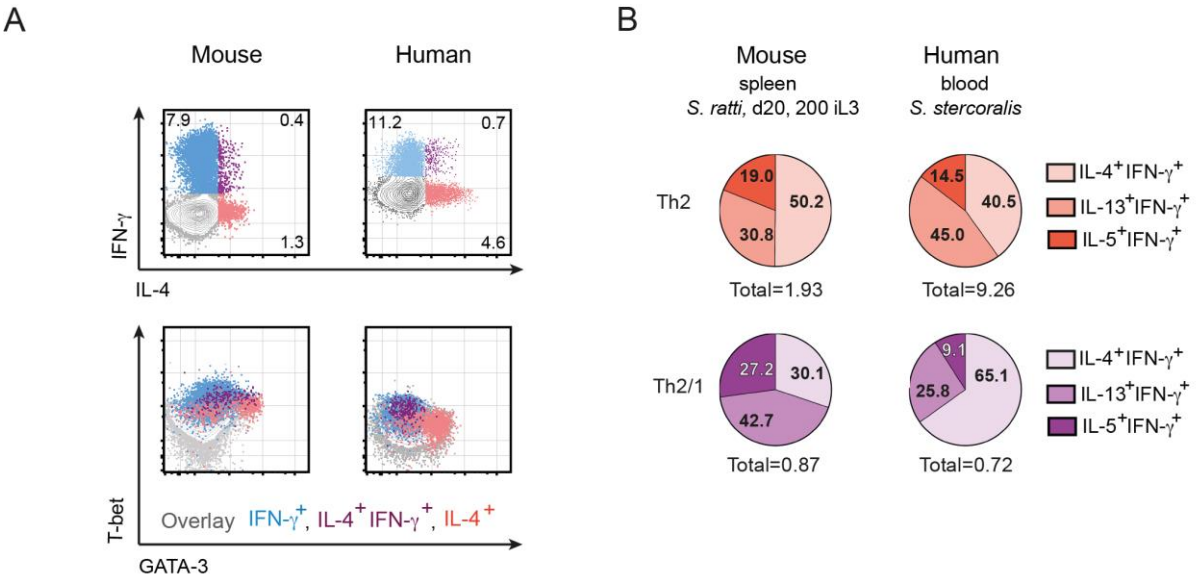

Fig. 3.15: Characteristics of human versus murine Th2/1 hybrid cells. (A) Exemplary contour plots derived from spleen of a mouse infected with 200 iL3 of *S. ratti* for d10 (top row) and PBMC of a patient infected with *S. stercoralis* displaying the cytokine expression of live CD4$^+$ T cells (top) indicating single producing IFN-γ (blue) and IL-4 (red) cells and IL-4$^+$IFN-γ$^+$ double producers (purple). (B) Pie charts represent proportions of Th2 (top) and Th2/1 cells (bottom) producing IL-4, IL-13 and IL-5 respectively. Numbers in pie charts report the mean proportion of the given subsets within the total Th2 cytokine expressing (Th2) or Th2 cytokine/IFN-γ co-expressing (Th2/1) cell pools. Data are derived from spleens of mice (200 iL3, d20) (left) and PBMC of humans (right) infected with *Strongyloides.* 'Total' reports the sum frequencies of cells expressing only Th2 cytokines and Th2 cytokines together with IFN-γ, respectively.

In summary, the second hypothesis of this work could be confirmed in showing that Th2/1 hybrid cells are not restricted to murine helminth infections, but also occur in helminth infected patients. *S. stercoralis* infection in humans was characterized by Th2 differentiation, in particular evident by GATA-3$^+$ and IL-13$^+$ cells, while frequencies of Th1 cells were indistinguishable from healthy controls. The distinct serum antibody profiles of infected patients comprised elevated levels of Th2 and Th1-related antibody isotypes, similar to what was seen in threadworm infected patients. Th2/1 cells were detected based on GATA-3/T-bet co-expression and the simultaneous expression of Th2 cytokines with IFN-γ. Such bifunctional human Th2/1 hybrid subsets were detectable in peripheral blood samples, a source relatively easily accessible for further analyses. However, similar levels of Th2 cytokine and IFN-γ co-producing cells were detected in infected and healthy individuals. In contrast to the findings in mice, human hybrids co-expressing IFN-γ were most prominent in the IL-4 producing T cell pool and present in lower numbers in the IL-13 and IL-5 populations. Furthermore, GATA-3 expression by human Th2 cytokine/IFN-γ producing Th2/1 cells was either undetectable or very low, while T-bet and IFN-γ were expressed on high levels. In fact, the per cell IFN-γ expression levels of hybrids often exceeded those of Th1 cells.

3.3. Characterization of T helper cell responses and the Th2/1 phenotype in patients with atopic dermatitis

Having established that Th2/1 cells occur in humans affected by asymptomatic threadworm infections three further questions arose: I) Are Th2/1 cell responses restricted to helminth infected patients or a generalized response of patients with Th2-associated disorders? Th2/1 hybrid cells were also detectable in currently uninfected Indian blood donors, which possibly had a previous infection with helminths, leading to a Th2 memory subset and therefore probably to Th2/1 cells as well. Hence, the next aim was to examine blood samples of donors affected by a non-parasitic Th2-driven disorder and find out whether these patients distinctly differed in terms of Th2 and Th2/1 cell responses. II) Are the observed differences between human and murine hybrids consistent comparing helminth-infected and allergic/atopic patients? III) Does the proportion of human Th2/1 cells within the total 'Th2-like' T cell population correlate to a distinct clinical disease phenotype and are higher Th2/1 cell proportions associated with a milder disease course and lower pathology?

To address these questions, peripheral blood cells of European patients afflicted with atopic dermatitis (AD) were assessed in comparison to those of healthy control subjects. This part of the work was conducted in cooperation with Prof. Dr. Margitta Worm, Department of Dermatology, Venereology and Allergology, Charité University Medicine, Berlin, Germany.

AD is considered as the most common skin disorder [95] with visually evident disease phenotypes, measurable at a clinical level by using the scoring method SCORAD [106], [140]. Patients enrolled in this study were allocated into two groups of severity based on this scoring scheme: Patients with mild AD (SCORAD < 25), and patients with moderate to severe AD (SCORAD > 25). Healthy donors served as controls. Furthermore, patients were selected for concomitant grass pollen allergy to possibly quantify antigen-specific Th2 and Th2/1 cells. To that end, PBMC were stimulated with a mixture of the immunodominant recombinant grass pollen allergens Phl p 1 and Phl p 5.01.

11-color flow cytometric analyses were performed at the Institute of Immunology, allowing for more comprehensive antibody panels. Hence, antibodies against CD45RO (allowing for the distinction between $CD45RO^+$ effector/memory cells and mostly naïve $CD45RO^-$ $CD4^+$ T cell populations), and TCR Vα24-Jα18 allowing for the exclusion of iNKT cells that potentially co-produce IL-4 and IFN-γ were included. Thus the cells of interest were defined more precisely. Foxp3, the key transcription factor of $CD4^+$ regulatory T cells (Treg), was included to investigate whether the patient groups differed in regulatory responses. Moreover, the skin-homing potential of circulating $CD4^+$ T cells was examined by staining cutaneous lymphocyte antigen (CLA), which is considered as a surrogate marker for lymphocytes prone for skin migration [111], [141]. In addition, the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) was included as an additional marker of Th2 cells beside GATA-3 [112], [142]. Furthermore, the pro-inflammatory cytokines IL-22 and IL-17 were analyzed, which play a role in skin disorders [35], [119].

3.3.1. Characteristics of the human study population – AD

Blood samples of healthy control donors, patients with mild AD (SCORAD < 25) and with a moderate to severe course of disease (SCORAD > 25) were examined. The number of participants in each group, the proportion of female and male donors and the average age were similar in all three cohorts (**Tab. 3.3**.). All participants were asked about their medical history. None of the patients suffered from a parasitic, bacterial or viral infection for at least four weeks before sampling. However, in some aspects the groups differed as shown in **Tab. 3.3**. For almost every patient of the 'moderate to severe group' (hereinafter referred to 'severe', '++'), AD onset was during their childhood, while such an early beginning of the disease occurred in only half of the patients with mild AD (hereinafter referred to 'mild', '+'). The aim was to include only patients with timothy grass pollen allergy, which was originally deemed an exclusion criterion in healthy control donors (hereinafter referred to 'HD', '-'). Due to the restricted AD sampling cohort, some participants were included despite the absence of this criterion (see 6.2.2.1.2). However, the three cohorts were affected by allergies to a different extent. 5 % of the healthy control group, but 63 % of patients with mild AD and as expected 100 % with severe AD stated an affliction with grass pollen allergy. Moreover, most of the patients with a mild (74 %) and severe (90 %) course of disease also had other allergies, mostly concomitant pollen allergies as birch (not shown). In contrast, only 20 % of healthy participants declared of having other allergies than against grass pollen. In these cases, allergic reactions were not directed against pollen, but rather to other agents such as nickel or pet hair (data not shown).

Tab. 3.3: Epidemiologic and clinical data of healthy controls and patients with AD

Parameter	Value for the group[a]		
	Control subjects	Patients with AD	
	Healthy (HD) **-** (n = 20)	**Mild** **+** (n = 19)	**Moderate – severe** **++** (n = 21)
Sex, no (%)			
Female	13 (65)	14 (65)	13 (62)
Male	7 (35)	5 (35)	8 (38)
Mean age (range [year])	34 ± 2,1 (18-51)	34 ± 2,0 (18 - 50)	33 ± 1,5 (22 - 51)
SCORAD score[b]	None	**14,4 ± 1,7 (0 – 24,8)**	**42,5 ± 2,9 (27,5 – 79,6)**
Onset of disease, no. (%)			
Since childhood	-	10 (53)	20 (95)
> 10 years	-	5 (27)	1 (5)
> 2 years	-	2 (10)	-
< 2 years	-	2 (10)	-
Grass pollen allergy, no. (%)	1 (5)	12 (63)	21 (100)
Other allergies, no. (%)	4 (20)	14 (74)	19 (90)

[a] Differences in the values for gender and age between healthy and AD patients were not significant.

[b] Significant differences between patient groups ($p < 0,0001$)

Hematological parameters, such as hemoglobin, number of red and white blood cells, number of platelets and the hematocrit values were similar in all study cohorts (**Tab. 3.4**).

Tab. 3.4: Hematological profile of healthy controls and patients with AD

Factor [a]	Mean (range) for the group			
	Control subjects	Patients with AD		
	Healthy (HD) **-** (n = 20)	**Mild** **+** (n = 19)	**Moderate – severe** **++** (n = 21)	***P*-value[b]**
Hb (g/dL)	14.02 (12.5 – 17)	14.24 (12 – 17.3)	14.68 (11.1 – 23.12)	NS
RBCs (10^6 /µL)	4.73 (4.2 – 5.2)	4.9 (4.2 – 5.8)	4.84 (3.9 – 5.3)	NS
WBCs (10^3 /µL)	6.31 (4.79 – 8.28)	6.86 (5.34 – 10.16)	7.41 (4.76 – 12.71)	NS
HCT (%)	41.85 (37.7 – 50.0)	42.54 (35.6 – 50.3)	42.59 (32.3 – 47.7)	NS
PLTs (10^3 /µL)	244.3 (164 – 362)	250.8 (157 – 355)	263.5 (171 – 331)	NS

[a] Hb, hemoglobin; RBCs, red blood cells; WBCs, white blood cells; HCT, hematocrit; PLTs,platelets.

[b] NS, not significant. (*p*-values were calculated by one-way ANOVA)

The counts of most leucocyte types such as lymphocytes, neutrophils, monocytes and basophils (**Fig. 3.16A-B, D-E,** respectively) did not differ between the three groups. Only the numbers of eosinophils increased gradually and significantly in patient cohorts (**Fig. 3.16C**), presumably driven by the Th2 responses in AD.

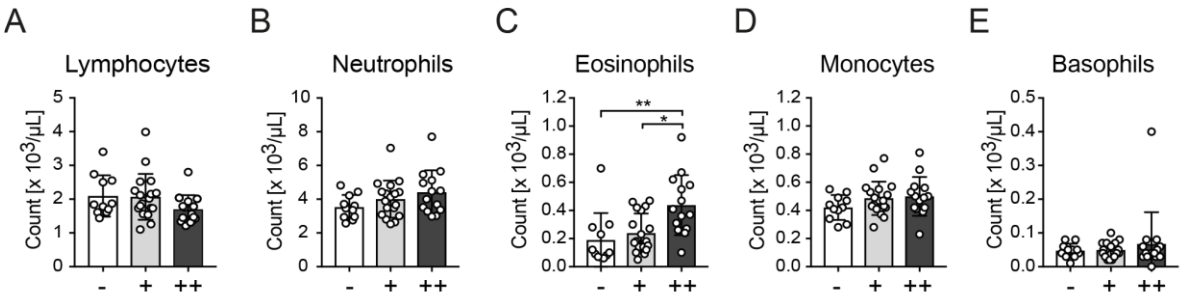

Fig. 3.16: Elevated numbers of eosinophils are associated with severe AD. Counts of lymphocytes (A), neutrophils (B), eosinophils (C), monocytes (D) and basophils (E) in white blood cells of healthy donors (-) and patients with mild (+) or severe (++) AD. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 12 (-), n = 18 (+), n = 15 (++). *P*-values were calculated by Kruskal-Wallis, **p* < 0.05, ***p* < 0.01.

Plasma samples were analyzed to assess the antibody levels. Comparing the three groups, a gradual increase of total IgE levels was detected. Healthy donors had the lowest levels, while severe AD patients displayed the highest levels (**Fig. 3.17A**). Regarding total IgG4 (**Fig. 3.17B**) and IgG1 (**Fig. 3.17C**), no significant differences were observed. Interestingly, patients with mild AD displayed a

significant increase in IgG3 compared to healthy donors and slightly elevated levels compared to severe patients (**Fig. 3.17D**).

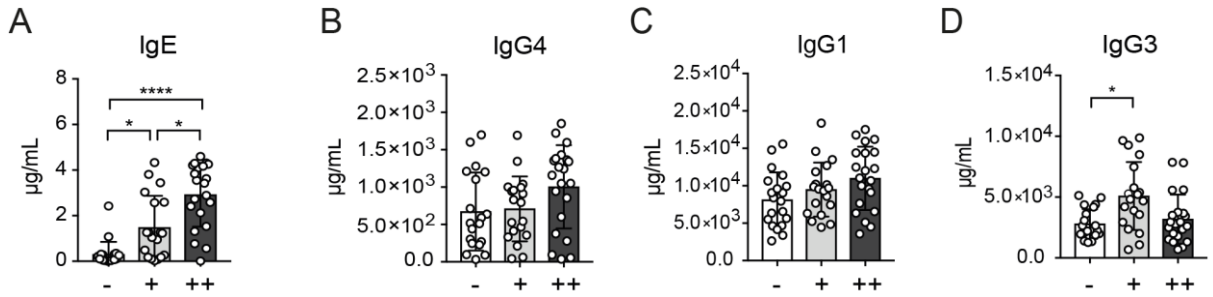

Fig. 3.17: Gradual increase of IgE level in plasma samples of AD patients. Levels of total IgE (A), total IgG4 (B), total IgG1 (C) and total IgG3 (D) of healthy donors (-), patients with mild (+) or severe (++) AD are shown. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 20 (-), n = 19 (+), n = 19 (++). *P*-values were calculated by Kruskal-Wallis (A,D) or one-way ANOVA (B, C) based on the results for normal distribution. *$p < 0.05$, ****$p < 0.001$.

Overall, the study groups differed in terms of disease phenotypes, but were otherwise healthy and comparable regarding demographic factors. Patients of both AD groups were characterized by significant higher eosinophil counts and gradually higher levels of total IgE with increasing severity of disease. A detailed comparison between all study groups with respect of the T helper cell responses is described in the following sections.

3.3.2. AD is associated with elevated Th2 responses

PBMC isolated from whole blood samples were analyzed for the T helper cell responses after stimulation with PMA/ionomycin by flow cytometry. The previously described gating strategy (Fig. 3.2.) was expanded here, as the AD-study included additional markers for a more precise analysis of the T helper cell subsets (**Fig. 3.18**). The effector/memory subpopulation ($CD45RO^+$) was included, while TCR $V\alpha24\text{-}J\alpha18^+$ cells (hereinafter referred to TCR-iNKT) were excluded, which allowed omitting iNKT cells. Finally, starting from live $CD3^+CD4^+CD45RO^+$ effector/memory $TCR\text{-}iNKT^{neg}$ cells, the Th2, Th1 and Th2/1 markers as well as other factors and cytokines were investigated.

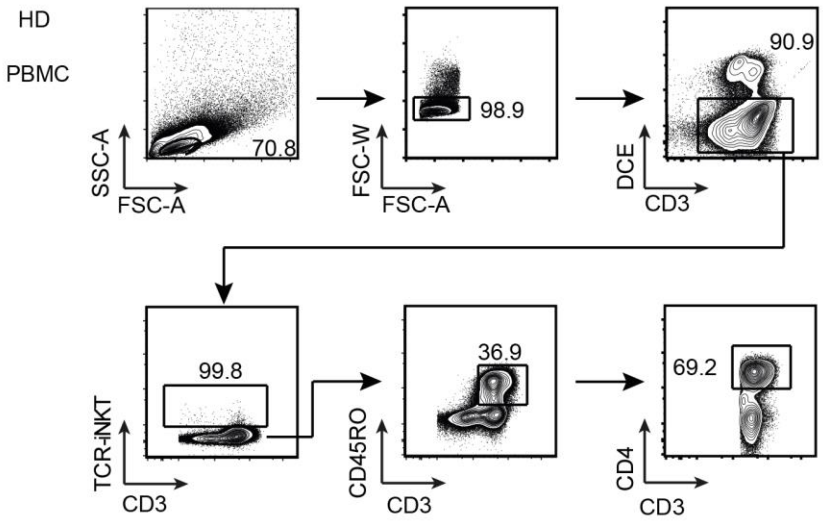

Fig. 3.18: Gating strategy for live CD4⁺ T cells as starting population for further analyses of T cell subsets in AD. Exemplary contour plots displaying cells isolated from PBMC of a healthy donor. Cells were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed by flow cytometry. The gating started on lymphocytes (top left), next on single cells (top middle), followed by plotting of CD3 vs. dead cells (top right) to include only live cells. The next gate was set on TCR-iNKT⁺ cells, but here events inside the gate were excluded. Hence, the number indicates the frequency of TCR-Vα24-Jα18⁻ non-iNKT cells (bottom left). Based on the TCR-iNKT⁻ subpopulation it was gated on CD3⁺CD45RO⁺ memory cells (bottom middle), followed by gating on CD4⁺ T cells (bottom right) as starting population for further analyses.

Patients with severe AD displayed significantly elevated frequencies of Th2 cells expressing IL-4, IL-13 and IL-5 compared to healthy donors (**Fig. 3.19A-C**), while the mean values for IFN-γ⁺ Th1 cells were similar in all groups (**Fig. 3.19D**). Both patient groups showed only slightly higher frequencies of the Th2 transcription factor GATA-3 compared to controls (**Fig. 3.19E**). Interestingly, the severe AD cohort displayed increased levels of the Th1 cells expressing T-bet (**Fig. 3.19F**). Moreover, patients with severe AD also displayed significant elevated levels of CD4⁺Foxp3⁺ compared to the other two cohorts (**Fig. 3.19G**). Next, the ratios of Th2 : Th1 cells were determined. High levels of Th2 cytokine producing cells in severe AD were reflected by an increased ratio of IL-13⁺ : IFN-γ⁺ cells (**Fig. 3.19H**, left). The ratios of IL-4⁺ : IFN-γ⁺, and IL-5⁺ : IFN-γ⁺ were similar in all groups (data not shown). The surprisingly high T-bet expression levels by CD4⁺ T effector memory cells in severe AD patients were reflected in relatively low GATA-3⁺ : T-bet⁺ cell ratios (**Fig. 3.19H**, right).

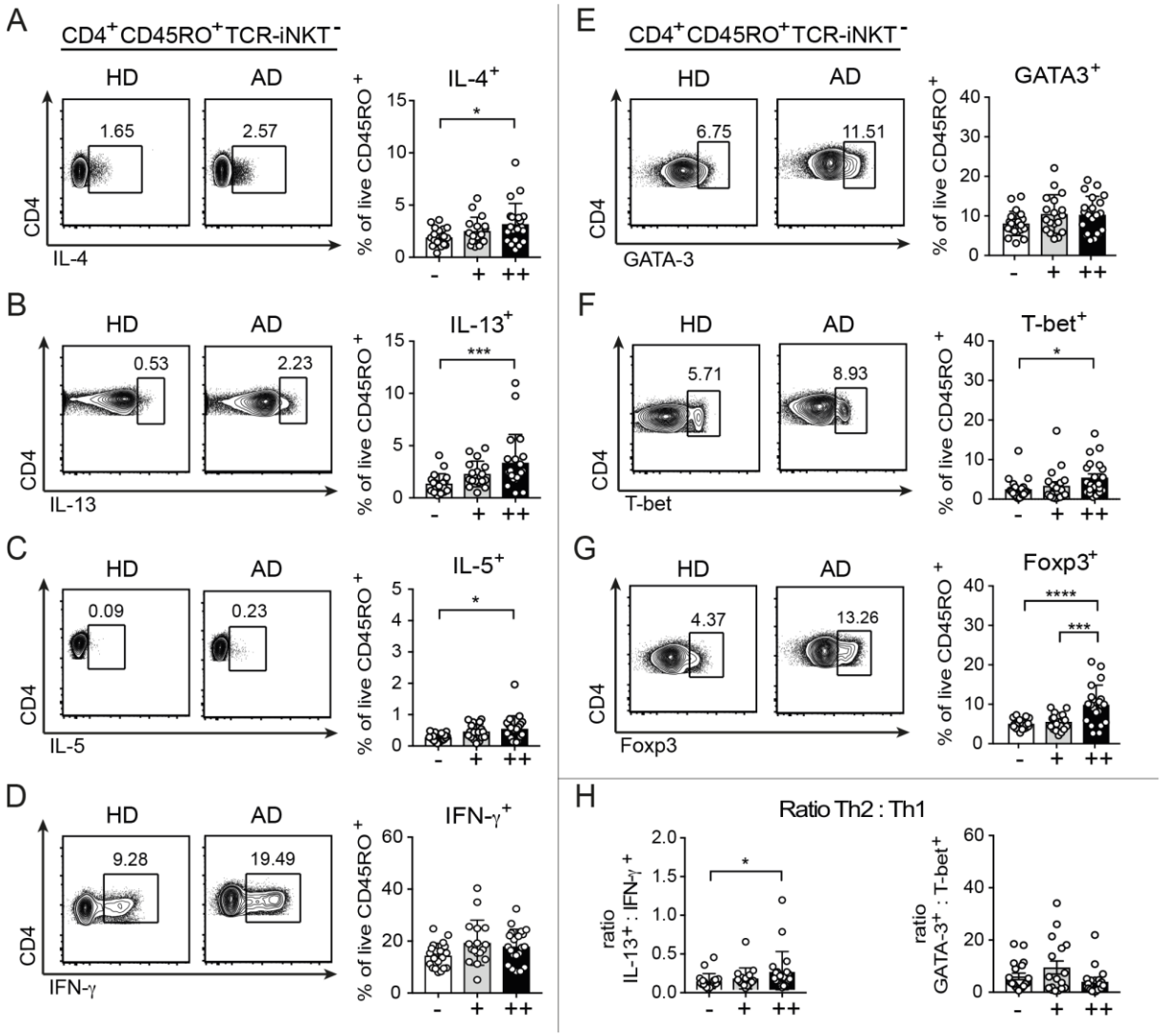

Fig. 3.19: AD severity correlates with Th2 cytokine bias. PBMC were stimulated with PMA/ionomycin for 4 h and stained IC for Th2 and Th1 cytokines and transcription factors as well as Foxp3 and were analyzed via flow cytometry within the live $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^-$ T cell subset (A-D) Left: Exemplary contour plots of T cells expressing the Th2 cytokines IL-4 (A), IL-13 (B), IL-5 (C) and the Th1 cytokine IFN-γ (D) of a healthy donor (HD) and an AD patient with severe AD. Right: Frequencies of the corresponding cytokines in healthy donors (-) and patients with mild (+) or severe (++) AD. (E-G) Left: Exemplary contour plots of T cells expressing the transcription factors GATA-3 (E), T-bet (F) and Foxp3 (G) of a healthy and an affected donor. Right: Frequencies of the corresponding transcription factors detected for the three study groups. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 20 (-), n = 18 (+), n = 21 (++). (H) Ratio of Th2 : Th1 single producing cells depicted for all cohorts based on cytokines (IL-13/IFN-γ, left) and transcription factors (right). The *p*-values were calculated by Kruskal-Wallis test (A-C, F) or one-way ANOVA (D, E, G) depending on the results for normal distribution. $*p < 0.05$, $***p < 0.005$, $****p < 0.001$.

Taken together, severe AD correlated with elevated Th2 cytokine expression. Interestingly, this cohort also displayed significantly increased frequencies of T-bet and Foxp3 expressing cells compared to HD. Mild AD patients displayed moderate expression levels of Th2 associated markers not reaching statistical significance compared to the HD cohort.

Next, circulating memory $CD4^+$ Th2 and Th1 cells were analyzed for their skin-homing potential based on CLA expression and surveyed for the expression of the Th2-associated surface molecule CRTH2.

3.3.3. Severe AD is marked by increased skin-homing Th2 cells, but CRTH2 as Th2 marker is subordinated to GATA-3 detection

Further investigations focused on the skin-homing (CLA^+) and the non-skin-homing cells (CLA^- ; in the following referred to as “systemic”) Th2 and Th1 subsets. First of all, the CLA expression was analyzed within the live $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^-$ population. Patients with severe AD displayed a significant increase of skin-homing $CD4^+CLA^+$ T cells compared to healthy donors (**Fig. 3.20A**), whereas mild AD patients displayed intermediate levels of CLA^+ T cells. Notably, healthy controls comprised considerable numbers of skin-homing cells allowing the investigation of skin-homing T cell phenotypes in absence of skin disorder. Next, Th2 and Th1 markers were assessed in the CLA^+ and CLA^- subsets (**Fig. 3.20B**). The frequencies of $CLA^+GATA\text{-}3^+$ and $CLA^+IL\text{-}4^+$ Th2 cells were at best slightly increased in severe AD (**Fig. 3.20B**, **Fig. 3.20C**). However, severely diseased patients had significantly increased levels of $CLA^+IL\text{-}13^+$ and $CLA^+IL\text{-}5^+$ cells compared to healthy controls (**Fig. 3.20C**). By contrast, mild AD patients displayed significantly elevated frequencies of $CLA^+IFN\text{-}\gamma^+$ Th1 cells compared to control individuals, whereas the frequencies for $T\text{-}bet^+$ cells were similar in all groups (**Fig. 3.20D**). Interestingly, severe patients exhibited significant lower levels of systemic $CLA^-GATA\text{-}3^+$ cells as well as slightly decreased frequencies of $CLA^-IL\text{-}4^+$ compared to mild AD patients (**Fig. 3.20B**, **Fig. 3.20E**). In all study cohorts, similar frequencies of the systemic $CLA^-IL\text{-}13^+$ and $CLA^-IL\text{-}5^+$ subsets were detected (**Fig. 3.20E**), in contrast to the observations made for the skin-homing population. The systemic CLA^- Th1 response was similar in all groups for $CLA^-T\text{-}bet^+$ and $CLA^-IFN\text{-}\gamma^+$ (**Fig. 3.20F**).

Strikingly, the skin-homing CLA^+ and the systemic CLA^- subpopulation displayed distinct differences regarding the expression of Th2 and Th1 markers. Th2 cells were enriched in the CLA^+ fraction in all patient groups compared to the CLA^- subset (**Fig. 3.20C** vs. **Fig. 3.20E**). Conversely, CLA^- cells comprised higher levels of Th1 cells than CLA^+ cells (**Fig. 3.20D** vs. **Fig. 3.20F**).

In summary, these data demonstrate that a considerable proportion of $CD4^+CD45RO^+$ skin-infiltrating CLA^+ T cells were present in patients as well as healthy donors, as described previously [35]. Especially patients with severe disease had high levels of skin-infiltrating $CD4^+$ T cells. Furthermore, severe AD was marked by a strong skin-infiltrating CLA^+Th2 response, while the mild AD cohort exhibited elevated levels of $CLA^+IFN\text{-}\gamma^+$ cells. In general, Th2 cells were enriched within the skin-homing CLA^+ population, while Th1 cells were enriched in the systemic CLA^- subset.

Consequently, a distinction into CLA^+ and CLA^- circulating T cells allows a more precise characterization of conventional Th2 and Th1 responses during AD. It was thus possible to characterize severe AD by a strong skin-infiltrating Th2 response. Hence, CLA^+ T cells isolated from blood probably delineate inflammatory events occurring in skin.

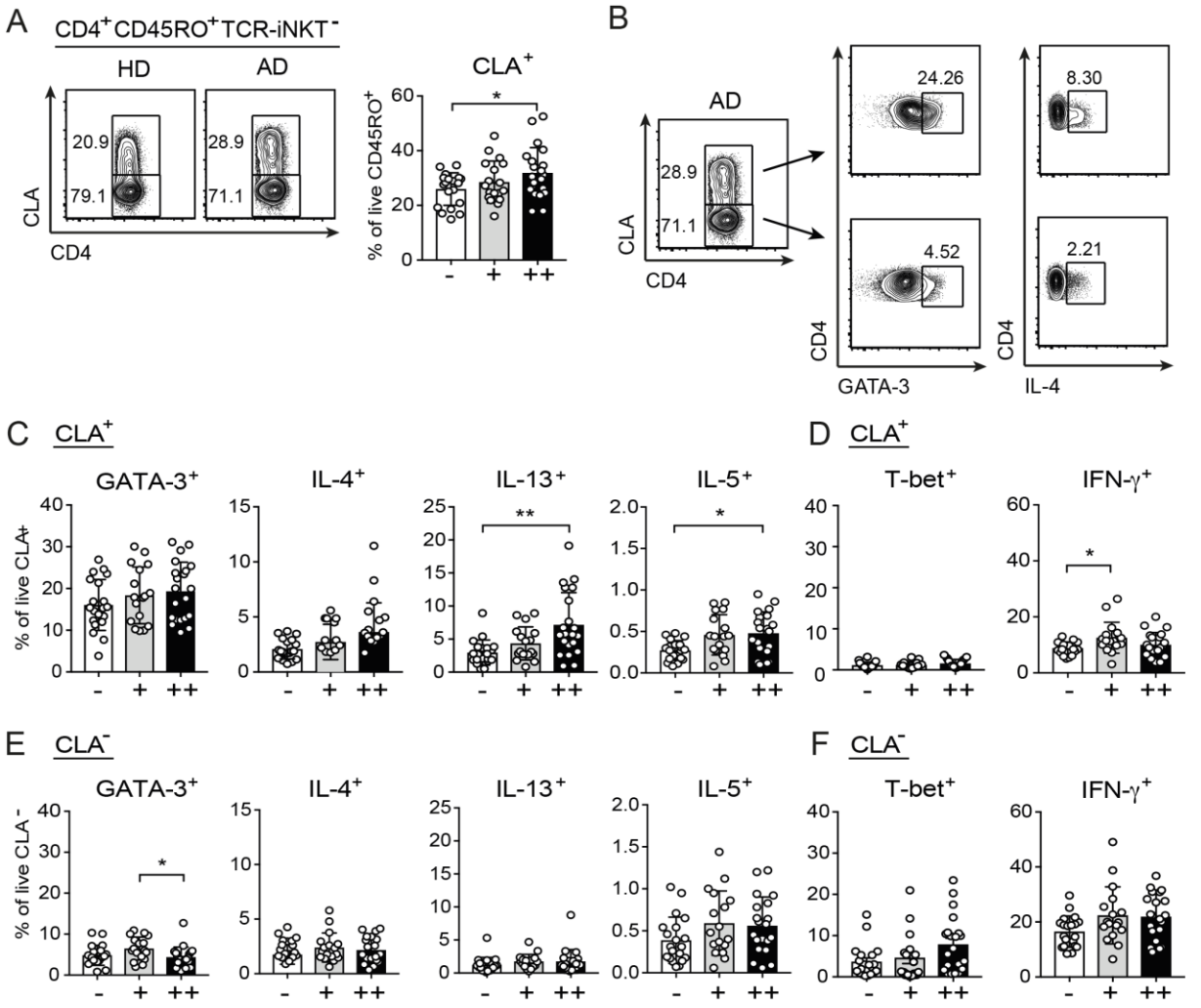

Fig. 3.20: Th2 cells are enriched in the skin-homing (CLA^+) subset. PBMC were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed for Th2 and Th1 cytokines and transcription within the $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^{neg}CLA^+$ and $CD3^+CD4^+CD45RO^+TCR^{neg}CLA^{neg}$ subsets via flow cytometry. (A) Left: Representative contour plots of the skin-infiltrating (CLA^+) and the systemic (CLA^-) subset within the live $CD4^+CD45RO^+$ population of a healthy donor (HD) and a severe AD patient (AD). Right: Frequencies of the CLA^+ subset within the $CD4^+CD45RO^+$ population in healthy donors (-), patients with mild (+) and severe (++) AD. (B) Exemplary FACS plots of a patient with severe AD showing the CLA^+ and CLA^- cells within the $CD4^+CD45RO^+$ subset (left). Further gating on $GATA\text{-}3^+$ (middle) and $IL\text{-}4^+$ (right) cells within the CLA^+ (top) or CLA^- (bottom) subset, respectively. (C-D) Frequencies of the indicated Th2 (C) and Th1 (D) markers detected within the CLA^+ population of the control (-) and patient groups (mild (+) or severe (++)). (E-F) Frequencies of the indicated Th2 (E) and Th1 (F) markers detected within the CLA^{neg} subset of the cohorts. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 20 (-), n = 18 (+), n = 19 (++). The *p*-values were calculated by the Kruskal-Wallis test $*p < 0.05$, $**p < 0.01$.

CRTH2 has been described as the most selective Th2-cell surface marker in humans [113] and was shown to promote Th2 cytokine production [143]. Since the human Th2/1 cells in threadworm-infected patients poorly expressed GATA-3, the question arose whether the expression of CRTH2 followed the same pattern than detected for GATA-3 or whether CRTH2 was more prominently expressed by human Th2/1 hybrids. Hence, CRTH2 was included to further characterize Th2 and Th2/1 cells in this study. First, the overall frequencies of $CRTH2^+$ T cells were determined in the study cohorts. Similar to the GATA-3 expression pattern, CRTH2 expression was detectable in the $CD4^+CD45RO^+$ population of all groups with slightly elevated levels in both patient cohorts compared to controls (**Fig. 3.21A**).

Next, the co-expression of IL-13 and GATA-3 with CRTH2 was analyzed. As expected, basically all IL-13$^+$ T cells simultaneously expressed GATA-3 (**Fig. 3. 21B**). The majority of CRTH2$^+$ T cells also co-expressed GATA-3 (**Fig. 3.21B**) expectedly, as CRTH2 expression is mediated by GATA-3 [113]. However, IL-13$^+$ cells displayed a shift in CRTH2 expression, but CRTH2 resolution was subordinate to the resolution of GATA-3 expression (**Fig. 3.21B**). Correlations between GATA-3/IL-13, GATA-3/CRTH2 or IL-13/CRTH2, respectively, confirmed these findings. Significant positive correlations were detected for IL-13 and GATA-3 (**Fig. 3.21C**) as well as for CRTH2 and GATA-3 (**Fig. 3.21C**). CRTH2 expressing cells correlated only weakly with IL-13$^+$ T cells (**Fig. 3.21C**).
Taken together GATA-3 detection resulted in a more comprehensive estimation of Th2 responses than CRTH2 detection. Hence, CRTH2 was not considered in further analyses.

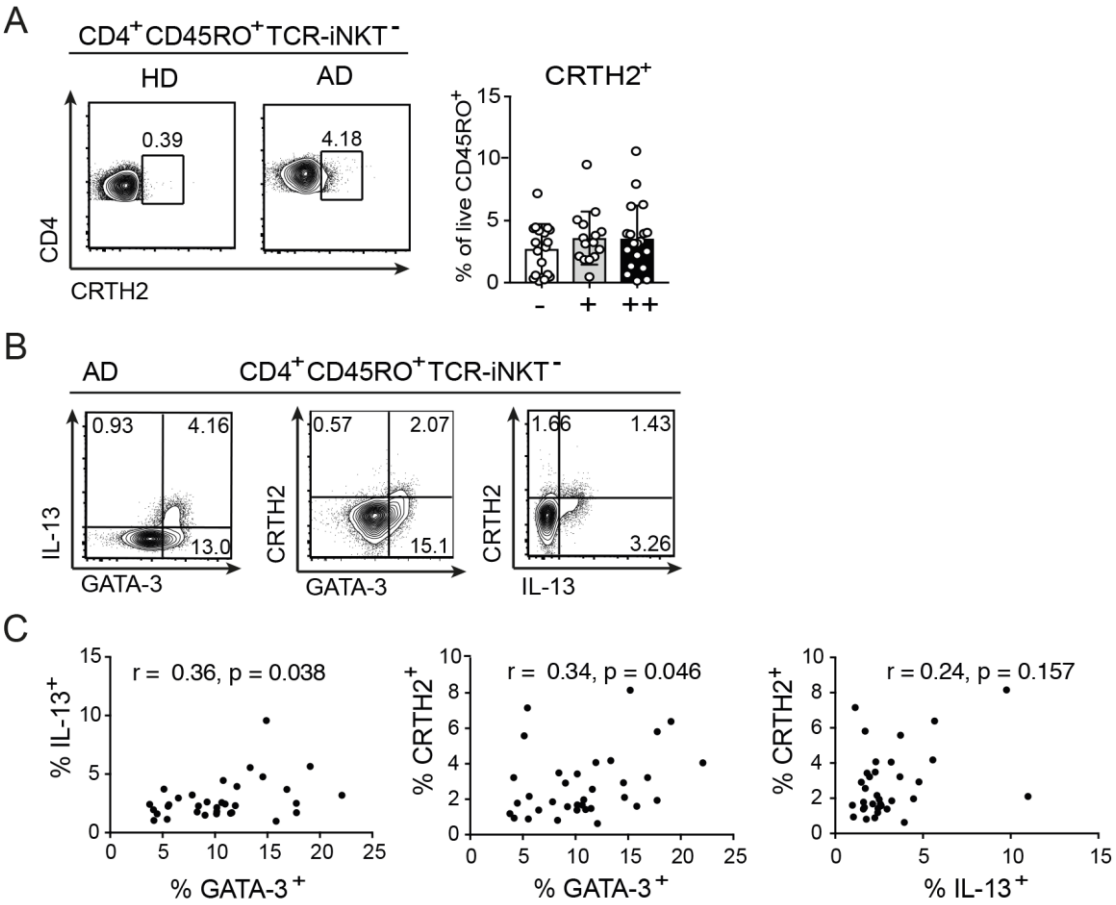

Fig. 3.21: CRTH2 as a Th2-related cell surface marker is not sufficient for an overall estimation of Th2 frequencies. PBMC were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed for Th2 marker (GATA-3, IL-13, CRTH2) within the CD4$^+$CD45RO$^+$TCR-iNKT^{neg} subset via flow cytometry. (A) Exemplary contour plots (left) of live CD4$^+$ T cells expressing CRTH2 in PBMC of a healthy donor (HD) and a severe AD-patient (AD). Frequencies of CRTH2 (right) in healthy donors (-) and patients with mild (+) or severe (++) AD are shown. The results are displayed as scatter plots with each circle representing a single individual. Depicted bars represent mean + SD of n = 18 (-), n = 15 (+), n = 19 (++). No significant differences between the cohorts were obtained by using one-way ANOVA. (B) Representative FACS plots of live CD4$^+$ T cells expressing GATA-3 and IL-13 (left), GATA-3 and CRTH2 (middle), IL-13 and CRTH2 (right) of an AD-patient. Numbers indicate frequencies of single and co-producing subsets as depicted. (C) A significant positive correlation is shown between frequencies of GATA-3 and IL-13 (left) as well as GATA-3 and CRTH2 (middle) in CD4$^+$ T cells of AD-patients (n = 32) ($p^* < 0.05$). No significant association between frequencies of IL-13 and CRTH2 (right) expressing cells in the same study population (n = 32). The Pearson correlational coefficient was used.

In summary, the inclusion of the skin-homing marker CLA allowed a more detailed analysis of conventional Th2 and Th1 responses. Circulating Th2 cells were more biased for skin homing in AD patients than healthy donors, while a large fraction of Th1 cells was detected in the non-skin-homing subpopulations of healthy and AD patients.
The surface marker CRTH2 only partially represented Th2 cells and was therefore not considered for further analyses of the Th2/1 phenotype.

3.3.4. Th2/1 cells occur in AD and appear enriched within the CLA$^-$ subset

The next analysis assessed if Th2/1 cells with similar characteristics to those of threadworm infected patients were present in the CD3$^+$CD4$^+$CD45RO$^+$TCR-iNKT$^-$ population of AD patients and if elevated Th2/1 cell levels were found in diseased blood donors. Indeed, also European patients with AD displayed cells co-producing IL-4/IFN-γ and, at lower levels, IL-13/IFN-γ (**Fig. 3.22A-B**). However, similar frequencies of Th2/1 cells were detected in patient and control groups, mirroring the findings of the human threadworm infection study. IL-5$^+$IFN-γ$^+$ T cells were almost absent in all groups, a finding probably related to the fact that IL-5 responses were generally low in all cohorts (**Fig. 3.22C**). Frequencies of GATA-3$^+$T-bet$^+$ subset were very low, but elevated in severe patients compared to controls (**Fig. 3.22D**). As observed in the Indian donors with threadworm infection ratios of Th2 : Th2/1 cytokine producers were similar in all groups (**Fig. 3.22E**).
Hence, CD4$^+$CD45RO$^+$ effector memory T cells from European participants with or without AD comprised Th2/1 cells at similar levels. Human cytokine Th2/1 cells appeared in different magnitudes with highest frequencies of IL-4$^+$IFN-γ$^+$ cells followed by IL-13$^+$IFN-γ$^+$ cells, while IL-5$^+$IFN-γ$^+$ cells were largely absent.

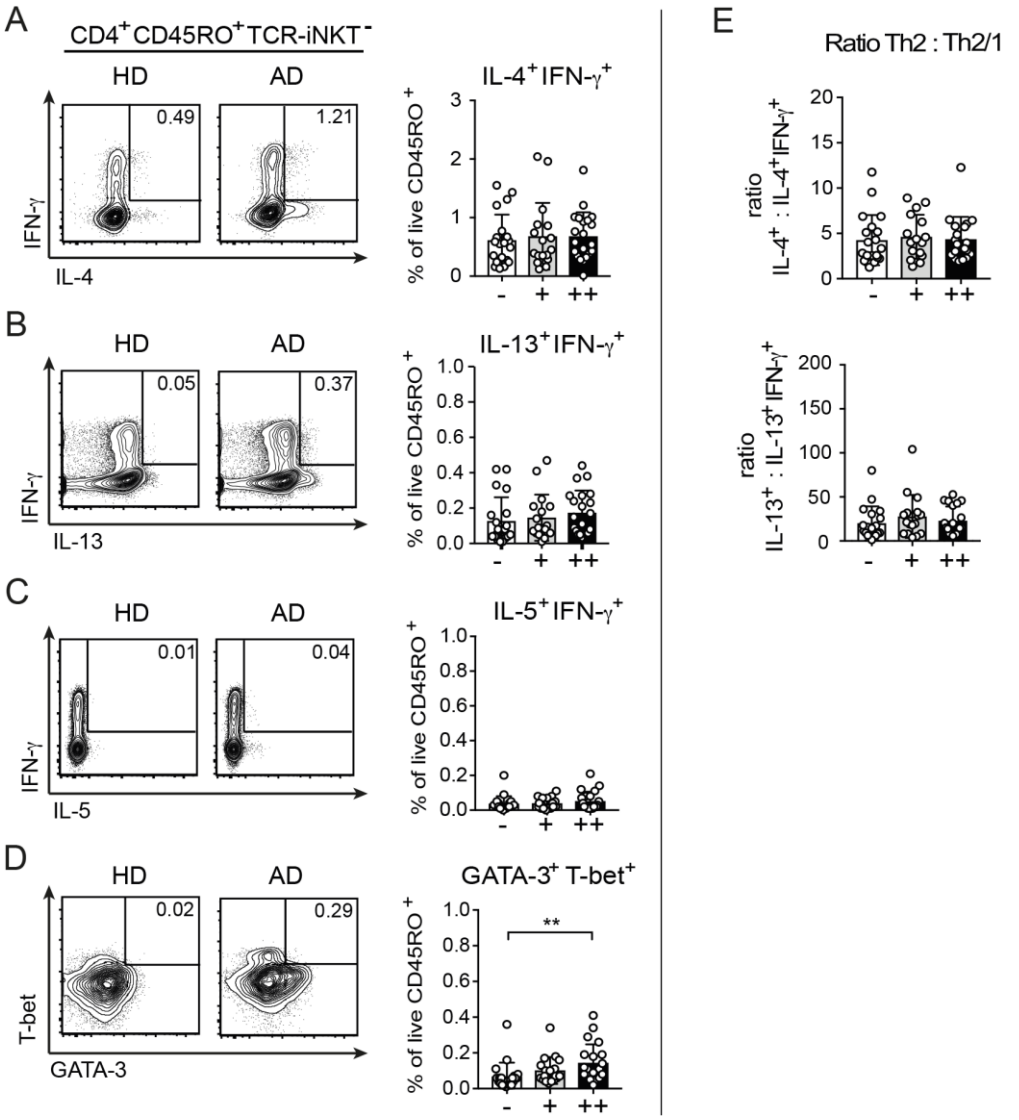

Fig. 3.22: Different disease phenotypes of AD induced Th2/1 cells. PBMC where stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed via flow cytometry based on the live $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^{neg}$ subset. (A-D) Left: Exemplary contour plots of PBMC co-producing IL-4/IFN-γ (A), IL-13/IFN-γ (B), IL-5/IFN-γ (C) and co-expressing GATA-3/T-bet (D) of a healthy (HD) and a patient with severe AD (AD). Right: Frequencies of the corresponding double-positive cells in healthy donors (-), patients with mild (+) and severe (++) AD. Results are displayed as scatter plots with each circle representing a single individual. Depicted bars represent mean + SD of n = 20 (-), n = 18 (+), n = 21 (++). (E) Ratios of Th2 : Th2/1 cells as indicated. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 17 (-), n = 16 (+), n = 18 (++). The *p*-values were calculated by Kruskal-Wallis test. $^{**}p < 0.01$.

Next, Th2/1 cells were analyzed within the skin-homing $CD4^+CD45RO^+CLA^+$ and systemic $CD4^+CD45RO^+CLA^-$ subset (**Fig. 3.23A**). Strikingly, $IL\text{-}4^+IFN\text{-}\gamma^+$ T cells as the most prominently detected Th2/1 cell type were enriched within the systemic CLA^- subpopulation (**Fig. 3.23A**, **B, C**, **E**), while $IL\text{-}13^+IFN\text{-}\gamma^+$ cells were detected at similar levels within the CLA^+ and CLA^- subset (**Fig. 3.23B-C**). A comparison of the study groups revealed similar Th2/1 cell levels within CLA^+ and CLA^- cells in all cohorts (**Fig. 3.23B-C**).

The $IL\text{-}4^+IFN\text{-}\gamma^+$ cells most prominently detected in the systemic $CD4^+CD45RO^+CLA^-$ subpopulation were also analyzed for their transcription factor profile. As seen in threadworm-infected patients, IFN-γ single producing cells clustered within the $T\text{-}bet^+$ population (**Fig. 3.23D**), while IL-4 single producers

expressed GATA-3 (**Fig. 3.23D**). IL-4$^+$IFN-γ$^+$ Th2/1 hybrid cells distinctly expressed GATA-3 at low and T-bet at high level (**Fig. 3.23D**). Similar to the observations in patients with nematode infection, Th2/1 cells expressed T-bet and IFN-γ at high, but GATA-3 at low levels (see Fig. 3.14A).

Finally, all study cohorts were analyzed for the IL-4$^+$IFN-γ$^+$ proportion within the total CLA$^+$IL-4$^+$ or CLA$^-$IL-4$^+$ populations (**Fig. 3.23E**). This again demonstrated the enrichment of Th2/1 cells in the systemic subset, but also revealed that the Th2/1 proportions in particular within CLA$^-$ Th2 cytokine expressing subset tended to decrease with disease severity in AD patients.

In conclusion, Th2/1 cells were present in AD patients irrespective of disease severity, but also occurred in healthy controls at levels indistinguishable from the AD cohorts when expressed as frequencies of all CD4$^+$ effector memory cells. Importantly, frequencies of IL-4$^+$ Th2/1 cells were higher within CLA$^-$ subset compared to the skin homing CLA$^+$ population. This finding argues against a prominent role of Th2/1 cells in dampening the overall intradermal Th2 cytokine production by expressing lower Th2 cytokine levels compared to Th2 cells. However, the finding that CLA$^+$ as well as CLA$^-$ cells of severe AD patients tended to comprise lower Th2/1 cell proportions fit the expectation that lower Th2/1 proportions are associated with more pronounced AD. The fact that hybrid cells were more prominent in the non-skin-homing subset deserves attention, as it suggests that these cells primarily perform effector functions at other sites of the body, but not in the inflamed skin itself.

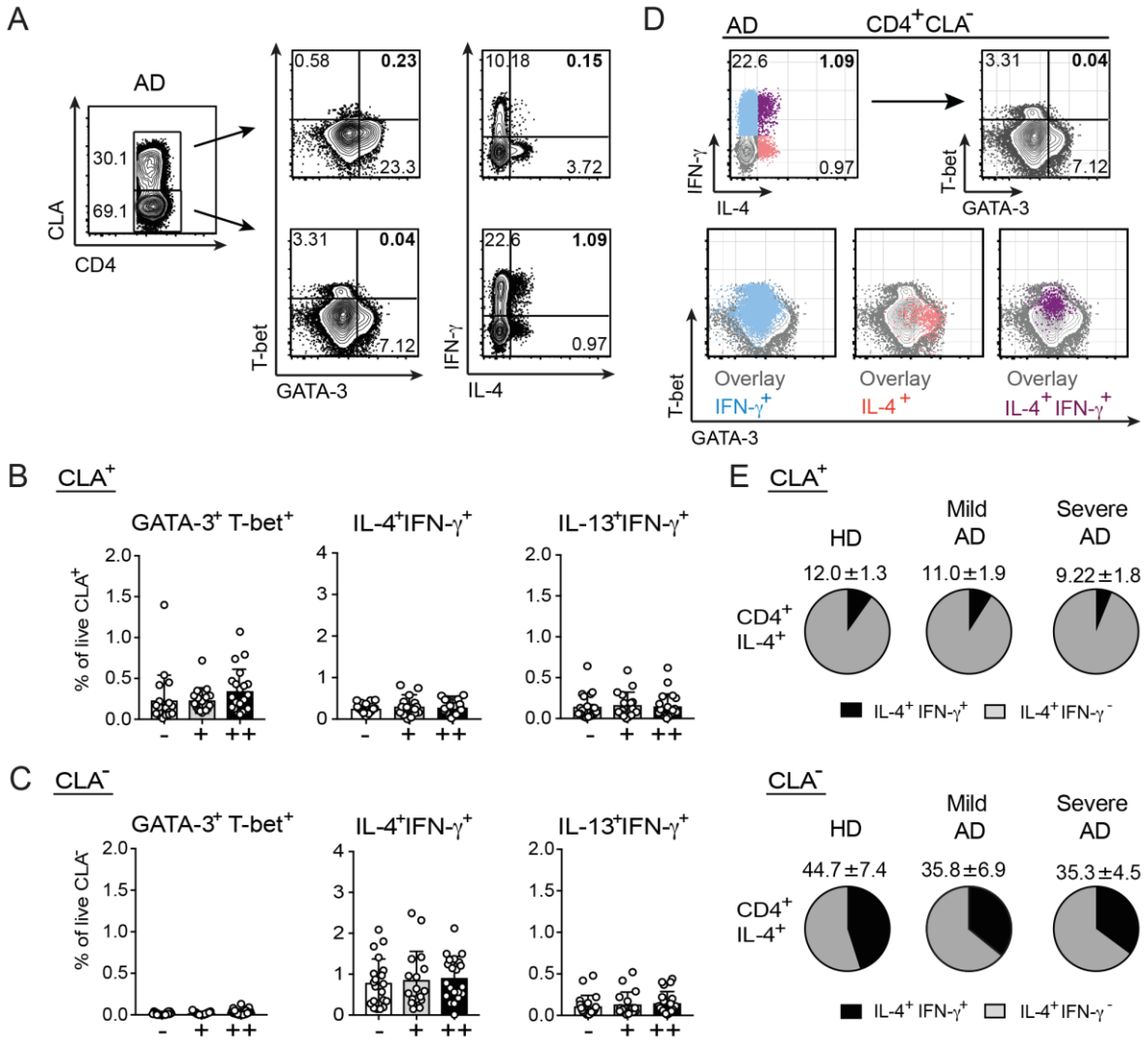

Fig. 3.23: The CLA$^+$ and CLA$^-$ subpopulation differ in Th2/1 levels. PBMC were stimulated with PMA/ionomycin for 4 h, stained intracellularly and analyzed for Th2/1 cells co-expressing Th2 and Th1 cytokines and transcription factors within the CD3$^+$CD4$^+$CD45RO$^+$TCR-iNKT^{neg}CLA$^+$ or CD3$^+$CD4$^+$CD45RO$^+$TCR-iNKT^{neg}CLA^{neg} subsets via flow cytometry. (A) Representative FACS plots of an AD patient displaying the CLA$^+$ and CLA$^-$ subsets based on the CD4$^+$CD45RO$^+$ population (left). Further gating on GATA-3 vs. T-bet (middle) and IL-4 vs. IFN-γ (right) within the CLA$^+$ (top) or CLA$^-$ (bottom) subpopulation. Numbers indicate frequencies of single (plain) and co-producing (bold) subsets. (B-C) Frequencies of Th2/1 cells as indicated within the CLA$^+$ (B) or CLA$^-$ (C) population of controls (-) and patients with mild (+) or severe (++) AD. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 20 (-), n = 17 (+), n = 19 (++). (D) Exemplary FACS plots derived from PBMC of an AD-patient depicting the cytokine expression within live CD3$^+$CD4$^+$CD45RO$^+$TCR-iNKT^{neg}CLA^{neg} (depicted as CD4$^+$CLA$^-$) T cells (top left) indicating single producers IFN-γ (blue) and IL-4 (red) as well as double-producing IL-4$^+$IFN-γ$^+$ cells (purple). The cytokine subsets were then analyzed for their respective GATA-3 and T-bet expression (top right) shown as overlays (lower row). The aforementioned color code was used. (E) Pie charts represent proportions of IL-4$^+$IFN-γ$^+$ Th2/1 cells (black) of the total IL-4$^+$ response (grey) within the CLA$^+$ (top) or CLA$^-$ (bottom) subpopulation. No significant differences were noted between the cohorts when calculated by Kruskal-Wallis test.

3.3.5. The skin-homing population of AD patients comprises several pro-inflammatory $CD4^+$ T cell subsets

Besides Th2 cytokines, IL-17 and in particular IL-22 are thought to play a pivotal role in the pathogenesis of AD [119], [144], [145]. Consequently, the production of these cytokines was assessed to provide a broader picture of AD pathogenesis. IL-17 and IL-22 producing T cells were examined within the skin-infiltrating $CD4^+CD45RO^+TCR\text{-}iNKT^{neg}CLA^+$ and systemic $CD4^+CD45RO^+TCR\text{-}iNKT^-CLA^-$ subpopulation (**Fig. 3.24A**). While CLA^+IL-17 producing cells were detected at similar levels in all cohorts (**Fig. 3.24B**), the frequencies of $CLA^+IL\text{-}22^+$ cells were significantly increased in patients with severe AD vs. control individuals (**Fig. 3.24B**). The levels of $IL\text{-}17^+$ and $IL\text{-}22^+$ cells within the CLA^- subset did not differ between the groups (**Fig. 3.24B**). A highly significant increase of skin-homing $CLA^+IL\text{-}22^+IL\text{-}13^+$ cells was seen in patients with severe AD compared to patients with mild AD and healthy donors (**3.24A-B**). By contrast, the frequencies of systemic $CLA^-IL\text{-}22^+IL\text{-}13^+$ cells were similar in all study cohorts and much lower than in the CLA^+ subset (**Fig. 3.24C**).

Hence, this work showed a highly significant increase in skin-homing Th2 cells co-producing IL-22 and IL-13 in patients with severe AD.

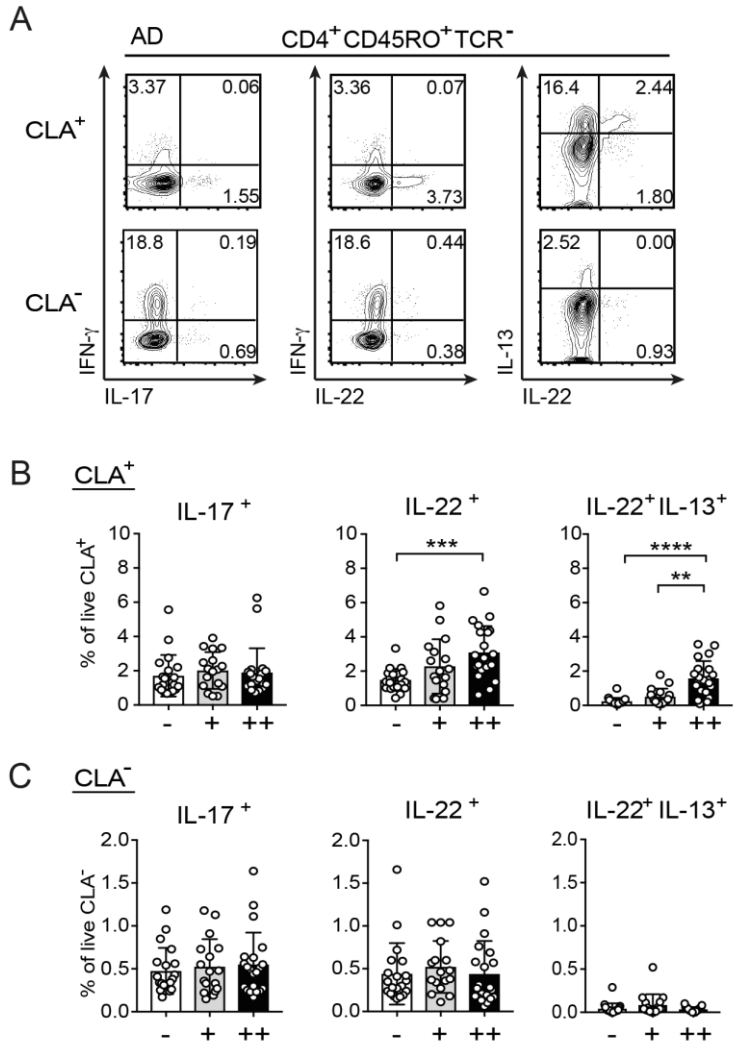

Fig. 3.24: Increased levels of pro-inflammatory cells in the skin-homing subset during severe AD. PBMC were stimulated with PMA/ionomycin for 4 h followed by intracellular staining and analysis for IL-17, IL-22, IL-13 and IFN-γ within the $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^{neg}CLA^+$ or $CD3^+CD4^+CD45RO^+TCR\text{-}iNKT^{neg}CLA^{neg}$ subsets via flow cytometry. (A) Exemplary FACS plots of an AD patient showing the expression of IL-17 vs. IFN-γ (left), IL-22 vs. IFN-γ (middle), IL-22 vs. IL-13 (right) within the skin homing CLA^+ (top row) and systemic CLA^- subset. (B-C) Frequencies of PBMC producing IL-17 (left), IL-22 (middle) and co-producing IL-13/IL-22 in the CLA^+ (B) or CLA^- (C) subpopulation. Circles represent individuals, horizontal lines and error bars represent mean + SD of n = 20 (-), n = 18 (+), n = 21 (++). The *p*-values were calculated by the Kruskal-Wallis test $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.005$.

3.3.6. Grass pollen specific $CD4^+$ T cells

As most AD patients suffered from concomitant grass pollen allergy, pollen antigen-specific responses were investigated to see if Th2/1 cells contributed to allergen-specific cytokine responses. PBMC were labeled with carboxyfluorescein succinimidyl ester (CFSE), stimulated for 7 days with a mixture of the immunodominant recombinant timothy grass pollen antigens *Phleum pratense* 5.01 and *Phleum pratense* 1 (hereinafter rPhl p 5+1) or SEB as a positive control (**Fig. 3.25A**). Unstimulated cells were kept as negative control. After 7 days, cells were stimulated again with the same antigens for 6 h and surveyed for cytokine production. Proliferated (allergen-specific) Th cells were identified by loss of the

CFSE label ($CFSE^-$). Based on the proliferated $CSFE^-CD4^+$ T cell population samples were analyzed for the expression of CLA, IL-13 and IFN-γ. Unfortunately, IL-13 production was undetectable after restimulation with grass pollen antigens or SEB (data not shown). Unstimulated controls comprised CLA^+, but no IL-13^+ cells, while IFN-γ^+ cells were detected in response to positive stimulation control SEB, but also Phl p 5+1 (**Fig. 3.25B**). PBMC stimulated with Phl p 5+1 displayed a distinct subset of CLA^+IFN-γ^+ cells (**Fig. 3.25B**). Activated $CD40L^+$ T cells were detected in similar frequencies in samples stimulated with SEB and Phl p 5+1 (**Fig. 3.25B**). However, a larger fraction of cells stimulated with grass pollen antigen expressed IFN-γ or co-expressed CLA and IFN-γ within the activated $CD40L^+$ T cell subset compared to the SEB control (**Fig. 3.25B**). Comparing AD patients with healthy donors, a slightly higher fraction of $CFSE^-$ cells was observed in patients (data not shown) and the frequencies of $CD40L^+$ T cells reacting to Phl p5+1 were slightly increased in samples of AD patients (**Fig. 3.25C**). Both cohorts displayed high frequencies of Phl p5+1 specific CLA^+IFN-γ^+ cells, the increase compared to unstimulated controls reaching significance in AD patients, but not in samples of healthy controls (**Fig. 3.25D**).

In summary, stimulation of PBMC of healthy and diseased donors with Phl p 5+1 was primarily performed to detect grass pollen allergen-specific Th2 and Th2/1 cells. Although cells from AD patients proliferated in response to the stimulation with Phl p 5+1, IL-13^+ cells were not detectable. Therefore, it was not possible to detect grass pollen specific human Th2/1 hybrid cells and analyze them further regarding activation status and preference of skin migration. Nevertheless, skin-homing Phl p 5+1 specific CLA^+IFN-γ^+ cells could be detected.

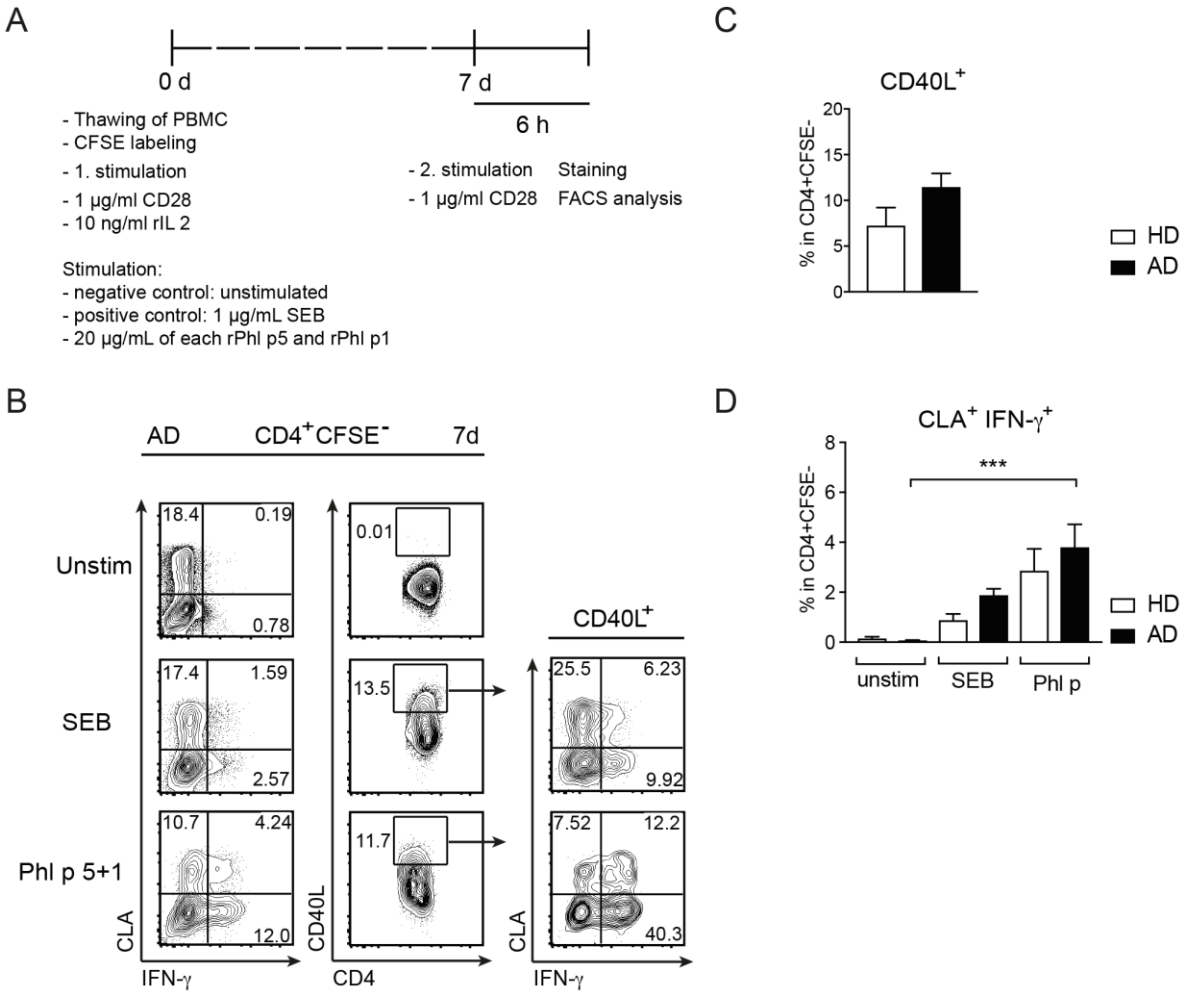

Fig. 3.25: Grass pollen specific response in AD patients. (A) Scheme of experimental procedure: PBMC were stained with the proliferation marker CFSE, kept unstimulated or were stimulated with SEB or Phl p 5+1 for 7 days and were then stimulated again for 6 h. Cells were stained intracellularly and analyzed for CLA, IL-13, IFN-γ and the activation marker CD40L within the $CFSE^{neg}CD3^{+}CD4^{+}CD45RO^{+}$ via flow cytometry. (B) Exemplary contour plots of an AD patient displaying the expression of CLA and IFN-γ (left column), CD4 and CD40L (middle column), CLA and IFN-γ within the $CD40L^{+}$ subset (right column) in an unstimulated sample (top row) and samples stimulated with SEB (middle row) or Phl p 5+1 (bottom row). (C) Frequencies of $CD40L^{+}$ cells of healthy donors (white) and AD patients (black) within the $CFSE^{neg}CD4^{+}$ population. (D) Frequencies of cells co-expressing CLA and IFN-γ within the $CFSE^{neg}CD4^{+}$ subset of healthy donors (white) and AD patients (black). All samples comprised > 300 $CLA^{+}IFN\text{-}\gamma^{+}$ cells. Data derived from two independent experiments. Results are shown as bar graphs representing mean + SD of n = 3 (HD), n = 5 (AD). The *p*-values were calculated by one-way ANOVA. **p* < 0.05, ****p* < 0.005.

3.3.7. Correlations between AD subtypes and inflammatory parameters

So far, this work shows that human Th2/1 cells exist in a non-parasitic, Th2-associated disease and that this phenotype occurs enriched within the non-skin-homing CLA^{-} population. However, Th2/1 hybrids were present irrespective of disease severity and could not be associated to a distinct clinical outcome based on this classification of patients into mild/severe AD cases. Therefore, patients were additionally stratified according to a second AD classification system: based on the total IgE-level, AD can be classified as extrinsic and intrinsic, representing approximately 80 % and 20 % of adult atopic patients, respectively [130]. Both subtypes share a similar clinical phenotype, while extrinsic AD is

characterized by high serum IgE levels and intrinsic AD patients exhibit normal serum IgE levels [99], [146]. Values of IgE levels greater then 150 IU/L define extrinsic AD and values lower than 150 IU/L define intrinsic AD. Patients previously grouped according to disease severity were here classified as extrinsic (n=30) and intrinsic (n=7) subjects. Both groups had a similar mean age of 33 vs. 36 and the SCORAD ranged from 0 - 79.6 in extrinsic AD vs. 6.5 - 33.3 in intrinsic AD. **Table 3.5** provides data of selected parameters comparing the AD subtypes. Patients with extrinsic AD displayed significantly increased eosinophil counts, elevated levels of IL-13$^+$ and IL-22$^+$IL-13$^+$ cells within the CD4$^+$ memory population compared to patients with intrinsic AD. However, both groups displayed similar frequencies of the other Th2 cytokines IL-4 and IL-5, Th2/1 cells (IL-4$^+$IFN-γ$^+$, IL-13$^+$IFN-γ$^+$) within the CD4$^+$ subpopulation (or further distinguished in skin-homing and systemic subsets) and the proportions of Th2/1 cells within the total Th2 response. Patients with extrinsic and intrinsic AD displayed also similar frequencies of total IL-17$^+$ and IL-22$^+$ cells within the CD4$^+$ population, pointing out the advantage of differentiating between CLA$^+$ and CLA$^-$ cells for analyzing the AD patient immune status in depth.
In conclusion, elevated frequencies of eosinophils, IL-13 and IL-22$^+$IL-13$^+$ T cells are associated with extrinsic AD, whereas Th2/1 cell frequencies do not differ between extrinsic and intrinsic AD patients.

Tab. 3.5: Mean frequencies of T cell subsets in patients with extrinsic vs. intrinsic AD

		Extrinsic vs. intrinsic AD (n = 30 vs. n = 7)	*p*
1.	Eosinophil (count)	0.37 vs. 0.19	0.02*
2.	CD4$^+$CLA$^+$	31.00 vs. 27.22	0.32
3.	CD4$^+$IL-4$^+$	3.1 vs. 2.0	0.13
4.	CD4$^+$IL-5$^+$	0.54 vs. 0.37	0.15
5.	CD4$^+$IL-13$^+$	3.16 vs. 1.67	0.02*
6.	IL-4$^+$IFN-γ$^+$ in total live CD4$^+$	0.71 vs. 0.56	0.73
7.	IL-13$^+$IFN-γ$^+$ in total live CD4$^+$	0.15 vs. 0.20	0.41
8.	IL-4$^+$IFN-γ$^+$ in total live CD4$^+$CLA$^+$	0.32 vs. 0.31	0.96
9.	IL-4$^+$IFN-γ$^+$ in total live CD4$^+$CLA$^-$	0.93 vs. 0.70	0.51
10.	Proportion of IL-4$^+$IFN-γ$^+$ within total CLA$^+$Th2 subset	10.85 vs. 14.68	0.56
11.	Proportion of IL-4$^+$IFN-γ$^+$ within total CLA$^-$Th2 subset	39.46 vs. 46.24	0.76
12.	IL-17$^+$ in total live CD4$^+$	1.12 vs. 0.81	0.38
13.	IL-22$^+$ in total live CD4$^+$	1.38 vs. 1.08	0.44
14.	IL-22$^+$IL-13$^+$ in total live CD4$^+$	0.48 vs. 0.19	0.04*

The *p*-values were calculated by Mann-Whitney *U* test. *p < 0.05.

Lastly, Th2/1 cells were analyzed regarding several parameters such as age, SCORAD and potentially inflammatory subsets (**Tab. 3.6**). Significant positive correlations were obtained between IL-4$^+$IFN-γ$^+$ hybrids and the eosinophil numbers, as well as the frequencies of T-bet$^+$, IL-4$^+$ and IFN-γ$^+$ cells. Interestingly, no correlation of IL-13$^+$IFN-γ$^+$ Th2/1 cells to eosinophils, Th1 or Th2 cells was detected.

However, a significant positive correlation was detected for IL-13 Th2/1 cells and age, meaning with increasing age the frequencies of IL-13$^+$IFN-γ$^+$ cells increased as well. No further significant associations were observed between Th2/1 cells and SCORAD (confirming previous results), plasma antibody levels, frequencies of Treg, IL-17-, IL-22-producing cells or the pro-inflammatory IL-22$^+$IL-13$^+$ subset.

Taken together, the correlation analysis was performed to possibly reveal an association of AD subtypes, apart from the stratification of cohorts by disease severity, and the hybrid subset to certain parameters. Extrinsic and intrinsic groups differed only regarding factors that are linked to IgE. Consistent correlations of Th2/1 cells to a certain parameter were not detected.

Tab. 3.6: Correlation analysis between circulating Th2/1 hybrid cells and selected parameters in patients with AD

		IL-4$^+$IFN-γ$^+$ r / p	IL-13$^+$IFN-γ$^+$ r / p
1.	Age	-0.08 / 0.63	0.54 / 0.0005 **
2.	SCORAD	0.02 /0.92	0,031 / 0.85
3.	Eosinophil (count)	0.38 / 0.03 *	0.18 / 0.33
4.	IgE	0.24 / 0.14	-0.01 / 0.92
5.	IgG4	0.23 / 0.15	0.27 / 0.11
6.	IgG1	0.14 / 0.41	0.26 / 0.11
7.	IgG3	0.15 / 0.37	-0.09 / 0.59
8.	GATA-3	-0.02 / 0.90	0.14 / 0.40
9.	T-bet	0.48 / 0.003 **	0.08 / 0.6487
10.	Foxp3$^+$	0.17 / 0.31	0.07 / 0.69
11.	IL-4	0.32 / 0.04 *	0.12 / 0.49
12.	IL-13	-0.03 / 0.87	0.19/ 0.25
13.	IFN-γ	0.58 / 0.0001 ***	0.16 / 0.34
14.	IL-17$^+$	0.17 / 0.31	-0.02 / 0.90
15.	IL-22$^+$	-0.018 / 0.91	0.024 / 0.89
16.	IL-22$^+$IL-13$^+$	-0.03 / 0.86	0.23 / 0.16

The Pearson correlational coefficient was used. *$p < 0.05$, **$p < 0.01$,***$p < 0.0001$.

In summary, the aim of this part of the work was to characterize the immune response of patients afflicted with AD and to possibly correlate the occurrence of human hybrid cells with distinct clinical disease phenotypes. Human hybrid cells were also detected in blood samples of European donors, confirming the third hypothesis that the occurrence of this phenotype is not a consequence of a nematode infection. However, as observed in the human *Strongyloides*-study (see 3.2.), Th2/1 cells were not restricted to diseased patients, but detected in similar frequencies in samples of healthy donors. Strikingly, Th2/1 hybrid cells appeared enriched in the non-skin-homing CLA$^-$ subset, while being rare in the skin-infiltrating CLA$^+$ subpopulation.

Regarding the patient cohorts, severe AD was characterized by elevated levels of Th2 cells, increased levels of Foxp3, IgE and elevated numbers of eosinophils. The mild cohort was defined by lower levels of IgE vs. the severe group, but high levels of IgG3 and high frequencies of skin-infiltrating $CLA^+IFN\text{-}\gamma^+$ cells.

3.4. *In vitro* generated human T cell lineages and their effect in a 3D skin model

With the previous parts of this work the existence of human Th2/1 cells was confirmed, but a clear functional role of this phenotype in a Th2-driven disorder using patient samples could not be assessed. Frequencies of Th2/1 cells were low, probably due to the limited sample volume. Sorting of Th2/1 cells for functional assays was not possible either. Even though Th2/1 cells were underrepresented in the skin-homing T cell population, the next aim was to determine a potential effector function *in vitro*, since human Th2/1 cells expressed Th2 cytokines at lower levels compared to Th2 cells. Hence, Th2/1 cells might have a lower pathological impact in a Th2-driven experimental skin inflammation.

It was previously shown that Th2 cytokines adversely affect the integrity of skin equivalents deficient in the epidermal protein filaggrin, which mimics the hallmarks of AD [134], [147]. Th2/1 and Th2 cells were generated *in vitro*, applied to a skin model and surveyed for their impact on the skin equivalents for atopic dermatitis. This work was conducted in cooperation with Prof. Sarah Hedtrich, Institute for Pharmacy, Freie Universität Berlin.

Skin equivalents were generated with primary fibroblasts and filaggrin deficient (FLG^-) keratinocytes (**Fig. 3.26A**) (kindly performed by the group of Prof. Hedtrich). The lack of the skin barrier protein FLG leads to impaired epidermal homeostasis [148], resulting in disturbed epidermal maturation [134], altered skin lipid composition and organization [138] mirrored by an impaired expression of e. g. the tight junction protein occludin [136] and an up regulation of thymic stromal lymphopoietin (TSLP), which reflects characteristics of AD. For the differentiation of T helper cell lineages, human naive $CD4^+CD45RO^+CD25^-$ cells were sorted and treated in repetitive rounds every six days with recombinant cytokines (IL-4, IL-12, IFN-γ) and neutralizing antibodies (anti-IL-4, IFN-γ or IL-12) to generate the desired cell lineage, Th1, Th2 or Th2/1, respectively (**Fig. 3.26A**). Comparing the T cell lineages with each other, cells of the Th1 condition comprised high frequencies of IFN-γ single producers (**Fig. 3.26B**). Interestingly, these cells also produced Th2 cytokines at low levels (**Fig. 3.26C**), despite multiple priming. A small proportion of Th1 cells co-produced Th2 and Th1 cytokines (**Fig. 3.26D**). In contrast, Th2 cells produced IL-4 and IL-13, but no IFN-γ (**Fig. 3.26B-C**). Indeed, the cells of the Th2/1 condition co-produced high frequencies of IL-4/IFN-γ and IL-13/IFN-γ, but displayed also low frequencies of Th1 and Th2 cytokine single producers (**Fig. 3.26B-D**).

Overall, the *in vitro* generation of the Th2/1 cell lineage was successful. The Th2/1 population comprised mainly cells co-producing Th2 and Th1 cytokines and low proportion of single producers compared to conventional cell lineages. Moreover, cells of the conventional Th2 and Th1 cell lineages produced the corresponding cytokine patterns.

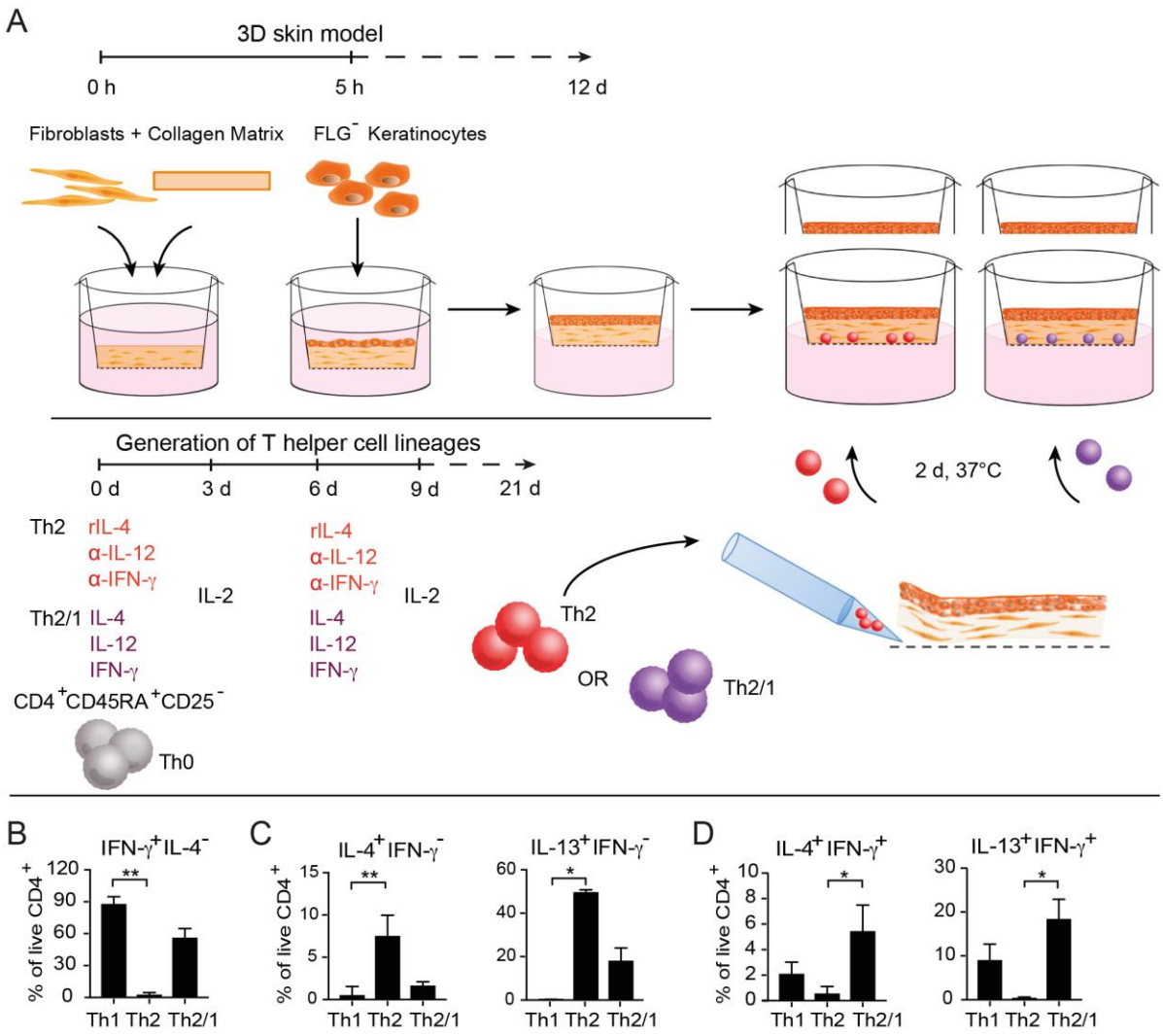

Fig. 3.26: *In vitro* generated human T helper cell lineages with distinct cytokine profiles were applied under 3D skin equivalents mimicking AD. (A) Scheme of generated 3D skin models and T helper cell lineages Th2 and Th2/1. Skin equivalents were generated from primary fibroblasts and filaggrin knock down (FLG$^-$) keratinocytes, which were cultured for 12 days (top row). For the generation of human T cell lineages naïve CD4$^+$CD45RA$^+$CD25$^-$ were treated every 6 days (in total 4 times) with polarizing recombinant cytokines and neutralizing antibodies accordingly to the respective T cell lineage. Cells were cultured for 21 days in total (Th1 not shown here). 0,375 x 10^6 activated Th2 or Th2/1 cells were then applied underneath skin equivalents and co-cultured for another 2 days followed by the subsequent analysis of the effects of the T cells lineages. (B-D) Frequencies of IFN-γ$^+$ Th1 (B), IL-4$^+$ or IL-13$^+$ Th2 (C) and IL-4$^+$IFN-γ$^+$ or IL-13$^+$IFN-γ$^+$ Th2/1 hybrid cells (D), which were analyzed via flow cytometry before co-culturing with skin equivalents. Bars represent mean + SD of n = 3-5 independent experiments from different donors. The *p*-values were calculated by the Kruskal-Wallis test. **p* < 0.05, ***p* < 0.01.

Next, the effects of the cell lineages were analyzed in a pilot test, by applying Th2 and Th2/1 cells underneath FLG$^-$ skin equivalents. After 2 days of co-culturing T cells with skin equivalents, the impact of the respective T cell lineages on the skin models were analyzed. First, the skin surface pH was measured (**Fig. 3.27A**) (performed by the group of Prof. Hedtrich). Skin equivalents co-cultured with Th2/1 cells or medium displayed pH value of 5.5 ranging in a slightly acidic pH milieu representing healthy skin [149], [150]. By contrast, the model incubated with Th2 cells displayed an increased pH value of 6.1, which is a typical characteristic of the skin of patients with atopic dermatitis [138].

Furthermore, the epidermis of the skin models was examined for skin barrier proteins. Skin

equivalents incubated with T cells showed a down regulation of occludin (OCLN) on mRNA level (**Fig. 3.27B**) suggesting an impairment of the barrier function due to produced cytokines. However, the immunofluorescence staining (performed by the team of Prof. Hedtrich) revealed a similar protein expression of OCLN in the skin equivalent incubated with Th2/1 cells and the control model without T cells (**Fig. 3.27C**). Interestingly, the occludin expression was clearly reduced in the Th2 condition compared to the Th2/1 condition or without cells (**Fig. 3.27C**).

Thymic stromal lymphopoietin (TSLP), another key player in AD pathogenesis [151] was analyzed. The epidermis of skin equivalents supplemented with T cells displayed a strong up-regulation compared to the model without cells (**Fig. 3.27B**).

Regarding the protein level the skin equivalent co-cultured with Th2/1 cells displayed also a similar TSLP expression as observed in the control skin equivalent, but were downregulated compared to the Th2 condition (**Fig. 3.27C**). These preliminary data suggested a different impact on the *in vitro* generated Th2 and Th2/1 cells in FLG deficient skin models. Th2/1 hybrid cells poorly induced skin inflammation, while Th2 cells had a strong impact.

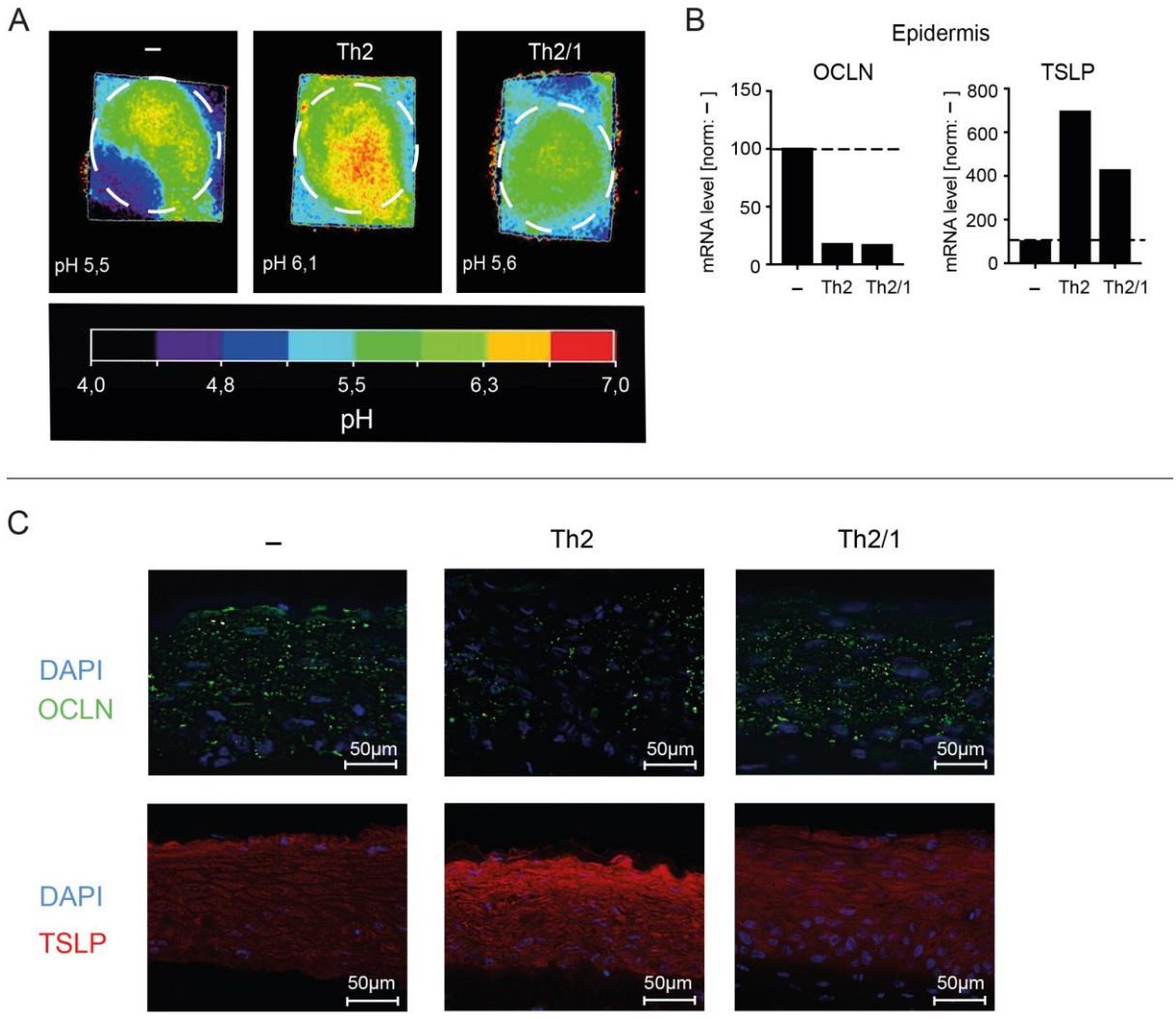

Fig. 3.27: *In vitro* generated Th2/1 cells are poor inducers of skin inflammation. Pre-trial of FLG$^-$ skin equivalents treated with medium only (-) or co-cultured with activated *in vitro* generated Th2 and Th2/1 cells for 2 days. (A) Skin surface pH measurement. (B) Relative mRNA expression of occludin (OCLN) and TSLP in epidermis. Quantification was performed via qPCR with GAPDH as house-keeping gene. (C) Immunostaining of FLG$^-$ skin equivalents for occludin (OCLN, green) and TSLP (red). Counterstained with DAPI (blue).

In addition, the expression levels of transcription factors of the generated T cell lineages were analyzed. Before T cells were transferred to skin equivalents, Th2 cells displayed elevated mRNA levels of GATA-3 (**Fig. 3.28A**), while Th1 cells (generated, but not applied to the skin models) expressed a significantly higher level of T-bet compared to the other cell lineages (**Fig. 3.28A**), as expected. Th2/1 cells expressed both transcription factors at intermediate levels compared to their conventional counterparts (**Fig. 3.28A**). After co-culture of T cell lineages with skin-models, T cells could be re-isolated from the dermis. This demonstrated the active migration of Th2 and Th2/1 cells into the skin equivalent. Re-isolated cells kept the transcription factor profile according to their respective T cell lineage (**Fig. 3.28B**). Even after such an extensive procedure starting with application of T cells, followed by co-culture with skin models and re-isolation, T cells produced cytokines. Generated Th2 cells expressed high frequencies of Th2 cytokines, while Th2/1 cells displayed higher levels of IFN-γ$^+$ and IL-13$^+$IFN-γ$^+$ cells (**Fig. 3.28C**). These preliminary data suggested that *in vitro* generated human T cell lineages were stable and maintained their phenotype.

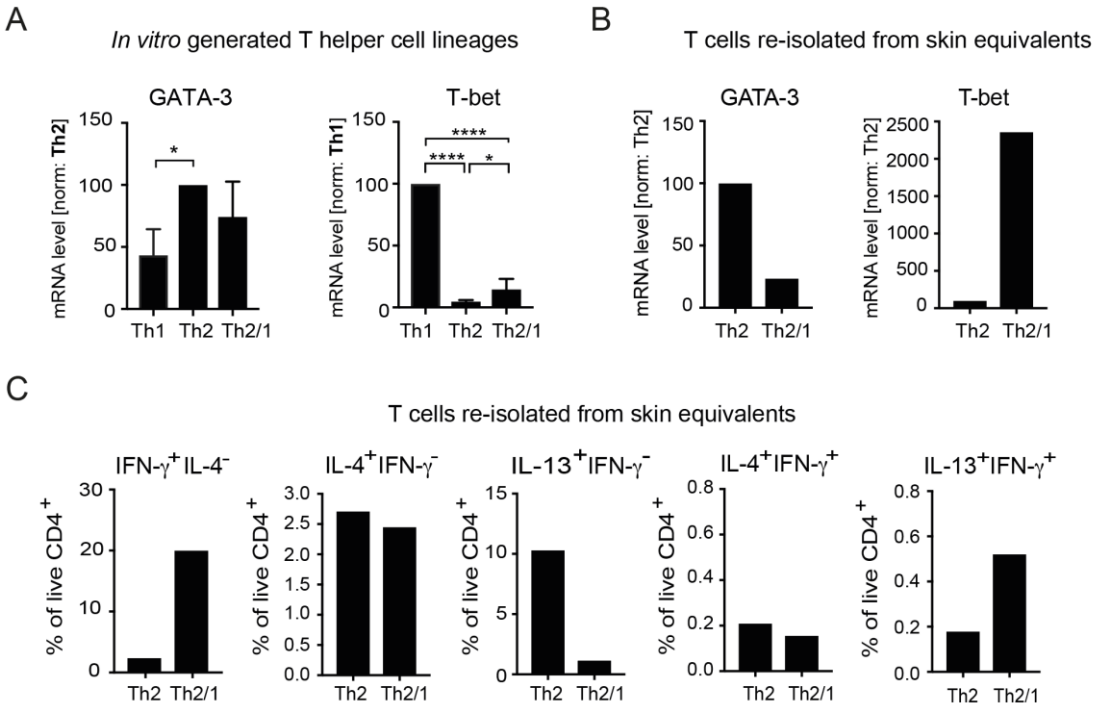

Fig. 3.28: *In vitro* generated human Th2 and Th2/1 cells seem to maintain their specific profile during co-culture with and isolation process from skin equivalents. Pre-trial of activated *in vitro* generated Th2 and Th2/1 cells co-cultured with FLG$^-$ skin equivalents for 2 days. (A-B) Relative mRNA expressions of GATA-3 and T-bet of *in vitro* generated T cells before (A) and after (B) co-culturing and re-isolation of T-cells from digested dermis of FLG$^-$ skin equivalents. Quantification was performed via qPCR with GAPDH as housekeeping gene. (C) Frequencies of T cells expressing IFN-γ, IL-4, IL-13 (left to middle) and co-producing IL-4$^+$IFN-γ$^+$ and IL-13$^+$IFN-γ$^+$ (middle right to right) after re-isolation from dermis of skin equivalents and stimulation with PMA/ionomycin. Flow cytometric analysis was performed with ≥ 0,6 x 10^5 re-isolated T cells per skin equivalent.

Taken together, human T helper cell lineages were generated successfully as characterized by distinct cytokine and transcription factor profiles. Th2 and Th2/1 applied to FLG$^-$ skin equivalents displayed different pro-inflammatory potential. Th2 cells increased the skin surface pH, downregulated the tight junction protein occludin and up-regulated TSLP compared to Th2/1 cells. These promising

preliminary results suggest Th2/1 hybrid cells as poor inducers of skin inflammation *in vitro*. Therefore, these data support the fourth hypothesis in terms of the low immunopathological potential of the Th2/1 phenotype, which needs to be re-confirmed by repeating this experiment and future studies.

4. Discussion

$CD4^+$ T cells differentiate into diverse cell lineages with distinct effector functions, based on their produced signature cytokines. Previous studies have reported that T helper cell subsets can acquire features of two T cell lineages, resulting in hybrid differentiation phenotypes [49]–[51], [152]. In particular, the Th2 and Th1 differentiation have long been considered as mutually exclusive, as cells differentiated under polarizing conditions express distinct patterns of the lineage specifying transcription factors GATA-3 and T-bet and the respective cytokines [22], [42]. Positive feedback mechanisms and reciprocal inhibition of the developmental programs reinforce and assure the efficient and mutually exclusive differentiation under the appropriate conditions [18], [38], [156]. However, studies have demonstrated that both human and murine T cell clones can co-express Th1 and Th2 cytokines [43]–[45]. More importantly, it was shown that murine $CD4^+$ T cells co-expressing Th1 and Th2 features developed naturally *in vivo* in response to the strongly Th2-polarized infection with the gastrointestinal nematode *H. polygyrus*. This Th2/1 phenotype was stably maintained for months and caused less immunopathology in transfer experiments compared to conventional Th1 and Th2 cells [48]. Based on these interesting findings this thesis investigated the Th2/1 phenotype in humans infected with the threadworm *S. stercoralis* analogous to the corresponding mouse model. This part of the present work addressed the key questions whether the occurrence of Th2/1 cells can be generalized to murine threadworm infections and whether the human $CD4^+$ T cell pool also comprises the Th2/1 hybrid phenotype with similar characteristics. Furthermore, in this work $CD4^+$ T cell responses were surveyed in humans affected by atopic dermatitis (AD). Here, patients were classified into two groups of disease severities aiming to reveal whether the Th2/1 subpopulation also occurs in a Th2-mediated inflammatory disorder other than helminth infection and whether the proportions of Th2/1 cells correlate to disease severities. Finally, *in vitro* generated human Th2/1 and Th2 cells were co-cultured with a skin model mimicking the hallmarks of AD. This trial aimed to shed light on a functional impact of Th2/1 cells compared to conventional Th2 cells in Th2-mediated diseases.

4.1. Th2/1 cells occur in murine and human strongyloidiasis

Infections with the human parasitic nematode *Strongyloides stercoralis* are an underestimated health risk with a high number of undetected cases, as the course of disease is usually asymptomatic [70], [71]. This infection may lead to lifelong persistence due to its autoinfection capacity. It can have devastating effects in immunosuppressed individuals due to corticosteroid treatment or infection with the human T lymphotrophic virus (HTLV-1) [73], [74]. Unlike in other nematode infections the spectrum of severity of infection is hard to quantify precisely [154]. The disease is thus categorized as „uncomplicated strongyloidiasis“, which is generally unrecognized by the patient, or life threatening „severe, complicated strongyloidiasis“ in immunocompromised patients [154]. The exact mechanism of the immune response to strongyloidiasis is not fully understood [73], but the importance of $CD4^+$

T cells for the adaptive response against *Strongyloides* spp. was shown especially for the protective anti-nematode immunity mediated by Th2 cells [72]. The present work expands the knowledge of Th2 cells and reports for the first time the natural occurrence of the recently described Th2/1 cells during murine and human threadworm infections.

It has been shown that experimental rodent and human infections with the threadworm *Strongyloides* spp. elicit conventional Th2 responses. Such infections with *S. ratti* or *S. stercoralis*, respectively, are both not only characterized by increased levels of Th2 cells, but also accompanied by elevated levels of IgE, IgG4 and blood eosinophils compared to uninfected healthy controls subjects [72], [81], [82], [84], [155], [156], [162]. These earlier findings are confirmed in the present work. Th2 cells are important for mediating resistance to the larvae in mice [157], [158] and are essential to protect against severe disease and hyperinfection in humans [73], [88]. Moreover, the current work shows that mice infected with the threadworm *S. ratti* displayed low signs of intestinal immunopathology (Fig. 3.1, [156]), mimicking the mostly asymptomatic course of threadworm infections in humans [154], [158], [159].

Th1 related IFN-γ activity negatively affects the control of intestinal nematode parasites [160]. The frequencies of Th1 cells expressing T-bet and IFN-γ were similar in blood samples of humans in both study cohorts, as reported earlier [81], while infected mice displayed a transiently increased IFN-γ production. Of note, alterations in Th1 responses during human threadworm infection have been described in patients co-infected with the virus HTLV-1, which induces a biased Th1 response with increased levels of IFN-γ and reduced production of Th2 cytokines [88], [161], [162]. Importantly, IFN-γ producing Th1 cells counteracting an efficient Th2 response are thought to cause the dissemination of *S. stercoralis* larvae and the development of severe strongyloidiasis in humans [71], [88]. Whether co-infection independent IFN-γ activity, e.g. by Th2/1 cells, restricts the control of *S. ratti* and, more importantly, long-lasting *S. stercoralis* infection by counteracting Th2 responses is unknown. The current study demonstrates the occurrence of the $CD4^+$ Th2/1 cells alongside elevated levels of Th2 cells during murine strongyloidiasis and shows that these cells produce IFN-γ, which might counteract the efficiency of Th2 responses and hence support host susceptibility. Evidence for such a scenario comes from a recently published study showing that mice with experimentally increased Th2/1 hybrid proportions are impaired in immune control of *H. polygyrus* infections [163].

The phenotype of the murine Th2/1 subpopulation induced by infections with *S. ratti* was similar to the subset described previously for mice infected with *H. polygyrus* and schistosomes [48]. During nematode infection the GATA-3^+T-bet^+ Th2/1 cells and cells co-expressing the Th2 cytokines IL-4/ IL-5/ IL-13 with IFN-γ displayed intermediate expression levels of Th2 markers and IFN-γ compared to the conventional cell subsets. In particular, the intermediate GATA-3 expression of murine IL-4^+IFN-γ^+ hybrid cells is consistent with previous studies [48], [55]. Moreover, Deaton *et al.* 2014 [55], reported intermediate methylation levels of the GATA-3 locus in murine IL-4^+IFN-γ^+ cells compared to Th1 and Th2 cells. These observations indicate that epigenetic mechanisms are involved in maintaining Th1 and Th2 identity [164], [165] and that DNA methylation is important for allowing the

co-existence of Th1 and Th2 characteristics [55]. Interestingly, Th2/1 cells induced by threadworm infections expressed higher levels of T-bet than Th1 cells, which probably reflect their highly activated status as effector cells compared to memory Th1 cells, which were generated before the nematode infection.

The experimental *S. ratti* infection model showed that the proportions of the Th2/1 subset within the total GATA-3 expressing Th2 population differed depending on the body compartment. The highest proportions of Th2/1 cells were detected in spleen and small intestinal tissue, while their proportions were lower in gut-draining lymph nodes and blood (Bock *et al.*, 2017 [166]). These findings were recapitulated in the recently published work on the *H. polygyrus* infection model [167]. It hence seems that the spleen might serve as an organ supporting the generation of Th2/1 cells, thereby modulating the anti-parasite immune response, which deserves attention in future studies. The relatively low levels of murine Th2/1 cells in blood compared to affected organs must be taken into account regarding the detection levels of Th2/1 cells in human blood samples, which is probably hampered by their poor representation in blood samples. The organ preference of Th2/1 cells for the spleen and small intestine in mice may offer an explanation for the overall low frequencies of Th2/1 cells detected in blood of patients infected with threadworms. Therefore, PBMC may not necessarily accurately reflect the proportion of Th2/1 cells in other compartments of the human body.

In comparison to infections with the nematode *H. polygyrus* and the trematode *S. mansoni* the proportions of Th2/1 cells in mice infected with *S. ratti* were relatively low [48], [55], [167].
Aspects possibly associated with different proportions of Th2/1 cells within the helminth-induced effector T cell pool might be the different routes of infection, differences in parasite burdens, but also the fine-tuning of host/parasite interactions. *H. polygyrus* larvae are applied orally (mimicking the oral uptake of larvae by free-living rodents) [48], whereas *S. ratti* larvae were injected in the hind footpad (mimicking the natural situation where the infective larvae enter the host via the skin) [84]. Thus, the experimentally induction of a *H. polygyrus* infection leads to a high larval up-take in contrast to the *S. ratti* infection where probably only a small fraction of injected larvae reached the small intestine. However, the current work showed that mice infected with 2000 *S. ratti* larvae displayed similar $CD4^+$ Th2 and Th2/1 cell levels as mice infected with a 10 fold lower infection dose. One could consider that the microbiota in the gut supports the induction of hybrids, due to organ-specific microbial signals, in contrast to the skin. But a recent study from my working group demonstrated that conventional and germ-free C57BL/6 mice infected with *H. polygyrus* developed Th2/1 cells, hence microbial signals were negligible for their induction [168]. Another reason could be the host specificity: Although mice are susceptible to *S. ratti* infections, rats represent the natural hosts. It was shown that rats infected with *S. ratti* exhibit quantitative increases in Th2 responses due to a higher parasite load and through time [155], [169], [170], whereas mice with a prolonged *S. ratti* infection did not display an overall increase of Th2 or Th2/1 frequencies. Therefore, it would be interesting to investigate whether the natural host species develops stronger Th2/1 responses compared to the murine host analyzed in this study, which would argue for good host adaptation in rats.

It was shown that *Strongyloides* spp. infect different host species with varying efficiency [171], but no studies directly compared infections and immune responses in different host species. However, the mouse strain C57BL/6, which was used in this study, is described as highly susceptible to *S. ratti* infections that induces patent but transient infections and were resolved spontaneously within three weeks [156], [172]. The parasite load and period of infection did have a minor impact on the proportions of Th2 and Th2/1 cells in *S. ratti* infected C57BL/6 mice. However, as addressed above, the proportion of Th2/1 cells within the total GATA-3 expressing Th2 population differed depending on the body compartment. While GATA-3$^+$T-bet$^+$ cells were present in significantly higher numbers in mice with a high worm dose and short period of infection, their levels in blood did not reach significance compared to uninfected controls in mice infected with lower burdens or a prolonged infection. Especially the infection status with low parasite load and prolonged infection, which is probably reflecting the state of disease in human strongyloidiasis, displayed only trends for Th2 and Th2/1 cell elevations in peripheral blood, whereas both cell types were significantly elevated in spleen and small intestinal tissues. Hence, the circulating murine T cell pool indeed comprised the Th2/1 phenotype, indicating blood as a suitable, but not optimal, source for further analyses of Th2/1 cells in humans, as access to other tissue samples is limited.

Indeed, the current study demonstrated that Th2/1 cells are also detectable in the peripheral blood of patients infected with the human threadworm *S. stercoralis,* although in low frequencies. The preferential location of Th2/1 cells in other organs seen in mice may explain the overall low frequencies of Th2/1 cells detected in blood of patients infected with threadworms. Therefore, PBMC may not necessarily reflect the proportion of Th2/1 cells in other compartments of the human body.
Still, the frequencies of GATA-3$^+$T-bet$^+$ T cells were significantly increased in infected blood donors compared to uninfected control subjects. This deserves attention, as most Th2/1 cells characterized by IFN-γ co-production with Th2 cytokines were marked by low GATA-3 expression, impeding the quantification of Th2/1 cells by the distinct transcription factor co-expression profile seen in mice. IL-4$^+$/IL-13$^+$ cells co-expressing IFN-γ were, however, readily detectable in infected patients, while IL-5$^+$IFN-γ$^+$ cells were present in very low numbers, reflecting the overall low IL-5 responses.

Unexpectedly, the Th2/1 phenotype was not restricted to the patient cohort and the levels of Th2/1 cells characterized by Th2 and Th1 cytokine co-expression were similar in infected and healthy individuals. Of note, it cannot be excluded that the endemic healthy controls recruited from the same rural areas in South India as the infected patients may have been infected with other Th2-inducing helminths earlier in life, leading to the formation of a Th2 memory cell population comprising the Th2/1 subset. In fact, unpublished experimental work from my working group suggests that Th2/1 cells induced in intestinal nematode infections might have an advantage in survival during memory formation, as their proportions in the GATA-3$^+$ population increases over time after parasite clearance. Hence, Th2/1 cells formed in previous helminth infections might survive better in humans and mask differences between currently infected and uninfected patients in areas where preceding contact to helminths cannot be excluded.

As samples of both uninfected and infected donors did not differ in their Th2/1 cell levels the attempt was made to assess, whether patients comprised parasite-specific Th2 and Th2/1 cells. Crude extract of *S. ratti* adult worms was used to stimulate PBMC from the study participants. Several studies have reported the usage of rodent *Strongyloides*, i.e., *S. ratti* and *S. venezuelensis* as heterologous antigens for diagnosis of strongyloidiasis [173]–[175]. Additional co-stimulatory reagents as CD49d/CD28 [81] and overnight resting of cells post thawing [176], [177] are supposed to be beneficial for an optimal measurement of antigen-specific $CD4^+$ T cells. In the present work both procedures were unsuccessful in detecting *Strongyloides*-specific Th2/1 cells. A possible reason for this outcome might be the loss of total cellular material in the freeze-thaw process resulting in a reduction of the anyway low number of antigen-specific $CD4^+$ T cells due to cryopreservation of PBMC, as reported by other studies [176], [178]. Nonetheless, the usage of cryopreserved PBMC was essential to standardize the stimulation and staining procedures for the long collection period of samples, thus, to ensure a better comparability of patient cohorts in all experiments.

The overall structure of the immune system in mice and humans is quite similar [179]. However, the species also differ considerably in some aspects of their adaptive immune response, even though the differentiation of Th1 and Th2 cells is considered to follow similar mechanisms [180], [181].
It hence may be not surprising that Th2/1 cells were detected in mice and human at lower levels than conventional Th2 cells and differed phenotypically in some features: (I) Murine Th2/1 hybrids were readily detectable by the co-expression of GATA-3 and T-bet, while the human Th cell pool comprised rather low levels of GATA-3^+T-bet^+ Th2/1 cells. (II) Murine Th2 and Th1 cytokine co-producing cells were marked by intermediate expression of GATA-3, while most human hybrids displayed a T-bet^+ but GATA-3 low/negative phenotype. (III) Whereas both murine and human Th2/1 cells expressed lower Th2 cytokine levels compared to Th2 cells, human Th2/1 hybrids were marked by high IFN-γ expression. Furthermore, *S. ratti* infected mice displayed similar frequencies of IL-4^+, IL-13^+ and IL-5^+ cells and all Th2 cytokine producing populations comprised similar proportions of IFN-γ^+ Th2/1 cells. Human patients infected with *S. stercoralis*, however, had relatively high frequencies of IL-4^+ and IL-13^+ cells, but far more IL-4^+IFN-γ^+ Th2/1 hybrids than IL-13^+IFN-γ^+ or IL-5^+IFN-γ^+ cells. These differences might be explained as follows. Whereas murine Th2/1 cells stably maintain the co-expression of GATA-3 and T-bet (at least for the time period surveyed in this study and for much longer periods in previous studies [48]), human Th2/1 cells may lose GATA-3 expression over time. This possibility should be surveyed in future *in vitro* experiments, where the GATA-3 expression by Th2/1 cells could be followed longitudinally. The difference in GATA-3 expression by murine and human Th2/1 cells might explain the finding that human Th2/1 cells expressed higher IFN-γ levels than murine hybrids. IFN-γ production is largely driven and quantitatively controlled by T-bet expression levels [22], [182]. GATA-3, however, actively represses IFN-γ expression [11], which probably leads to the relatively low IFN-γ expression by murine hybrids. The poor GATA-3 expression in human hybrids probably allows the unabated IFN-γ production in human Th2/1 cells [153], [183]. The finding of Th2 cytokine production by human Th2/1 cells despite the poor/lacking GATA-3 expression may be explained by a Th2/1 hybrid transition from a GATA-3^+ to a GATA-3 low/negative state during their development. This might lead to an open chromatin structure and hence accessibility

of the Th2 cytokine loci and the maintenance of Th2 cytokine production via accessory transcription factors such as STAT5 and STAT6 [184]. As addressed above, these speculations could be tested in future *in vitro* trials.

Of note, the conditional deletion of GATA-3 from established murine Th2 cells negatively affects the maintenance and IL-5 and IL-13, but not IL-4 production [185], [186].

CD4 memory cells may reduce the expression of GATA-3 and T-bet, while cytokine expression is still possible via other, accessory transcription factors and facilitated by the open chromatin structure of cytokine loci [184]. Still, it is surprising that the high T-bet expression (shown to repress the production of Th2 cytokines in Th1 cells [187], [188]) by human Th2/1 cells does not more efficiently repress the production of IL-4, IL-13 and IL-5 in human hybrids largely lacking GATA-3 expression. Analyzing IL-4$^+$IFN-γ$^+$ cells derived from mice primary infected with e. g. *H. polygyrus* (surviving in infected mice for up to several months) might reveal whether the transcription factor profile of murine Th2/1 cells also shifts to a GATA-3 low/negative state at least in some of the Th2/1 cells.

As addressed above, IL-4$^+$IFN-γ$^+$ cells were the most frequent Th2/1 hybrid population in *S. stercoralis*-infected patients, followed by the IL-13$^+$IFN-γ$^+$ cells, while IL-5$^+$IFN-γ$^+$ cells were present at low levels. This can be partially explained by the fact that the overall IL-5 response was very low in most patients. However, IL-13$^+$ cells were highly prominent, but comprised fewer IFN-γ$^+$ hybrids compared to IL-4$^+$ cells. Prussin *et al.* 2010 [189] and Upadhyaya *et al*. 2011 [66], have demonstrated that human Th2 responses are heterogeneous and composed of two distinct subpopulations that differ by IL-5 expression. A minority expresses IL-5 in addition to IL-4 and IL-13. The majority does not express IL-5, but produces IL-4 and IL-13. IL-5$^+$ Th2 cells are considered as being the most highly differentiated Th2 cells, as their differentiation requires multiple rounds of antigenic stimulus [66]. However, this hierarchical expression of Th2 cytokines and the fact that GATA-3 is critical to enable IL-5 expression may explain the rare IL-5$^+$ cells co-producing IFN-γ in humans.

Murine IL-5$^+$ cells were largely characterized as IL-4$^-$ and therefore may reflect differences in Th2-differentiation between mice and human [66], [190]. Why the proportion of IL-4$^+$IFN-γ$^+$ and IL-13$^+$IFN-γ$^+$ differs in human remain to be elucidated, as IL-4 and IL-13 single producers were detected at similar levels. Again, this might be explained by the lack of GATA-3 expression in most Th2/1 cells, as GATA-3 may be more essential for IL-13 maintenance then for IL-4 production, as addressed above.

Studies with volunteers to determine the immunoregulation of nematode infections or chronic inflammatory disorders may provide insight into whether recently infected individuals display GATA-3$^+$T-bet$^+$ cells resembling more closely the murine Th2/1 phenotype.

Apart of possible time-dependent changes in the murine and human Th2/1 phenotype, the role of other transcription factors like c-Maf, selectively expressed in murine Th2 cells, and Ets-related molecules (ERM), selectively expressed in murine Th1 cells [191], might play a role. A previous study on *in vitro* generated human Th1 and Th2 cells provides evidence that these transcription factors are expressed in a broadly analogous manner compared to murine cells [44]. However, theses data indicate differences between murine and human Th1/Th2 differentiation. While GATA-3 mRNA is present in resting naive cells, it is slightly downregulated upon acute activation. Moreover, this study

shows that c-Maf is present in both Th1, but more highly expressed in Th2 cells. Therefore, these transcription factors should be assessed in future studies on the instruction of Th2/1 cells.
As a recent study shows that strong Th2/1 responses in murine nematode infections restrict the control of the parasites [192]. Other helminth infections in humans should be surveyed for the proportions of Th2/1 cells to see if also the human IL-4^{+}IFN-γ^{+} T cells regulate the efficiency of Th2 responses in helminth infections. As hybrid cells are also induced in mice infected with *S. mansoni* [48], [193] it would be interesting to investigate the impact of increased vs. decreased hybrid proportions on the parasite fitness as well as pathology.

Interestingly, the present work shows a distinct plasma antibody profile in the human infected cohort. Expectedly, strongyloidiasis patients displayed elevated levels of Th2-associated antibody classes (IgE, IgG4) due to strongyloidiasis [174], [194]. However Th1-associated IgG3 antibody levels were also significantly increased in the infected group. IgG3 is a potent pro-inflammatory antibody with high affinity to the activating Fcγ receptor I (FcγRI), which plays a central role for the control of bacterial infections [195], [196]. Both IgG3 class switching by B cells and FcRI expression by human myeloid cells are positively regulated by IFN-γ [197]. Considering the relatively high proportion of Th2/1 cells in the spleen, it is conceivable that the IFN-γ production by Th2/1 cells generated in such infections might contribute to the diversification of antibody responses. This may assure that IgG3 primarily involved in opsonization of pathogens and usually associated with viral and bacterial infections, is also present in a Th2-associated infection [154], [198].

The present study detected a reduced neutrophil count in the patient cohort, a finding shared with one previous study [199], but contradicted by several reports that described elevated numbers of neutrophils in blood in immunocompetent patients with strongyloidiasis [200]–[202]. In mice neutrophils are necessary for the protective adaptive immune response to *S. stercoralis* larvae, which may also play a similar role in humans [158], [203], [204]. It is known that neutrophils are recruited to sites of infection during tissue invasion by helminths [203]–[205]. Reduced circulating neutrophil numbers therefore could be the consequence of neutrophil recruitment to the small intestine, the site of infection. The reason for the disparate findings in different studies on blood neutrophil counts remains unclear, but may be related to different sampling procedures, cell storage or differences in the study cohort such as parasite burdens and time point of infection.

In summary, this part of the current work confirmed the first and second hypothesis that Th2/1 cells are induced in highly diverse helminth infections in mice and that they also occur in humans. Interestingly, both murine and human Th2/1 hybrids expressed intermediate levels of Th2 cytokines, but human hybrids expressed high levels of IFN-γ. Hence, murine and human Th2/1 cells displayed similarities, but clearly differed in terms of phenotypic as well as functional features. These differences should be taken into account when extrapolating functional results of the Th2/1 subset from mouse models to humans. By sharing effector functions with Th1 cells, Th2/1 cells may also act as a provision against hampered responses to co-infecting pathogens in face of a strongly Th2-biased immune status or counteract Th2 driven immunopathology. Furthermore, this raised the question

whether the human hybrid phenotype may efficiently counter-regulate an overt Th2 bias in allergic disorders.

The next part of this work addressed the questions whether the occurrence of the human Th2/1 phenotype can be expanded to the non-parasitic Th2-mediated inflammatory disorder atopic dermatitis.

4.2. $CD4^+$ T cell responses and Th2/1 cells in atopic dermatitis

The next part of this work assessed if pro-inflammatory markers, disease severities or atopic dermatitis (AD)-subtypes can be linked to the levels of Th2/1 cells. AD is considered a primarily T cell-driven disease with an imbalance of Th2 and Th1 cytokine expression [67], [116], [206]. The complex pathophysiology of AD and its wide spectrum of the clinical phenotypes, based on severity of disease, age of onset, serum level of IgE, ethnic background and others [76], [97], [106], is challenging with respect to an appropriate treatment and prevention. The identification of reliable biomarkers is needed to stratify this highly complex disease with into more homogenous subgroups, which is crucial for a successful development of new therapeutic options and the implementation of personalized medicine in patients with AD [97], [207]. Hence, the investigation of the heterogeneity of Th2 responses in parallel to several other $CD4^+$ T cell subsets may provide more insights into the immunopathology of this inflammatory skin disease. In this study, 11-color flow cytometric antibody panels were applied to assess T helper cell phenotypes. Furthermore, this work examined whether a moderate inflammatory response in mild AD patients was associated with high proportions of Th2/1 cells, regulatory T cells (Treg) and lower levels of the inflammatory Th17 and Th22 cells.
Several previous studies have described contradictory results, such as increases or decreases in Th1 or Th2 cell levels in peripheral blood of patients with AD. However, these studies were conducted across children and adults, a variety of background treatments and different ethnic populations [35]. The study cohorts analyzed in the current work ensured a profound assessment of the examined $CD4^+$ T cell fraction as the groups were similar in mean age, gender distribution and ethnic background.
All immune parameters were first analyzed based on the disease severity of patients, which were grouped into mild or moderate to severe inflammation. The disease severity is visually evident and measurable at a clinical level [76], [106] and was estimated using the scoring system SCORAD.
For further analyses of selected immune parameters, patients were stratified into intrinsic vs. extrinsic AD status (normal vs. elevated total IgE levels, respectively). Moreover, factors that can influence the immune response, such as age of onset of AD [76], concomitant allergies and other diseases as well as medication were recorded and tested, but did not show an overall relevant impact. The stratification of AD by disease severity or total IgE levels revealed in significant differences in certain Th cell levels between the cohorts and will be discussed in the following.

All T helper cell subsets were investigated within the memory $CD4^+CD45RO^+$ T cell subpopulation in

peripheral blood samples of European AD patients and healthy controls. Once more, the human Th2/1 phenotype was the subset of particular interest. Indeed, cells co-expressing Th2 and Th1 cytokines were also detected in this study cohort. This confirms the third hypothesis that human Th2/1 cells also occur in Th2-associated disorders not related to helminth infections. Similar to the observations described for the human *Strongyloides*-study, IL-4$^+$IFN-γ$^+$ cells were found most frequently, followed by IL-13$^+$IFN-γ$^+$ cells, while IL-5$^+$IFN-γ$^+$ were barely present. On one hand, these findings demonstrate that Th2/1 hybrid cells appear independently of the ethnic background. On the other hand, these findings support the concept of the hierarchical expression of Th2 cytokines, as seen previously in the threadworm study (section 4.1.). Frequencies of Th2/1 cells were detected at similar levels in healthy control subjects and AD patients. Furthermore, patients with mild and severe AD did not differ in the Th2/1 proportions. Therefore, the fourth hypothesis that the proprotion of human Th2/1 cells correlates with distinct disease phenotypes and is associated with low immunopathology, could not be confirmed. Patients classified into intrinsic vs. extrinsic AD also displayed similar Th2/1 levels.

Since both classifications of AD subtypes led to a similar outcome and since no correlations between the frequencies of IL-4$^+$IFN-γ$^+$ and IL-13$^+$IFN-γ$^+$ cells and other immune parameters were detectable, the concept of human Th2/1 proportions, as a factor contributing to immune regulation in Th2-associated infections could not be verified.

However, a strongly positive correlation was observed between IL-13/IFN-γ co-producers and age. Distinct age-related differences in the human immune response have been reported [208], [209], implying that other factors than the examined immunological markers play a role here.

A very interesting observation resulted from analyzing the Th2/1 phenotype within skin-homing vs. the non-skin-homing “systemic” CD4$^+$CD45RO$^+$ subpopulation. This classification comprised different proportions of Th subsets, thus providing detailed insights into T cell activity in AD.

Skin-infiltrating T cells are characterized by the expression of CLA (cutaneous lymphocyte antigen) and represent the main population in AD skin lesions [95], [210], [211]. CLA is expressed on the surface of CD45RO$^+$ memory T cells recirculating between inflamed skin and blood [111], [212]–[214]. Therefore CLA is considered as a reliable surrogate marker of inflammatory events occurring in the skin and the evaluation of CLA$^+$ T cells in the blood may eliminate the need for skin biopsies [111].

In the present work, elevated frequencies of CLA producing CD4$^+$CD45RO$^+$ memory T cells were detected in the severe cohort vs. healthy donors, while similar levels of CLA$^+$ cells were detected in the mild AD group vs. the control cohort as well as patients with extrinsic vs. intrinsic AD. The observation that patients with severe AD have increased levels of skin-infiltrating CD4$^+$CLA$^+$ cells are in line with previous studies [35], [111], [215], [216]. Although an elevated CLA expression characterizes severe AD, healthy donors also comprised a considerable number of CLA$^+$ T cells that allows to investigate skin-infiltrating T cells in the absence of a skin disorder.

Strikingly, IL-4$^+$IFN-γ$^+$ Th2/1 cells were enriched within the systemic CLA$^-$ subpopulation in all study cohorts (classified by disease severity and IgE status, extrinsic vs. intrinsic) compared to the skin-infiltrating CLA$^+$ subset. Comparing all study cohorts for the IL-4$^+$IFN-γ$^+$ proportion within the total CLA$^-$IL-4$^+$ populations (Fig. 3.23.E) the Th2/1 phenotype showed the tendency to decrease in AD patients, indicating that Th2/1 cells do not contribute to inflammation. Moreover, the systemic Th2/1 cells expressed GATA-3 at low and T-bet at high levels even more distinctly as observed for the

expression profile of Th2/1 cells based on the $CD4^+$ T cell subset in the human threadworm study. In contrast to IL-4/IFN-γ co-producers, the levels of $IL\text{-}13^+IFN\text{-}\gamma^+$ Th2/1 cells were similar within the CLA^+ vs. the CLA^- subset and between the study cohorts. Hence, the inclusion of the CLA marker allowed for a highly detailed survey of systemic versus skin-prone T helper cell subsets and provided interesting insights in the homing behavior of Th2/1 cells.

In order to find out whether Th2/1 cells contribute to allergen-specific responses, AD patients of this study were enrolled for concomitant timothy grass pollen allergy. Pollen is one exacerbating factor in AD and therefore allergen-specific T helper cells play an important role in this skin disease [217], [218]. Since the antigen-specific Th cell population only accounts for a small percentage of Th cells, it is difficult to phenotype these cells without prior selection or expansion [219]. To increase the low numbers of grass pollen-specific $CD4^+$ T cells, PBMC of AD patients and healthy controls were cultured in the presence of the major timothy grass pollen allergens Phl p 5.01 and Phl p 1, resulting in the proliferation and expansion of Th cells. CLA in combination with CD40L, an early activation marker on antigen-reactive $CD4^+$ T cells [220], [221] was used to reveal whether activated Phl p 5+1-specific cells are prone to migrate to the skin or not. As $IL\text{-}13^+CLA^+$ cells are considered as a marker for AD severity [35] and as high levels of $CLA^+IL\text{-}13^+$ cells were detected in AD patients in this work, the attempt was made to detect antigen-specific IL-13-, IFN-γ-single-producing and co-producing cells. Cells of healthy donors and AD patients proliferated in response to Phl p 5+1 and high levels of $CLA^+IFN\text{-}\gamma^+$ cells were detected in the proliferated fraction. Expectedly, AD patients displayed slightly increased levels of activated $CD40L^+$ cells, whereas the frequencies of $CLA^+IFN\text{-}\gamma^+$ cells were similar in patients compared to healthy donors. Unfortunately, no $IL\text{-}13^+$ cells were detected, therefore Phl p-specific $IL\text{-}13^+IFN\text{-}\gamma^+$ Th2/1 cells could not be analyzed. Hence, the existence of allergen- (as well as helminth-) specific Th2/1 cells remains to be demonstrated.

Besides the Th2/1 phenotype, this work focused on Th2 cell levels in the study cohorts. It is widely accepted that a critical characteristic of the pathogenesis of AD is a predominant Th2 phenotype [98], [94]. In particular IL-4 and IL-13 are known to decrease the expression, production, and function of genes in the epidermal differentiation complex (EDC) that contribute to barrier function, as such as filaggrin (FLG), involucrin and loricrin, leading to skin desquamation and increases in transepidermal water loss (TEWL) [222]–[224]. Moreover, Th2 cytokines act directly on keratinocytes and can inhibit some antimicrobial peptides (AMPs), which in turn increases the susceptibility to microbial invasions into the skin [206]. The analyses of AD patients in the current work showed an increased Th2 response compared to healthy donors, confirming previous studies [225]–[227]. Elevated levels of Th2 cells were accompanied by an increase of eosinophil counts, total IgE levels, total IgG4 levels (in the severe cohort) as well as an increased Th2/Th1 ratio in patient cohorts compared to healthy donors. These parameters increased gradually with increasing SCORAD indicating a correlation of the intensity of the Th2 response with the severity of the inflammation. These results are consistent with the current view that Th2 cells play a critical role in the initiation of inflammation [85], [227], [228] by attracting eosinophils and causing subsequent tissue damage by eosinophil mediators or activated macrophages [229]. In particular, the comparable high IL-13 levels in severe and extrinsic AD patients

in the current work correspond to the known role of IL-13 in IgE production [78], [225], [230]. Besides the increased levels of IL-13, severe AD patients also displayed elevated levels of IL-4 and IL-5 producing cells compared to healthy donors. In contrast, the levels of these cytokines were similar in intrinsic vs. extrinsic patients. These data are in line with the findings of Suárez-Fariñas *et al.* 2013 that reported also similar levels of Th2 cells in intrinsic vs. extrinsic AD lesions. Additionally, they described a marked Th2 activation (high levels of IL-4/IL-13 expression) in skin lesions of both cohorts, suggesting that the Th2 bias is not the sole cause of high IgE levels in extrinsic disease [130], even though the IgE production is associated with the presence of elevated Th2 cytokine levels.
IL-13 and IL-4 share common receptor components and exert similar effects to some extent [206], [231]. The expression of IL-4 is associated with IgE-production [225], [226], [231] but plays a minor role in the induction of IgE synthesis compared to IL-13 [230], [232]. Following activation of T cells, IL-13 is produced for a much longer time than IL-4 [231]. This may explain the relatively low levels of $IL\text{-}4^+$ cells in AD patients. In addition to IL-4 and IL-13, AD patients displayed elevated levels of $IL\text{-}5^+$ cells and eosinophil counts, confirming previous studies implicating IL-5 in eosinophil development, activation and proliferation in AD [206], [233].

In terms of the skin-migratory function, Th2 cells appeared enriched in the skin-homing CLA^+ subpopulation and were detected at increased levels in AD patients compared to healthy donors. In particular, IL-13 producers were prone to migrate to skin, while IL-4 producers expressed CLA especially in severe AD patients. $IL\text{-}5^+$ cells were detected at similar fractions on the skin-homing and non-skin-homing proportion. In contrast to the skin-infiltrating subset, the levels of Th2 cytokine producers in the systemic CLA^- population were similar in all study cohorts. The present work shows that the IL-13 subset appeared to be the Th2 marker revealing the main differences in disease severity with a high proportion of skin-infiltrating cells. The enrichment of Th2 cells within the skin-homing subset, primarily the elevated levels of $IL\text{-}13^+CLA^+$ in severe AD confirm other studies [118], [141], [211]. These observations together with the positive correlation of $IL\text{-}13^+$ skin-homing T cells with disease severity and IgE status [35], [146], confirms the usage of $IL\text{-}13^+CLA^+$ as a marker for AD severity [35] and the use of anti-IL-13-antobodies in therapy [100]. Interestingly, it was reported that not only adult AD patients display an enrichment of Th2 cells, but already children with AD had elevated levels of Th2 cells within the CLA^+ fraction, highlighting a Th2 dominance in early AD [141] and thus emphasizes its importance as a suitable biomarker.

In addition to the already discussed Th2 markers, the chemoattractant receptor–homologous molecule expressed on Th2 lymphocytes (CRTH2) was included in this study, which has been described as the most reliable cell surface marker for Th2 cells in humans [114], [142]. The analysis of $CRTH2^+$ cells as an additional Th2 marker was included to verify the low GATA-3 expression in human Th2/1 cells.
It has been reported that CRTH2 (the type 2 prostaglandin D2 receptor) is the only surface marker selectively associated with single circulating $CD4^+$ (as well as $CD8^+$) T cells able to produce and enhance the expression of the Th2 cytokines (IL-4, IL-13 and IL-5), is associated with GATA-3 up-regulation and is increased in PBMC of patients suffering from atopic dermatitis [112], [113], [234], [235]. Moreover, CRTH2 has been described to mediate the recruitment of Th2 cells to the sites of

allergic inflammation [235] and to the lung [236]. While this work confirmed a positive correlation between CRTH2 and GATA-3 expression, no correlation was detected between $CRTH2^+$ and $IL\text{-}13^+$ T cells. Why these contradictory results were obtained, remains elusive. The experimental settings of the previous studies were comparable with the current work: PBMC of healthy controls and mild, moderate and severe AD patients were stimulated with PMA/ionomycin or antigen-specific, respectively, and surface stainings were performed [112], [235].
Taken together, in the current work GATA-3 represents a better marker for Th2 cells, which correlates positively with $IL\text{-}13^+$ cells. Including CRTH2 did not result in beneficial information for the analysis of the Th2/1 subset and was not considered for further investigations.

Furthermore, Th1 cells were investigated in AD patients and healthy controls. Previous studies have suggested that IFN-γ and IL-12 are involved in chronic lesions in AD [117], [237] and thus contribute to the pathogenesis of AD. The IFN-γ production in skin lesions is supposed to modify the function of keratinocytes [206], by strongly inducing IL-22 on keratinocytes and both cytokines together favoring the induction of epidermal hyperplasia [124]. Previous studies investigating PBMC from AD patients reported an expansion of Th2 cytokine-producing cells with a concomitant decrease of IFN-γ [86], [238], [239]. In contrast to these observations, similar levels of $IFN\text{-}\gamma^+$ T cells in all groups were detected in the present work. Historically, AD is considered as a biphasic disease, with a predominant Th2 response in acute inflammation, and a switch to a Th1 response in chronic disease [122], [229]. A recent study of paired non-lesional, acute and chronic skin lesions from AD patients demonstrated that the onset of acute AD is characterized by Th2 and Th22 activation with intensification of these pathways (rather than a switch to a primarily Th1 polarization) in chronic disease, where an increase in Th1-related markers was also detected [117]. These observations made by Gittler *et al.*, 2012 [117] and the results of the Th1 markers of AD patients in the present study, are in line with a chronic inflammation in patients.
The classification into CLA^+ and CLA^- revealed a different composition also for Th1 cells. In contrast to Th2 cells, Th1 cells appeared to be enriched in the systemic CLA^- subpopulation. The reduced levels of skin-homing IFN-γ producers vs. the non-skin-homing subset have been described in previous studies as well [35], [141], [240]. While the present work detected similar levels of the $CLA^+IFN\text{-}\gamma^+$ subset in severe AD patients and controls, confirming previous studies [35], [141], others reported a decrease of this subset in AD patients compared to control donors [226], [238], [241].

Besides Th2 and Th1 cells, further subsets as Th17 and Th22 cells contribute to skin inflammation. Previous studies reported that IL-17-producing Th cells are abundant in AD skin lesions [115], [117], [128], [145]. Data on circulating Th17 cells are rare and, again, controversial. Koga *et al.*, 2008 [128] reported a positive correlation between Th17 frequencies and AD severity in Japanese populations. By contrast, Czarnowicki *et al.* 2015 [35], did not support the disease-associated relevance of Th17 cells, because no differences in IL-17 frequencies were observed between patients with AD and control subjects of European American populations. However, ethnic differences have been described to contribute to phenotypic variation of AD [120], [129], [242]. The present study displayed an enrichment of IL-17-producing cells in the skin-homing population and confirms similar expression

levels of IL-17$^+$ cells between the healthy group and both AD cohorts, confirming the findings described for AD in European Americans [35]. Moreover, a low IL-17 expression in chronic AD skin has been correlated to reduced expression of key antimicrobial peptides (AMPs) by keratinocytes, potentially accounting for the susceptibility to skin infections [119], [243] and this downregulation of AMPs in AD patients was proposed to result from the inhibitory effects of Th2 cytokines [244], [245] .
While IL-17 plays an important role in psoriasis [148], [246], [247], the current and previous data indicate a limited role of IL-17$^+$ Th cells in particular in chronic-phase AD [243]. Moreover, the circulating IL-17$^+$ T cells do not seem to reflect Th17 responses in skin. Furthermore, a correlation between the levels of IL-17 producing CD4$^+$ Th cells with Th2/1 subsets were not detected.

Frequencies of skin-homing IL-22$^+$ cells revealed a significant increase in severe AD patients compared to controls. An up-regulation of IL-22 in AD skin lesions was recently shown [142], [244] and the enrichment of IL-22-producing CD4$^+$ T cells within the CLA$^+$ population is in line with previous data [141]. These data suggest that the AD blood phenotype greatly reflect the activation of IL-22$^+$ cells in skin lesions, additionally to Th2 cells. Therefore, both Th2 and Th22 cells might represent a suitable biomarker in blood for AD severity. Interestingly, the expansion of the IL-22 subset is not seen in young children and might be influenced by immune development, disease chronicity, or recurrent skin infections [141].
The expression levels of IL-22$^+$ cells in the non-skin-homing CLA$^-$ fraction were similar in all study cohorts, but lower compared to the CLA$^+$ population. As observed for other T cell markers presented in this work, no correlation between the levels of IL-22$^+$ cells and Th2/1 subsets were detected *ex vivo*. Nevertheless, the current and previous data support a role for IL-22 in AD disease chronicity and not initiation of disease, since the Th22 response in pediatric AD was reported to be comparable with healthy controls [141].

In keeping with recent reports by Teraki *et al*., 2013 [246] and Czarnowicki *et al*., 2015 [35], which identified high fractions of IL-22/IL-13 co-producing cells in AD patients, the current study also detected significantly higher frequencies of IL-22$^+$IL-13$^+$ Th cells in severe AD. Strikingly, the IL-22$^+$IL-13$^+$ subset was enriched in the skin-homing CLA$^+$ population and was significantly increased in severe AD compared to healthy and mild AD donors. Most of the other parameters tested in this study showed only a gradual change between the groups of controls vs. mild AD vs. severe AD. Moreover, elevated frequencies of IL-22$^+$IL-13$^+$ Th cells were obtained for patients with extrinsic vs. intrinsic AD supporting the previously described positive correlation of IL-22$^+$IL-13$^+$ Th cells with IgE-levels. That indicates the relevance of circulating IL-22$^+$IL-13$^+$ Th cells in AD. Negative correlations between the pro-inflammatory IL-22$^+$IL-13$^+$ cells and the Th2/1 subsets, as a potential subset with low immunopathology, were not detected.
Similarly to AD, elevated levels of peripheral IL-22$^+$CD4$^+$ T cells have been also reported for patients with psoriasis [246]. Remarkably, it has been demonstrated that the mean frequencies of the IL-22/IL-13 co-producing subset in psoriatic patients were similar to healthy donors and significantly decreased compared to the analyzed AD cohort of the same study. Thus, IL-22 producers may be

heterogeneous and vary in their cytokine production in different inflammatory skin diseases [246]. That reinforces that this ‘‘double-positive’’ population might play a key role in AD pathogenesis.

In addition to the conventional Th2- and Th1-related markers, the key transcription factor of regulatory T cells (Treg), Foxp3 was examined. $Foxp3^+$ T cells are considered to be critical for the downregulation of inflammatory processes and the maintenance of peripheral tolerance in the skin allergic reaction [75]. In this study, patients with severe AD showed elevated frequencies of $CD4^+CD45RO^+Foxp3^+$ T cells compared to mild AD patients and healthy donors. The expansion of $Foxp3^+$ T cells in severe AD patients confirms previous studies [212], [248]–[250] indicating that strong inflammation leads to Treg expression not necessarily sufficient for the control of immunopathology. Although Treg have been demonstrated to suppress the production of pro-inflammatory cytokines in various immune cells, including Th1, Th2, and Th17 cells, the role of Treg cells in AD remains controversial [206], [248]. It has been reported that flow cytometric analyses of Treg may lead to false-positive results as activated T cells without regulatory function may transiently upregulate the expression of Foxp3 [248]. The detection of DNA-methylation in the Foxp3-promotor region ‘TSDR’ (Treg-specific demethylated region) is believed to be the most robust marker to detect Treg and is proven to be superior compared to antibody-staining methods [250], [251]. Taken together, the levels of $Foxp3^+$ T cells were associated with disease severity.

In summary, this comprehensive study provided detailed insights in the immune responses across patients with mild/moderate/extrinsic/intrinsic AD by investigating Th cell marker for Th1, Th2, Th17, Th22 cells and Treg as well as CLA and CRTH2. In particular the skin-homing subset of circulating Th cells seems to play an important role as it comprised the major fraction of Th2 cytokine producing, $IL\text{-}22^+$ and $IL\text{-}22^+IL\text{-}13^+$ cells resulting in the main differences between the study cohorts. Severe AD was characterized by an overall elevated Th2 response and the expansion of circulating skin-homing Th cells producing Th2 cytokines, IL-22 and the $IL\text{-}22^+IL\text{-}13^+$ double positive population. Patients with mild AD displayed elevated IgG3 and $CLA^+IFN\text{-}\gamma^+$ levels, but barely any other significant differences compared to healthy controls or patients with severe AD. Therefore the circulating $IL\text{-}22^+$, $IL\text{-}13^+$ and $IL\text{-}22^+IL\text{-}13^+$ within the CLA^+ subsets seem to be a suitable biomarker for disease severity.
Expectedly, patients with IgE-mediated extrinsic AD were characterized by a significantly increased eosinophil count, elevated levels of IL-13 and IL-22 single- and IL-22/IL-13 double-producing Th cells, confirming a previous study [35].
$IL\text{-}17^+$ cells appeared also enriched in the CLA^+ population, but were expressed at similar levels in all study cohorts (grouped by disease severity and IgE-status) as described previously [35].

In contrast to these Th subsets, Th1 and $IL\text{-}4^+IFN\text{-}\gamma^+$ Th2/1 cells were enriched in the systemic CLA^- population and did not seem to target the skin, and their frequencies were similar in the study groups. In contrast to $IL\text{-}4^+IFN\text{-}\gamma^+$ hybrids, $IL\text{-}13^+IFN\text{-}\gamma^+$ cells were detected at similar levels within the CLA^+ and CLA^- proportion, suggesting a more pro-inflammatory role. Why these two Th2/1 subtypes appear differently in their skin-homing capacity remains to be investigated. However, the differing composition of the skin-homing and systemic subsets highlights the relevance of such a classification for a better

understanding of the pathogenesis of AD. Moreover, CLA^+ T cells have been suggested to represent peripheral biomarkers in other inflammatory diseases as psoriasis and skin tumors [214]. The easy access to these T cells from peripheral blood during active disease or disease flares creates an opportunity for *ex vivo*, less invasive, translational approaches that might be particularly beneficial in pediatric patients with AD, where skin sampling is particularly challenging [141]. Altogether studying polarized, activated circulating CLA^+ memory T cell subsets might provide a surrogate to skin phenotyping [141] and might be best targeted for therapeutic approaches [35].

While the circulating Th2/1 subsets occurred independently from disease severity, different features were detected regarding their subtypes. Considering the observed immunological differences described by others e. g. acute vs. chronic AD and lesional vs. circulating expression of effector cytokines, further investigations of the Th2/1 phenotype are required. Even though, no clear function of the human Th2/1 subpopulation could be assessed *ex vivo*, the third hypothesis of this work is confirmed: human Th2/1 cells appear independently of nematode infections. The Th2/1 subtypes showed different features. IL-13^+IFN-γ^+ cells correlated positively with age, while the IL-4^+IFN-γ^+ subset was enriched within the CLA^- proportion, and correlated positively with eosinophil count and frequencies of Th1 cell. These findings suggest that several factors might be involved in the naturally occurrence of the human Th2/1 phenotype. This complexity of the experimental setting, due to the heterogeneity of AD and different features of Th2/1 subtypes needs to be simplified in *in vitro* experiments to assess a potential functional role of the Th2/1 phenotype.

4.3. The human Th2/1 hybrid phenotype – a comparison of both studies

Investigating PBMC of patients afflicted by two different Th2-mediated disorders the human Th2/1 phenotype was detected with some similar features to the murine subset: The Th2/1 phenotype was represented by cells co-expressing Th2 cytokines with IFN-γ and mostly present as IL-4^+ and IL-13^+ cells. Hybrid cells appeared alongside an elevated Th2 response, irrespective of the parasite load and duration of infection in mice or overall strength of the Th2 response in humans (e.g. mild AD vs. severe AD). However, species-specific differences were found as well: while murine IL-4^+IFN-γ^+ cells expressed GATA-3, IL-4 and IFN-γ at intermediate levels compared to the conventional Th cell subsets, human IL-4^+IFN-γ^+ cells were marked by the expression of high IFN-γ and T-bet levels and low GATA-3 levels. Moreover, human hybrids appeared in different magnitudes with IL-4^+IFN-γ^+ cells most frequently detectable, followed by IL-13^+IFN-γ^+ cells, while IL-5^+IFN-γ^+ cells were rarely present.

Interestingly, in particular the human IL-4^+IFN-γ^+ cells appeared enriched in the non-skin-homing CLA^- population, arguing for a role of these cells elsewhere in the body. Another difference between the species was found regarding the co-expression of transcription factors: the murine Th2/1 phenotype comprised a distinct population of GATA-3^+T-bet$^+$ cells, whereas human GATA-3^+T-bet$^+$ cells were much less prominent. Apart from the comparison of murine and human Th2/1 cells, this work revealed a positive correlation of IL-4^+IFN-γ^+ Th cells with eosinophil counts and levels of Th1 cells as well as IL-13^+IFN-γ^+ cells with age. Further factors that can influence the likelihood of Th2/1 differentiation, such as sex, ethnical background (Indian vs. European study populations) or the type of infection

(triggered by parasite vs. exacerbation of AD by grass pollen) do not seem to have an effect on Th2/1 cell proportions.

In order to simplify the complex context of the obtained results and to assess a potential lower immunopathological impact of the human Th2/1 phenotype, compared to Th2 cells, a functional *in vitro* experiment was conducted.

4.4. Potential impact of *in vitro* generated human Th2/1 cells

In the final part of this work, the attempt was made to assess a potential effect of *in vitro* generated human Th2/1 cells on a 3D skin model mimicking the hallmarks of atopic dermatitis. Therefore parameters important in the AD pathogenesis were investigated, such as skin surface pH, tight junction protein, and TSLP. In a first step, the questions had to be tackled whether the human Th2/1 phenotype can be induced *in vitro* and, if so, whether it displays the same characteristics as naturally occurring Th2/1 cells.

It has been previously shown that naïve Th cells from mice can directly commit to stable T-bet and GATA-3 co-expression by simultaneous initiation of the Th1 and Th2 differentiation programs resulting in an intermediate production of Th2 and Th1 cytokines compared to their conventional counterparts [10], [48]. To generate human Th2/1 cells and conventional Th2 and Th1 cells, in this work naïve human $CD4^+$ T cells were repeatedly treated with recombinant cytokines and neutralizing antibodies and thereby successfully directed to the differentiation of the given subsets. Hence, also human naïve Th cells can differentiate into a Th2/1 phenotype co-producing IL-4/IFN-γ and IL-13/IFN-γ. Small fractions of the Th2/1 hybrid population produced only IFN-γ or IL-4, reflecting a common finding reported earlier for differentiated Th cell populations. Cytokine production by T cell populations is generally heterogeneous [252], [253], thus, the independent decision of restimulated hybrid Th2/1 cells to produce IL-4 and/or IFN-γ rather reflects the probabilistic cytokine expression behavior of classic Th2 and Th1 cell populations [48]. Interestingly, low frequencies of cells of the Th1 condition also co-expressed Th2 and Th1 cytokines, maybe suggesting a higher flexibility of Th1 cells. In contrast, Th2 cells comprised only IL-4 single-producing cells.

In successfully inducing human Th2/1 cells *in vitro*, this analysis revealed a close link of Th2/1 cells with the Th1 phenotype. These results support the findings of the human *ex vivo* studies that showed similar features of Th2/1 cells with conventional Th1 cells, despite the Th2-mediated disease setting.

The functional impact of the *in vitro* generated Th cell lineages were then examined in filaggrin-deficient (FLG^-) skin equivalents eliciting the hallmarks of AD. Filaggrin is a key component of terminal differentiation and skin barrier function that also involves pH regulation and epidermal hydration [254]. The effect of mutations in the filaggrin gene [148], [255] and the contribution of a Th2-polarized immune response in the pathogenesis of AD are well established [256].

However, it is still under debate, whether the initial trigger, such as Th2 cytokines, leading to a down-regulation of filaggrin or other skin barrier proteins, or whether an immune dysregulation developed from skin barrier abnormalities [257]. Hönzke *et al*., 2016 [147] demonstrated that a compensatory up-regulation of skin barrier and tight junction proteins can initially balance a lack of filaggrin. FLG^- skin

equivalents treated with the Th2 cytokines IL-4 and IL-13 showed disturbed counterregulation, which resulted in weakened skin barrier and increased skin surface pH [147], characteristic for AD skin lesion [258]. In the present work a pilot-test was performed with differentiated and activated $CD4^+$ Th2 or Th2/1 cells that were applied directly underneath FLG^- skin equivalents. Skin models without Th cells served as negative controls. The co-culture with Th2 cells resulted in an increased skin surface pH compared to the Th2/1 condition and the negative control, supporting the findings previously described for the effect of Th2 cytokine treatment [147]. Importantly, filaggrin deficiency alone does not cause the increase in skin surface pH often seen in AD patients, which may rely on compensatory feedback mechanism [138]. The tight junction protein occludin was detected with reduced mRNA levels in skin equivalents co-cultrered with the Th cell lineages vs. the control model. In contrast, the analysis by immunohistochemistry revealed a decreased protein expression only for the Th2 condition vs. the Th2/1 condition and control. These data are in line with the reduced expression of occludin in the presence of Th2 cytokines [147] but this has also been described in the epidermis of filaggrin-deficient patients, which might be one factor contributing to the skin barrier impairment in these subjects [136]. An even stronger effect of Th2 vs. Th2/1 cells was detected for TSLP. The skin equivalent co-cultured with Th2 cells displayed an up-regulated mRNA level and increased protein expression. Enhanced TSLP level in the presence of Th2 cytokines were described by Hönzke *et al*., 2016 [147]. TSLP derives from keratinocytes and is considered to play a key role in the AD pathogenesis [151] in driving allergic responses, skin remodelling and itch [98]. These preliminary data suggest a different pro-inflammatory potential of Th2 and Th2/1 cells, while Th2/1 cells seem to be poor inducers of skin inflammation *in vitro*. Beside these findings, the present work indicates the migratory activity of differentiated CD4 Th cells into the dermis, as Th2 and Th2/1 cells were re-isolated from the dermis equivalent. These data are in line with Wallmeyer *et al*., 2017 [259] demonstrating that activated naïve CD4 Th cells exclusively migrate in FLG^- skin equivalents due to direct stimulation by TSLP. By contrast, it was shown that naïve CD4 T cells do not migrate in filaggrin normal skin equivalents despite enhanced TSLP levels following T cell exposure suggesting a specific role of the filaggrin deficiency that requires further investigations [259]. Moreover, the present study implies that the generated Th cell lineages are stable phenotypes. Even after the extensive experimental procedure including co-culture with skin equivalents and re-isolation from those, differed the Th cell lineages regarding their transcription factor and cytokine profile. For naïve CD4 Th cells a shift from Th1/Th17 to Th2/ Th22 polarization directed by TSLP was observed [259].

In summary, the present work demonstrated for the first time the successful induction of the Th2/1 phenotype *in vitro* as well as the first application of differentiated CD4 Th cells in FLG^- skin equivalents. These data allowed preliminary insights into the interaction between skin components that are difficult to detect *in vivo* due to the myriad of potentially confounding factors within this biologically complex setting. While *in vitro* generated Th2 cells induced an inflamed phenotype in the FLG^- skin equivalent, Th2/1 cells seem to have a lower inflammatory potential. These promising findings suggest that the Th2/1 phenotype is a poor inducer of skin inflammation *in vitro*, in contrast to Th2 cells and therefore seem to support the fourth hypothesis of the present work. However, these preliminary data have to be interpreted with caution and further investigations are required to confirm the lower immunopathological impact of the Th2/1 phenotype.

5. Conclusion and perspectives

The present thesis extends the knowledge of the Th2/1 phenotype in mice and human. The first finding of the current work is that Th2/1 cells also appear alongside Th2 cell responses in mice with patent threadworm infections. Thereby neither an increased parasite burden nor a longer duration of infection has a significant impact on hybrid frequencies. However, the organs affected by the infection differ in Th2/1 cell levels. Hybrid cells are most prominent in the small intestine and spleen, but also detectable in peripheral blood.

An important new finding of this thesis is the detection of human Th2/1 cells. The presented data show for the first time that the human peripheral $CD4^+$ T cell pool comprises Th2/1 cells alongside elevated Th2 responses. PBMC of patients infected with a Th2-driving nematode infection and patients suffering from atopic dermatitis display Th2/1 cells, co-producing Th2 cytokines together with IFN-γ. However, Th2/1 hybrid cells are also detectable in healthy individuals.

Furthermore, this work demonstrates similarities, but also differences between the murine and human Th2/1 hybrid phenotype with respect to transcription factor and effector cytokine expression profiles. The Th2 cytokine production by murine and human Th2/1 cells is lower compared to conventional Th2 cells. In mice, Th2/1 cell levels are elevated during nematode infection. IFN-γ^+ cells co-produce the different Th2 cytokines in similar proportions, whereas human hybrids are dominated by IL-4/IFN-γ co-producing cells with similar Th2/1 cell levels in patients and healthy donors. Remarkably, murine hybrid cells express elevated T-bet levels compared to Th1 cells and intermediate levels of GATA-3 compared to Th2 cells. In contrast, human Th2/1 cells express T-bet as well as IFN-γ at high levels, but barely GATA-3.

Of note, the AD-study demonstrates that most of the Th2/1 cells circulating in blood lack the skin-homing marker CLA, whereas the pro-inflammatory Th2, single-producing IL-22 and IL-13 as well as co-producing IL-22^+IL-13^+ subsets are enriched in the skin-homing population.

In this work, differentiated human Th2 and Th2/1 cells were applied underneath a filaggrin deficient skin model mimicking the hallmarks of AD, for the first time. These promising preliminary data suggest that the Th2/1 hybrid phenotype is a poor inducer of skin inflammation.

In conclusion, the murine and human $CD4^+$ T cell pool comprises the Th2/1 phenotype simultaneously expressing Th2 and Th1 cell markers, but also displays species-dependent features. Previous studies reported that murine Th2/1 cells cause less pathology than Th2 or Th1 cells [48]. In using blood samples for the human *Strongyloides*-study and AD-study such an effect of hybrid cells was not observed. However, using biopsies of affected organs would be beneficial to reveal an impact of human Th2/1 hybrid cells in immune responses. Preliminary data of *in vitro* generated human Th2/1 cells provide first indications of a lower immunopathological potential, a finding deserving further investigations.

To gain deeper insights in the characteristics and functions of murine and human Th2/1 cells, the limitations of the current study needs to be conquered. First, the blood volume of study participants was a limiting factor. In order to analyze various parameters and thus to ensure a good

characterization, different experimental approaches had to be carried out. That means samples had to be split, resulting in lower cell numbers of the anyway rare Th2/1 subset. Since human Th2/1 cells were characterized by unspecific stimulations in this work, further investigations therefore may focus on antigen-specific detection. However, it remains to be investigated whether just the cell number was too low in this experimental setting or whether other aspects using frozen PBMC impede the detection of antigen-specific Th2/1 cells. To increase the lymphocyte numbers for investigations on the functional impact of Th2/1 cells, participants may undergo lymphapheresis.

To address the key question, which role do human Th2/1 cells play in immune responses, screening a panel of further markers might provide more insights. In addition to that, analyses of locally affected tissue will be beneficial, since no correlations between analyzed markers and the hybrid phenotype were detected using blood samples.

In addition to the presented data here, further work of the group showed a TCR-independent production of murine Th2/1 cells when triggered by IL-12 and IL-8 [166]. Hence Th2/1 cells might counteract an excessive Th2-bias of immune responses like Th1 cells, even without being activated via antigen recognition.

Furthermore, previous studies reported that IL-18 is involved in the expansion of T cells co-producing Th2 and Th1 cytokines in mice infected with *S. mansoni* [260] or *S. venezuelensis* [261]. In addition to that, it has been shown that the inflammasome NLRP3 triggers IL-18 production and limits Th2 responses to helminth infections [262]. The inflammasome NLRP3 secretes also IL-1ß, which suppress IL-25 and IL-33 production leading to a reduced Th2 response and maintaining chronicity in infections with *H. polygyrus* [263]. Whether the activation of inflammasomes, by infectious larvae penetrating tissue or tissue irritation by adult worms is a decisive factor for the induction of Th2/1 cells and thereby limit Th2 responses against intestinal nematodes remains to be investigated. Besides helminth infections, IL-18 plays a role in inflammatory skin diseases. Increased serum levels of IL-18 has been associated with disease severity in AD [264]. Therefore, further work may examine the interaction of Th2/1 cells with inflammasomes in regulating adaptive Th2 and Th2/1 immune responses.

Beside the analysis of cytokines, other transcription factors then T-bet and GATA-3 might be considered for further investigations. For example, the transcription factor promyelocytic leukemia zinc finger (PLZF), which plays an important role in the exertion of the functions of NKT cells that can co-produce IL-4 and IFN-γ and might be involved in the plasticity of committed T cells, such as Th1 and Th2 cells [193].

It was shown that murine IL-4$^+$IFN-γ$^+$ differs in DNA methylation at the *Ifng* and *Il4* loci when compared with single-producing IFN-γ and IL-4 cells, demonstrating that they represent a distinct effector cell population [55]. Whether also human Th2/1 cells differs in DNA methylation in contrast to the conventional Th2 and Th1 cells remain to be investigated. The question, whether murine and human CD4$^+$ Th2/1 cells differ in other regulatory elements of effector functions remains to be elucidated.

As described previously, infections with *H. polygyrus* induce the differentiation of CD4$^+$ T cells in the Th2/1 phenotype in mice [48]. Current work of the group explores if the preferential location of

H. polygyrus-induced Th2/1 cells in spleen and the organs directly affected by parasites are associated with specific effector functions.

The identification of selective surface markers would be beneficial for sorting of naturally occurring Th2/1 cells of humans and mice for functional assays. Using a cytokine secretion assay for IL-4 or IL-13 and IFN-γ followed by mRNA isolation would allow to analyze the transcription patterns and may verify two findings of the current work: Firstly, the low GATA-3 expression of human Th2/1 cells in contrast to murine Th2/1 cells; secondly, the differential expression levels of CLA in human IL-4$^+$IFN-γ$^+$ vs. IL-13$^+$IFN-γ$^+$ cells. In addition, the data on the human Th2/1 phenotype obtained by flow cytometry remains to be investigated at mRNA level. To determine mRNA levels (of GATA-3, T-bet, IFN-γ, IL-4, IL-13) in Th2/1 cells in comparison to conventional Th2 and Th1 subsets a PrimeFlow RNA assay may be conducted. This assay would allow the simultaneous detection of RNA targets in combination with immunophenotyping for cell surface and intracellular proteins and might lead to a better understanding of the features of the Th2/1 phenotype in comparison to conventional Th2 and Th1 cells. Multiplex assays or single cell transcriptomic analysis might reveal peculiarities of Th2/1 cells with respect to the production of cytokines and regulatory elements. Furthermore, the assessment of inflammatory plasma markers such as acute-phase proteins or MMP-1, TIMP-4, and HO-1, which differ significantly in patients with threadworm infection vs. control subjects [265] might be linked to differences in the composition of the CD4$^+$ T cell effector populations characterized in the present study and might also correlate with disease severity/progression and T helper cell responses in atopic disorders.

Functional assays are required to gain more insights in a pro- or anti-inflammatory potential of the human Th2/1 hybrid phenotype. Therefore, the *in vitro* assay with skin equivalents should be repeated as demonstrated above, including analyses of normal skin models and FLG$^-$ skin equivalents co-cultured with Th1 cells. Apart from the analysis of Th2/1 cells, the effect of *in vitro* generated IL-22$^+$ and IL-22$^+$IL-13$^+$ CD4$^+$ T cells co-cultured with skin equivalents would be intriguing. These skin-homing Th subsets occurred with elevated levels in blood and skin and therefore might be used as biomarker for diagnostic and therapeutic tools.

Furthermore, co-cultures of human Th cell lineages (Th2, Th1 or Th2/1) with B cells might show if hybrid cells are able to support B cell isotype switching to IgG3/IgG4/IgE, the antibody isotypes detected in elevated concentrations in blood of threadworm infected and AD patients. Co-cultures with macrophages would elucidate whether human Th2/1 cells are able to activate macrophages, as observed in mice. It has been reported that CD4$^+$ T cells and group 2 innate lymphoid cells (ILC2s) cooperate to mediate type 2 immune responses in mice [266]. Hence, future investigations may examine the “crosstalk” of conventional Th subsets and the Th2/1 cells with these innate cells, which play a role in helminth infections and AD.

Finally, comparative analyses of the Th2/1 phenotype in affected tissues and PBMC would reveal a potential role of the human Th2/1 hybrid phenotype by e. g. analyzing lesional and non-lesional skin biopsies with blood samples of AD patients. Another aspect that should be examined more closely is the impact of the age on the Th2/1 cell proportions. The present work indicated a positive correlation of Th2/1 cells (IL-13$^+$IFN-γ$^+$) with age in participants of the AD-study. This finding needs to be verified by further studies.

Taken together, this study shows that Th2/1 cells exist in helminth infected mice and humans as well as in patients with atopic dermatitis. Whether these cells ‘passively’ regulate Th2 responses due to their lower Th2 cytokine expression or actively cross-regulate an overt Th2 bias by e. g. the production of IFN-γ or by so far uncharacterized other factors, remains to be established. The discovery of naturally occurring Th2/1 cells in living organisms inspires to further scientific questions and may represents the beginning of a new interesting area in the field of T cell biology.

6. Materials and methods

6.1. Materials

6.1.1. Laboratory equipment

Accu-jet pro pipette	Brand, Wertheim, Germany
Act-5 Diff hematology analyzer	Beckman Coulter, Brea, CA, USA
CASY® cell counter, model TT	Innovatis, Roche, Mannheim, Germany
Cell incubator	Thermo Fisher Scientific, Schwerte, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Centrifuge 5804R	Eppendorf, Hamburg, Germany
Centrifuge 5454R	Eppendorf, Hamburg, Germany
Cryotome Leica CM 1510	Leica Biosystems, Nussloch, Germany
FACSAria™ III cell sorter	BD Bioscience, San Jose, CA USA
FACSCanto™ II flow cytometer	BD Bioscience, San Jose, CA USA
FastPrep® 24 homogenizer	MP Biomedicals, Santa Ana, CA, USA
Fluorescence microscope, BZ-8000	Keyence, Neu-Isenburg, Germany
HydroSpeed® microplate washer	Tecan, Männedorf, Switzerland
Lamina flow, Scanlaf, Mars Safety 2	LaboGene, Lynge, Denmark
Lamina flow, Heraeus LB-732-C	Thermo Fisher Scientific, Boston, MA, USA
LightCycler® 480 II	Roche, Basel, Switzerland
Microscope, Inverted, Primovert	Zeiss, Göttingen, Germany
Pipette, adjustable (10, 200, 1000 µl)	Eppendorf, Hamburg, Germany
Pipette, multichannel	Brand, Wertheim, Germany
Synergy HT plate reader	BioTek, Vermont, USA
Temperature regulator SC100 for FACSAria™ III	Thermo Fisher Scientific, Boston, MA, USA
TissueLyzer	Quiagen, Hilden, Germany
Vacuum pumping unit, PC 3004 Vario	Vacuubrand, Wertheim, Germany
ViviSens system for 2D pH imaging	PreSens, Regensburg, Germany
VortexGenie® 2	Scientific Industries, Bohemia, NY, USA
Waterbath Julabo	Sigma-Aldrich, Steinheim, Germany
Waterbath shaker, Brunswick™ Innova® 3100	Eppendorf, Hamburg, Germany

6.1.2. Consumables

48-well cell culture plates	Costar, Corning Inc., Corning, NY, USA
96-well cell culture plates (flat-, round-, cone-bottom)	Costar, Corning Inc., Corning, NY, USA
96-well NUNC ELISA plates (flat bottom)	Fisher scientific, Schwerte, Germany
C-chips, disposable hemocytometer	NanoEntek, Seoul, Korea

Cell strainer (40, 70 µm)	BD Bioscience, San Jose, CA USA
Cryotubes	Greiner bio-one, Frickenhausen, Germany
Dissection instruments	KLS Martin, Freiburg, Germany
Eppendorf tubes (1.5 and 2 mL)	Eppendorf, Hamburg, Germany
Falcon CellStar tubes (15 and 50 mL)	Greiner bio-one, Frickenhausen, Germany
Flow cytometry tubes	Sarstedt, Nümbrecht, Germany
Lithium heparin vacutainer	BD, Heidelberg, Germany
Microhematocrit capillary tubes, heparinized	A. Hartenstein Laborbedarf, Würzburg, Germany
Micro tubes K3 EDTA (1,3 mL)	Sarstedt, Nümbrecht, Germany
Needles (18G, 20G)	Braun, Melsungen, Germany
Petri dishes (92 and 35 mm)	Eppendorf, Hamburg, Germany
Pipette tips (10, 200,1000 µL)	Greiner bio-one, Frickenhausen, Germany
Stripettes (10, 25, 50 mL)	Costar, Corning Inc., Corning, NY, USA
Syringes (1, 5, 10 mL)	Braun, Melsungen, Germany
Transfer pipettes (3,5 mL)	Sarstedt, Nümbrecht, Germany

6.1.3. Chemicals and biological reagents

Ammonium chloride (NH_4Cl)	Sigma-Aldrich, Steinheim, Germany
Bovine serum albumin (BSA)	AppliChem, Darmstadt, Germany
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Aldrich, Steinheim, Germany
Collagenase D	Sigma-Aldrich, Steinheim, Germany
Collagenase P	Sigma-Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	AppliChem, Darmstadt, Germany
DNase I	Sigma-Aldrich, Steinheim, Germany
Dithioerythritol (DTE)	Sigma-Aldrich, Steinheim, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethylendiamintetraacetat (EDTA)	AppliChem, Darmstadt, Germany
Fetal calf serum (FCS)	PAN-Biotech, Aidenbach, Germany
Ficoll sodium diatrizoate (density: 1,077 g/mL)	ICN Biomedicals, Aurora, OH, USA
Fixation/ permeabilization concentrate	eBioscience, San Diego, CA, USA
Fixation/ permeabilization diluent	eBioscience, San Diego, CA, USA
Formaldehyde 37 %	Carl Roth, Karlsruhe, Germany
Gentamycin	AppliChem, Darmstadt, Germany
HANKS Buffered Salt Solution (HBSS)	PAN-Biotech, Aidenbach, Germany
HEPES	PAN-Biotech, Aidenbach, Germany
Human serum, type AB	Biochrom, Berlin, Germany
ImmunoCult™ Human CD3/CD28 T Cell Activator	STEMCELL Technologies, Cologne, Germany
Isoflurane	Abbott, Ludwigshafen, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany

L-glutamine	PAN-Biotech, Aidenbach, Germany
Liberase, TL	Roche, Basel, Switzerland
CD3 monoclonal antibody (purified, clone OKT3)	eBioscience, San Diego, CA, USA
CD28 monoclonal antibody (purified, clone CD28.2)	BD Bioscience, Heidelberg, Germany
CD49d/CD28	BD Biosciences, San Diego, CA
MEM non-essential amino acids (NEAA)	PAN-Biotech, Aidenbach, Germany
Pancoll human (density: 1,077 g/mL)	PAN-Biotech, Aidenbach, Germany
Penicillin/ streptomycin (10 000 U/mL; 10 mg/mL)	PAN-Biotech, Aidenbach, Germany
Percoll (density: 1,13 g/mL)	GE Healthcare, Uppsala, Sweden
Phosphate-buffered saline (PBS)	PAN-Biotech, Aidenbach, Germany
Potassium bicarbonate ($KHCO_3$)	AppliChem, Darmstadt, Germany
Recombinant Phl p 1 Batch #DS13-74	Allergopharma, Reinbek, Germany
Recombinant Phl p 5.01 Batch #LP22-90	Allergopharma, Reinbek, Germany
RPMI-1640	PAN-Biotech, Aidenbach, Germany
Sodium bicarbonate ($NaHCO_3$)	Carl Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium pyruvate ($C_3H_3NaO_3$)	PAN-Biotech, Aidenbach, Germany
Sulfuric acid (H_2SO_4)	Carl Roth, Karlsruhe, Germany
SYBR Green I Masterplus kit	Roche, Penzberg, Germany
Tissue freezing medium	Leica, Biosystems, Nussloch, Germany
Trypan blue (0.4 %)	Sigma-Aldrich, Steinheim, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
Water (molecular biology grade)	AppliChem, Darmstadt, Germany

6.1.4. Commercial kits

Foxp3 staining buffer set	eBioscience, San Diego, CA, USA
IFN-γ Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IgE Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IgG1 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IgG3 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IgG4 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IL-4 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IL-5 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
IL-13 Ready-Set-Go, human	eBioscience, San Diego, CA, USA
innuPREP RNA kit	Analytik Jena, Jena, German
iScript™ cDNA Synthesis Kit	BioRad, Hercules, CA, USA

6.1.5. Growth factor, cytokines and neutralizing antibodies

IL-2, recombinant, human	Peprotech, New Jersey, USA
IL-4, recombinant, human	Peprotech, New Jersey, USA
IL-12, recombinant, human	Peprotech, New Jersey, USA
IFN-γ, recombinant, human	Peprotech, New Jersey, USA
Anti-IL-4 (MAB304), neutralizing, human	R&D Systems, Minneapolis, MN, USA
Anti-IL-12 p70 (MAB219), neutralizing, human	R&D Systems, Minneapolis, MN, USA
Anti- IFN-γ (MAB285), neutralizing, human	R&D Systems, Minneapolis, MN, USA

6.1.6. Stimulators and inhibitors

Brefeldin A	eBioscience, San Diego, CA, USA
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany

6.1.7. Buffers and media

6.1.7.1. Isolation and culture of murine cells

RPMI complete (cRPMI)	RPMI-1640 100 U/mL Penicillin, 100 µg/mL Streptomycin 10 % FCS 1 mM L-glutamine
Wash RPMI (wRPMI)	RPMI-1640 100 U/mL Penicillin, 100 µg/mL Streptomycin 1 % FCS
CMF (calcium/magnesium free)	HBSS (w/o Ca^{2+} and Mg^{2+}) 2 % FCS 10 mM HEPES 25 mM $NaHCO_3$
CMF/DTE	CMF 8 % FCS (= final 10 %) 0.154 mg/mL DTE
HBSS/EDTA	HBSS 10 % FCS 15 mM HEPES 5 mM EDTA
HBSS/HEPES	HBSS 15 mM HEPES
Complete RPMI – siLP	RPMI (w/o $NaHCO_3$) 5 % FCS 15 mM HEPES 100 µg/mL gentamycin
Complete RPMI – siLP digest	RPMI complete siLP 0.1 mg/mL Liberase, TL 0.1 mg/mL DNase I

Collagenase digestion solution	PBS 0.1265 U/mL Collagenase D 0.15 mg/mL DNase I
ACK lysis buffer (pH 7. 2)	Distilled water 150 mM NH_4Cl 0.1 mM $KHCO_3$ 0.1 mM Na_2-EDTA

6.1.7.2. Isolation and culture of human PBMC

Complete RPMI (cRPMI)	RPMI-1640 5 % Human serum 100 U/mL Penicillin, 100 µg/mL Streptomycin 1 mM L-glutamine 1 mM MEM non-essential amino acids 1 mM sodium pyruvate
Freezing medium	cRPMI-1640 5 % Human serum (= final 10 %) 10 % DMSO

6.1.7.3. Flow Cytometry

FACS staining buffer	PBS 0.2 % BSA
Fixation/permeabilization concentrate	Foxp3 staining buffer set (eBioscience)
Fixation/permeabilization diluent	Foxp3 staining buffer set (eBioscience)

6.1.8. Antibodies and dyes used for flow cytometry

The antibodies and dyes that were used for immunophenotyping of murine and human cells, sorting of naïve T cells, stains of *in vitro* generated human T cells and cells re-isolated from skin equivalents are indicated in the table below.

Tab. 6.1: Antibodies and dyes used in flow cytometry

Antibody	Dilution	Fluorochrome	Clone	Company
Anti-human CD3	1 : 20	Alexa 700	OKT3	BioLegend
Anti-human CD3	1 : 400	Biotin	OKT3	eBioscience
Anti-human CD4	1 : 20	PerCP	SK3	BD Bioscience
Anti-human CD4	1 : 20	eVolve 605	SK3	BD Bioscience
Anti-human CD8	1 : 200	FITC	G42-8	BD Bioscience
Anti-human CD14	1 : 20	PE-Cy7	M5E2	BD Bioscience
Anti-human CD19	1 : 160	FITC	HIB19	eBioscience
Anti-human CD25	1 : 20	APC	BC96	eBioscience
Anti-human CD45RA	1 : 20	eFluor 450	HI100	eBioscience
Anti-human CD45RO	1 : 20	FITC	UCHL1	eBioscience
Anti-human CD45RO	1 : 50	PE-Cy7	UCHL1	eBioscience
Anti-human CD154 (CD40L)	1 : 10	VioBlue	5C8	Miltenyi Biotec
Anti-human CLA	1 : 20	PerCP-Cy5.5	HECA-452	BioLegend
Anti-human CRTH2	1 : 20	Brilliant Violet 421	BM16	BioLegend
Anti-human Foxp3	1 : 20	eFluor 450	236A/E7	eBioscience
Anti-human GATA-3	1 : 20	eFluor 660	TWAJ	eBioscience
Anti-human IFN-γ	1 : 20	Brilliant Violet 510	4S.B3	BioLegend
Anti-human IFN-γ	1 : 20	eFluor 450	4S.B3	eBioscience
Anti-human IFN-γ	1 : 50	FITC	4S.B3	eBioscience
Anti-human IL-4	1 : 400	PE	MP4- 25D2	BD Bioscience
Anti-human IL-5	1 : 250	PE	TRFK5	BD Bioscience
Anti-human IL-5	1 : 250	APC	TRFK5	BioLegend
Anti-human IL-13	1 : 250	PE	JES10-5A2	BD Bioscience
Anti-human IL-13	1 : 20	APC	JES10-5A2	BD Bioscience
Anti-human IL-17A	1 : 20	PE-Cy7	BL168	BioLegend
Anti-human IL-22	1 : 20	eFluor 450	22URTI	eBioscience
Anti-human T-bet	1 : 100	PE-Cy7	eBio4B10	eBioscience
Anti-human TCR Vα24-Jα18	1 : 20	PE-Dazzle 594	6B11	BioLegend
Anti-mouse CD4	1 : 250	PerCP	RM4-5	BD Bioscience
Anti-mouse GATA-3	1 : 50	eFluor 660	TWAJ	eBioscience
Anti-mouse IFN-γ	1 : 200	eFluor 450	XMG1.2	eBioscience
Anti-mouse IL-4	1 : 100	PE	11B11	BD Bioscience
Anti-mouse IL-5	1 : 300	PE	TRFK5	eBioscience
Anti-mouse IL-13	1 : 200	Alexa488	eBio13A	eBioscience
Anti-mouse T-bet	1 : 100	PE-Cy7	eBio4B10	eBioscience
Fixable cell viability dye (DCE)	1 : 1000	eFluor 780	-	eBioscience
Fixable cell viability dye (DCE)	1 : 500	eFluor 506	-	eBioscience
Streptavidin	1 : 100	Brilliant Violet 510	-	BD Bioscience

6.1.9. Software

Adobe Photoshop CS3 (v. 10.0)	Adobe Systems Incorporated, San Jose, USA
BZ image analysis software	Keyence, Neu-Isenburg, Germany
FlowJo (v. 9.8)	Tree Star, Ashland, OR USA
Inkscape (v. 0.92.2)	Inkscape, California, USA
Mendeley (v. 1.17.12)	Mendeley, London, UK
GraphPad Prism (v.7)	GraphPad Software, San Diego, USA
ViviSens AnalytiCal2	PreSens, Regensburg, Germany

6.2. Methods

6.2.1. Animal experimentation

6.2.1.1. Ethics statement, mice and parasites

Animal experiments were performed in accordance with the National Animal Protection Guidelines and approved by Federal Health Authorities of the State of Hamburg (permission number 55/13). Female C57BL/6 mice were kindly provided by Dr. Minka Breloer, Bernhard Nocht Institute for Tropical Medicine (BNI), Hamburg, Germany, and were kept in individually ventilated cages under specific pathogen-free (SPF) conditions. Mice were infected by subcutaneous injection of 200 or 2000 *S. ratti* infective stage 3 larvae (iL3) in the hind footpad, as described in Eschbach *et al.*, 2010 [84], and sacrificed by isoflurane inhalation followed by cervical dislocation at day 10 or day 20 p.i. at 8 – 10 weeks of age. Naïve female C57BL/6 mice served as uninfected controls.

The *S. ratti* life cycle was maintained in Wistar rats, as described previously [84], at the BNI. Marie-Luise Brunn (Breloer's group) from BNI, kindly examined the parasite burdens of infected mice. Faeces of mice were collected at day 6 p.i. and *S. ratti* 28S ribosomal RNA was quantified by using real-time quantitative PCR, as described in Nouir *et al*. 2012 [267].

6.2.1.2. Preparation of single cell suspension

6.2.1.2.1. PBMC

For the isolation of PBMC, whole blood was collected using microhematocrit tubes from the peri-orbital sinus into a micro tube containing EDTA. Blood samples were then diluted 1:2 with cRPMI medium for murine cells, layered onto Pancoll in 2 mL Eppendorf tubes and centrifuged at 800 x *g* at RT, without break or accelerator for 10 min. Then the interphase was collected and cells were suspended in cold PBS supplemented with 0.2 % BSA and centrifuged at 400 x *g* for 10 min. After repeating this washing step cells were suspended in cRPMI medium.

6.2.1.2.2. Lung cells

First, lungs were perfused and rinsed with 0.9 % NaCl until the tissue turned white. Lungs were then rinsed in ice-cold cRPMI medium, transferred onto a petri dish and cut in small pieces (~ 2 mm). Subsequently tissues were added to 10 mL collagenase digestion solution and incubated under horizontal shaking in a tube shaker water bath (250 rpm, 37 °C) for 1 h. Following this, the digested lung tissues were forced through 40 µm cell strainer under sterile conditions to obtain single cell suspensions, washed with cRPMI medium and centrifuged at 400 x *g* for 10 min at 4 °C. Cell pellets were then re-suspended in 10 mL of ACK lysis buffer and incubated for 5 min on ice to lyse erythrocytes. Finally, the tissues were washed twice, centrifuged at 400 x *g* for 10 min at 4 °C and re-suspended in 5 mL cRPMI medium.

6.2.1.2.3. Spleen and mesenteric lymph node cells

Spleens and mesenteric lymph nodes (mLN) were isolated and placed in cold wRPMI medium. Tissues were then forced through 70 µm cell strainers to obtain single cell suspensions. Erythrocytes in spleen samples were lysed in 3 mL of ACK buffer for 3 min on ice and were then washed twice with wRPMI medium. Cells of spleen and mLN were finally resuspended in cRPMI medium.

6.2.1.2.4. Small intestinal lamina propria cells

Small intestinal lamina propria (siLP) cells were isolated as described earlier [268]. The small intestine was excised and placed in ice-cold CMF medium. This was then flushed with 20 mL cold CMF using a 20 G needle before removal of mesenteric fat and Peyer's patches. After opening the intestine longitudinally, excess mucus was scraped off using forceps. Tissues were thoroughly washed in HBSS/HEPES buffer, then cut into 1 cm pieces and placed in 20 mL of CMF/DTE buffer. These intestinal pieces were incubated under horizontal shaking in a tube shaker water bath (200 rpm, 37 °C) for 15 min. This incubation step was repeated twice. Next, the tissue pieces were added to 20 mL of HBSS/EDTA buffer and incubated under vigorously shaking at RT for 15 min. This step was repeated three times. To remove residual EDTA, tissues were rinsed in wRPMI medium, subsequently transferred to 10 mL of 37 °C cRPMI-siLP medium for digestion and incubated under continuous shaking in a tube shaker water bath at 37 °C, 200 rpm for 30 min. After incubation, the samples were vortexed vigorously to disrupt the remaining tissue pieces. Suspensions were forced up and down through an 18 G needle, filtered over a 70 µm cell strainer and washed twice with HBSS/HEPES buffer. Cell suspensions were layered onto a percoll gradient (3 mL 40 %, 3 mL 70 % percoll), centrifuged and the prepared lamina propria cells were finally recovered from the 40/70 % interface. These cells were then washed in complete RPMI-siLP medium.

6.2.1.3. Cell count and *in vitro* culture

Cells of all tissues were resuspended in cRPMI medium, counted using a CASY automated cell counter and then adjusted to a concentration of 1×10^7 cells/mL. Aliquots of 200 µL of these cell suspensions were then plated on round-bottom 96 well tissue culture plates. In order to detect intracellular cytokines, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL) and incubated at 37 °C for 30 min, 5 % CO_2. Brefeldin A (10 µg/ml) was then added to inhibit cytokine export and cells were stimulated at 37 °C for further 2.5 h. Unstimulated cells were kept as negative control. After a total incubation of 3 h, cells were washed twice with FACS staining buffer, stained and analyzed by flow cytometry (6.2.3.).

6.2.1.4. Histology

Dr. Anja Kühl from the Research Center ImmunoSciences (Charité University Medicine, Berlin) kindly performed the histological staining, image acquisition and scoring of murine samples. In order to achieve this, proximal small intestinal tissue samples (0,5 - 1 cm) were first fixed in formalin and

stained with hematoxylin and eosin (H&E) to assess inflammatory infiltrates. Periodic acid Schiff reaction (PAS) was performed for goblet cell quantification.

Images were acquired by using the AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany) at 100 x magnification and enteritis was scored as described earlier [269]. Histological scoring was performed according to a two-parameter scale ranging from 0 to 4: (I) Appearance of intestinal architecture, (0: normal; 1: slight blunting of villi; 2: moderate blunting of villi; 3: strong blunting of villi and crypt hyperplasia; 4: strong villus atrophy, crypt hyperplasia). (II) Signs of inflammation (0: no signs; 1: mild leucocyte infiltration; 2: moderate leucocyte infiltration; 3: marked leucocyte infiltration; 4: mucin depletion, strong bowel wall thickening, ulceration). All evaluations were performed blinded.

6.2.2. Human study populations

6.2.2.1. Ethics statements and study populations

6.2.2.1.1. Patients infected with *Strongyloides stercoralis*

All participants were examined as part of a natural history study protocol approved by Institutional Review Boards of the National Institute of Allergy and Infectious Diseases, USA and the National Institute for Research in Tuberculosis, India (ClinicalTrials.gov identifiers NCT00375583, and NCT00001230). Written informed consent was obtained from all participants. Blood and plasma samples were kindly provided by Dr. Subash Babu, NIH-NIRT-ICER, National Institute for Research in Tuberculosis, Chennai, India.

A study cohort with a total of 74 participants was examined comprising of 34 clinically asymptomatic, *S. stercoralis*-infected individuals (Inf) and 40 uninfected, healthy individuals with endemic normal status (EN) from Tamil Nadu, South India. All individuals were recruited from a rural population by screening individuals for helminth infection by stool microscopy and serology, which was conducted by the team of Dr. Subash Babu. All individuals from the age of 18 to 65 years who were willing to give blood and stool samples for examination were included in this study. Individuals who had previously undergone an anti-helminth treatment or had a history of other helminth or HIV infections were excluded from this study. *Strongyloides* infections were diagnosed by the presence of IgG antibodies to the recombinant *Strongyloides* antigen, NIE, as described previously [173], [270]. None of the individuals had lymphatic filariasis (verified by the TropBio Og4C3 ELISA; Trop Bio Pty. Ltd, Queensland, Australia) other intestinal helminths (confirmed by stool microscopy) or suffered from acute tuberculosis (verified via QuantiFERON TB Gold-in-Tube ELISA; Cellestis).

All infected individuals were treated with single doses of ivermectin and albendazole. All uninfected individuals were negative for anti-*Strongyloides* NIE-antigen, filarial infection, other intestinal helminths and acute Tuberculosis.

The data of demographic and clinical characteristics are shown in Tab. 3.1, section 3.2.

6.2.2.1.2. Patients affected by atopic dermatitis

The Charité Ethical Committee approved this study (EA1/124/16). The European participants affected by atopic dermatitis (AD) and healthy donors (HD) were recruited in cooperation with Prof. Dr. Margitta Worm, Department of Dermatology, Venereology and Allergology, Charité University Medicine, Berlin, Germany. Written informed consent was obtained from all participants and all procedures were performed in accordance with the Declaration of Helsinki [271]. Approval was given to take blood samples and skin biopsies, but no volunteers for skin biopsies were recruited. A study cohort with a total of 60 participants was examined comprising 40 patients with atopic dermatitis (AD) and 20 healthy control donors. To evaluate the severity of disease, the SCORAD (scoring of atopic dermatitis) index [272] was assessed for each patient by a physician at the Comprehensive Allergy Center Charité. This scoring method consists of the interpretation of the extent of AD, the intensity and subjective symptoms (like puritus and sleeplessness) [272]. Patients were classified into two groups: (1) mild AD (SCORAD < 25) with 19 individuals or (2) moderate to severe AD (SCORAD > 25) with 21 patients.

For correlation analysis (section 3.4.6) patients were stratified into the extrinsic and the intrinsic AD subtypes. This classification distinguishes patients according to high and low serum total IgE levels, while the clinical phenotype is similar. IgE levels greater than 150 IU/L were defined as extrinsic AD (n = 33) and values lower than 150 IU/L were defined as intrinsic AD (n = 7).

Inclusion criteria for all participants were the age of 18 to 65 years and the willingness to give blood. Patients enrolled in this study were diagnosed with atopic dermatitis and were affected by seasonal allergic rhinitis and concomitant timothy grass pollen allergy. The presence of a grass pollen allergy was not documented for all patients in an allergy passport or medical record. When documentation was not available, patients were enrolled in this study based on their statement to suffer from grass pollen allergy. A verification of an existing grass pollen allergy by a prick test or the determination of allergen-specific IgE (by e.g. ImmunoCAP™) was not possible here. Finally, all patients suffering from AD were considered in this study due to low cohort size. Exclusion criteria in this study were an ongoing systemic immunomodulatory treatment, systemic immune suppression, an allergen-specific immunotherapy (SIT) and simultaneous participation in another clinical trial when tested for medicinal products.

Each participant was interviewed for their medical history, which included the onset of AD (for patients), presence of timothy grass pollen allergy as well as other allergies and diseases.

A group of 33 AD patients declared to suffer from additional allergies, mostly against pollen as birch and hazel but also against pet hair and house dust mites. A small group of four healthy control individuals reported allergies against nickel, pet hair, seafood and one control donor was affected by a timothy grass pollen allergy.

Some patients received topic medication containing cortisone (4) or underwent allergen-specific immunotherapy (SIT) including birch and grass pollen (17), which had been completed at least two years prior to sampling for the current study. Few patients were afflicted with diseases as asthma (4), hypothyreosis (3) or colitis ulcerosa (1).

Patients or control individuals did not suffer from a bacterial, viral or parasitic infection and were not vaccinated for at least two weeks before sampling. Control subjects had no medical history of inflammatory diseases, no family history of atopy and, except one donor, no grass pollen allergy.
The demographic and clinical data are shown in Tab. 3.3, section 3.4.

6.2.2.2. Hematological parameters

Hemograms of the Indian study population were performed by the team of Dr. Subash Babu at the NIH-NIRT-ICER, Chennai, India, using the Act-5 Diff hematology analyzer (Beckman Coulter, Brea, CA, USA). Blood counts of the European study population were carried out at the routine clinical laboratories of Charité University Medicine, Berlin, Germany.

6.2.2.3. ELISA

Plasma levels of total IgE, IgG1, IgG3 and IgG4 were evaluated using Ready-Set-Go! ELISA kits as per the manufacturer's instructions. All samples were tested in duplicates.
In addition to the flow cytometric analysis, PBMC derived from the Indian study cohort were either stimulated with 2 µg/mL anti-CD3/CD28 antibodies, 1 µg/mL SEB as positive control or 20 µg/mL *S. ratti* crude antigen or kept unstimulated as negative control. After five days of incubation at 37 °C, 5 % CO_2 cell culture supernatants were taken and stored at -20 °C. The concentrations of IL-4, IL-5, IL-13 and IFN-γ were measured via Ready-Set-Go! ELISA kits.

6.2.2.4. Sampling, PBMC isolation and cell count

Whole blood (10-20 mL) was collected in a lithium heparin vacutainer by a trained person. Heparinized blood was centrifuged at RT, at 720 x *g* for 10 min, then plasma was recovered and stored at -20 °C until further usage. Taken volumes were refilled with cRPMI medium for human cells.
For PBMC isolation, blood was layered onto the lymphocyte separation medium Ficoll sodium diatrizoate (for Indian samples) or Pancoll (for European samples) and was centrifuged at RT, at 1260 x *g* for 20 min using no brake and no accelerator. The interphase was collected and washed with cold PBS supplemented with 0.2 % BSA. Cells were centrifuged at 4 °C, at 400 x *g* for 10 min, washed again and centrifuged at 4 °C at 200 x *g* for 10 min to remove platelets. Then cells were counted using trypan blue stain (Indian samples) or a CASY automated cell counter (European samples) and adjusted to a maximum of 20 x 10^6 cells/mL in freezing medium. Isolated PBMC were stored at -80 °C until usage. Minor changes in processing of samples of both studies are due to differences in laboratory equipment. However, both procedures lead to comparable quality and cell yield of PBMC.

6.2.2.5. *In vitro* cell culture

Cryopreserved human cells were thawed gently, washed twice with cRPMI medium, centrifuged at 4 °C at 400 x *g* for 10 min and cultured in cRPMI medium. Cells were counted by trypan blue exclusion and adjusted to 10 x 10^6 cells/ml.

6.2.2.5.1. Stimulation with PMA/ionomycin

For unspecific stimulations, 2 x 10^6 cells/well were placed on round bottom 96-well cell culture plates. In order to detect intracellular cytokines PBMC were stimulated with 25 ng/ml PMA and 0,5 µg/ml ionomycin or kept in medium as negative controls. Cell cultures were incubated at 37 ºC for 30 min, 5 % CO_2. To enforce intracellular cytokine accumulation, 10 µg/ml Brefeldin A Solution was added to each well and cells were incubated for further 3.5 h. Finally, cells were washed twice with FACS staining buffer, stained and subjected to flow cytometric analysis (see 6.2.3.).

6.2.2.5.2. Stimulation with *Strongyloides* crude extract

In order to assess whether the blood of patients infected with *Strongyloides* comprised nematode-specific Th2/1 effector cells, 2 x 10^6 cells/well were stimulated with 20 µg/ml crude extract of *S. ratti* adult worms, kindly provided by Dr. Minka Breloer (BNI, Hamburg, Germany). Cell cultures were incubated at 37 ºC for 24 h, 5 % CO_2, in combination with CD49d/CD28 as co-stimulatory reagents. As positive controls, cells were stimulated with PMA/ionomycin or kept unstimulated as negative control. Brefeldin A solution was added for further 6 h of stimulation. Cells were washed twice with FACS staining buffer, stained and followed by flow cytometric analysis (6.2.3.).

6.2.2.5.3. Stimulation with recombinant *Phleum pratense*-antigen

Patients with AD and concomitant timothy grass pollen (*Phleum pratense*) allergy were analyzed for allergen-specific immune responses. For antigen-specific T cell expansion, cells were first labeled with 5 mM CFSE (10 µl/ 1 x 10^7 cells) in PBS at RT and kept in the dark for 8 min. Cells were serially washed with 15 mL and with 10 mL of cRPMI medium and then centrifuged at RT and 400 x *g* for 10 min. Next, cell numbers were determined by using trypan blue staining and adjusted to 1 x 10^6 cells/ml in cRPMI. The CFSE labeling was validated via flow cytometry then 0,5 x 10^6 cells/well were placed on 48 well cell culture plates and stimulated with 20 µg/mL of each of the recombinant major allergens Phl p 1 and Phl p 5.01 (a kind gift from Allergopharma). For each donor, aliquots of unstimulated cells were used as negative control and cells that were stimulated with 1 µg/mL of SEB were treated as positive controls. Each well was supplemented with 1 µg/ml of anti-CD28 to induce a co-stimulatory signal and 10 ng/ml of IL-2 as growth factor. Duplicates were performed for each donor and condition. After 7 days of incubation at 37 °C and 5 % CO_2, cells were stimulated again with the respective agents for 30 min. Then Brefeldin A solution (10 µg/ml) was added to samples and incubated for further 5.5 h at 37 °C. Cells were then washed with PBS and counted. 5 x 10^6 cells/well were placed on conical bottom 96-well cell culture plates and were subsequently stained and analyzed by flow cytometry (6.2.3.).

6.2.3. Staining and flow cytometric analysis

Antibodies and dyes used for surface and intracellular staining of murine and human lymphocytes are reported in table 6.1. Eight-colour flow cytometric antibody panels were used for the analyses of conventional Th1, Th2 and Th2/1 cells in murine and human strongyloidiasis as well as for the *in vitro* generation of human T cell lineages. Several 11-colour flow cytometric antibody panels were established for the investigation of T cell subsets for the AD-study. The lineage and functional markers used in the respective analysis are summarized below:

T cell response in *Strongyloides* infection:

Murine $CD4^+$ lymphocytes: DCE, αCD4, αGATA-3, αT-bet, αIL-4, αIL-5, αIL-13, αIFN-γ

Human $CD4^+$ lymphocytes:

Unspecific stimulation: DCE, αCD3, αCD4, αCD45RO, αCD154 (CD40L), αGATA-3, αT-bet, αIL-4, αIL-5, αIL-13, αIFN-γ

Strongyloides-specific stimulation: DCE, αCD3, αCD4, αCD154 (CD40L), αGATA-3, αT-bet, αIL-4, αIFN-γ

T cell response in patients with atopic dermatitis:

Unspecific stimulation: DCE, αTCR Vα24-Jα18, αCRTH2, αCD3, αCD4, αCD45RO, αCLA, αGATA-3, αT-bet, αFoxp3, αIL-4, αIL-5, αIL-13, αIFN-γ, αIL-17A, αIL-22

Phl p 1+5-specific stimulation: DCE, αCD3, αCD4, αTCR Vα24-Jα18α, αCLA, αCD154 (CD40L), αGATA-3, αT-bet, αIL-13, αIFN-γ, CFSE

In vitro generation of human Th1, Th2 and Th2/1 cells:

Sorting strategy: $CD4^+CD25^-CD45RA^+$

Test stain to ensure that sort is pure: DCE, αCD45RO, αCD8, αCD14, αCD19

Test stain during *in vitro* generation,

re-isolation of T cells from skin models: DCE, αCD4, αGATA-3, αT-bet, αIL-4, αIL-5, αIL-13, αIFN-γ

6.2.3.1. Surface staining

Cell surfaces were stained with fixable viability dyes (DCE), to exclude dead cells (Tab. 6.1.). Further markers surface staining were anti-CRTH2, antibodies used for sorting of T cells and for their test stainings (see above). Surface stainings were performed in FACS staining buffer at 4 °C, kept in the dark for 10 min, and washed twice with FACS buffer. Exceptions from this procedure are stainings

with αCD25, which were performed at RT for 25 min or αCRTH2, which were performed at 37 °C for 10 min, followed by the staining for dead cell exclusion (DCE) .

6.2.3.2. Intracellular staining

Intracellular targets such as cytokines, transcription factors, but also the early T cell activation marker CD154 (CD40L) and CLA were stained intracellularly. Cells were fixed at 4 °C for 30 min in the fixation/permeabilisation buffer using the Foxp3 staining buffer set according to the manufacturer's instructions. After fixation, cells were washed twice in permeabilization/wash (hereinafter: perm./wash) buffer, and stained intracellularly in perm./wash buffer at 4 °C for 30 min. Finally, cells were again washed twice with perm./wash buffer. Subsequently, samples were re-suspended in FACS buffer and acquired via a FACSCanto™ II or a FACSAria™ III (eight- or 11-colour, respectively). Unstained samples were used as negative controls to estimate the background noise. FMO (fluorescence-minus-one) controls were used as guidance for gate placement. The data were anaylzed using FlowJo software.

6.2.3.3. Flow cytometric analysis

Different $CD4^+$ T cell subsets were examined based on the following gating strategy using FlowJo software: Starting with lymphocytes, which were identified due to their cell size and granularity by plotting FSC-A/SSC-A, followed by excluding cell aggregates using FCS-A/FCS-W (area/width). From the obtained single cell population, dead cells were eliminated by plotting DCE/$CD4^+$ (mouse) (see Fig.3.2, section 3.1.) or DCE/$CD3^+$ (human). Murine life $CD4^+$ T cells were then analyzed for the different subpopulations. Human life cells were gated on $CD3^+$/$CD4^+$ for identification and characterization of T cell subsets.

6.2.4. *In vitro* generation of human T cell lineages

PBMC were isolated from whole blood as described above (6.2.2.4.), stained with anti-CD4, anti-CD25, anti-CD45RO (see 6.2.3.1.). The naïve $CD4^+CD45RO^+CD25^-$ subset was then sorted using a FACSAria™ III. The obtained naïve cells were washed in cRPMI medium and counted via trypan blue staining. To ensure a successful sort, test stains were performed with cells before and after sort (see 6.2.3.).

Wells of a 96-well flat-bottom cell culture plate were coated at 4 °C over night with anti-CD3 and anti-CD28 antibodies (each 10 µg/mL) for each T cell lineage. Then the coated wells were washed twice with PBS and once with cRPMI medium. 2×10^5 naïve cells/well were seeded in triplicates for each T cell lineage.

Cells were repeatedly treated with polarizing recombinant cytokines and neutralizing antibodies corresponding to the T cell lineage for the following 21 days (see scheme of *in vitro* generated T cell lineages Fig. 3.25, section 3.5). To obtain Th1 cells, cultures were treated with rIL-12, rIFN-γ (10 ng/ml each) and neutralizing anti-IL-4 monoclonal antibodies (10 µg/ml). For the generation of the Th2 cell

lineage, rIL-4 (10 ng/ml), neutralizing anti-IL-12 (10 µg/ml) and neutralizing anti-IFN-γ antibodies (10 µg/ml) were added. Th2/1 cells were generated by adding rIL-4, rIL-12 and rIFN-γ (10 ng/ml each) without adding neutralizing antibodies. In addition, rIL-2 (10 ng/ml) was added to all cultures. This procedure was repeated every six days (d0, d6, d12, d18). During the incubation at 37 ºC, 5 % CO_2 for 21 days in total, medium was changed at latest every three days with cRPMI supplemented with the growth factor rIL-2 (10 ng/ml). Cells were monitored via microscopy at a two-day-interval and split if necessary. Cells then received fresh cRPMI supplemented with 4 ng/ml rIL-2.
Directly before each round of treatment with cytokines and neutralizing antibodies and after 21 days of incubation, 1 x 10^6 cells/ donor/ condition were taken, stimulated with PMA/ionomycin (as described in 6.2.2.5.1.) and were analyzed regarding their profile of transcription factors (T-bet, GATA-3) and cytokines (IFN-γ, IL-4, IL-13) to monitor the differentiation of the T cell lineages.

6.2.5. Generation of 3D skin equivalents and co-culture with T cells

The skin model approach was conducted in collaboration with Prof. Dr. Sarah Hedtrich, Institute for Pharmacy, Freie Universität Berlin. Leonie Verheyen from the Hedtrich group kindly performed the generation of filaggrin-deficient (FLG^-) skin equivalents (Ø 5 mm), according to previously published procedures [134], [138], [147]. In brief, primary human keratinocytes were transfected with FLG^- specific siRNA and were added to primary fibroblast in a collagen matrix. The skin equivalents were cultured for 12 days with media changes every second day. *In vitro* generated Th2 and Th2/1 cells were activated via ImmunoCult Human CD3/CD28 T cell activator according to the manufacturers instructions. Cells (0,375 x 10^6) were then applied underneath the skin models, directly onto the cell culture insert membrane and were cultured for 2 days (see scheme in Fig. 3.25, section 3.5). Skin equivalent treated with cRPMI medium only served as a negative control.

6.2.5.1. Skin surface pH measurements

After co-culturing of skin equivalents with Th2 or Th2/1 cells for two days, the skin surface pH was kindly measured by Stefan Hönzke (Hedtrich group). Optical sensor foil for pH imaging containing pH indicator microparticles (fluorescein isothiocyanate) and reference microparticles (ruthenium(II)-tris (4,7-diphenyl-1,10-phenanthroline) [147] were applied to skin equivalents [138] incubated with cells or medium as a control. Foil was allowed to slowly adapt to the model. Then a RGB image was recorded using a VisiSens system for 2D pH imaging and calculations were performed with corresponding ViviSens AnalytiCal2 software.

6.2.5.2. Immunofluorescence

Protein expression of occludin and TSLP was analyzed via immunofluorescence. Skin equivalents were embedded in tissue freezing medium, shock frozen in liquid nitrogen and stored at -80 °C until usage. The equivalents were cut to vertical slices of 7 µm with a freeze microtome. Leonie Verheyen from the Hedtrich group kindly performed the immunofluorescence staining according to the standard

protocol, as described earlier [259]. Slices were analyzed using a fluorescence microscope and BZ analysis software.

6.2.5.3. RNA extraction, reverse transcription and real-time quantitative PCR

The gene expression analyses of skin proteins and master regulatory transcription factors were performed in cooperation with Leonie Verheyen from the Hedtrich group.

For the gene expression analyses of occludin and TSLP, the epidermis of skin models was removed gently, frozen and grinded for 30 s at 25 Hz using TissueLyzer. For gene expression analyses of the master transcription factors (GATA-3, T-bet) dermis and cells from T cell lineages were directly lysed in lysis buffer using the innuPREP RNA Mini kit as per the manufacturers instructions. Extracted RNA was reverse transcribed to cDNA using iScriptTM cDNA Synthesis Kit. qPCR was performed on a LightCycler480 II using the SYBR Green I Masterplus kit. The primer sequences employed are as listed in Tab. 6.2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping gene.

Tab. 6.2 Primer sequences for qPCR

Gene	Primer sense 5'-3'	Primer antisense 5'-3'
GAPDH	CTCTCTGCTCCTCCTGTTCGAC	TGAGCGATGTGGCTCGGCT
OCLN	TGCATGTTCGACCAATGC	AAGCCACTTCCTCCATAAGG
TSLP	CCCAGGCTATTCGGAAACTCAG	CGCCACAATCCTTGTAATTGTG
TBX21	TTGAGGTGAACGACGGAGAG	CCAAGGAATTGACAGTTGGGT
GATA3	GAACCGGCCCCTCATTAAG	ATTTTTCGGTTTCTGGTCTGGAT

6.2.5.4. Digestion of skin equivalents

In order to estimate whether the applied *in vitro* generated T cells maintained their characteristics in co-cultures with skin equivalents, T cells were re-isolated from the dermal compartment. To achieve this and to possibly characterize $CD4^+$ T cells derived from skin biopsies of AD patients, a skin digestion protocol was established using porcine skin (licence number L0363/08). Dermis of each skin equivalent was placed in cRPMI supplemented with 1 mg/mL collagenase P and 0.1 mg/mL DNase and incubated while gentle shaking at 37 °C for 30 min. Using a pipette, the tissue was then homogenized further and filtered through a 40 µm cell strainer. The obtained cell suspension was then washed with PBS containing 2 mM EDTA and 0,2 % BSA and again washed with PBS supplemented with 2 % BSA. Cells were counted by trypan blue exclusion test, placed in cRPMI and rest at 37 °C over night, 5 % CO_2. Re-isolated Th2 and Th2/1 cells were stimulated with PMA/ionomycin (6.2.2.5.1.) and analyzed for the transcription factor and cytokine profiles (6.2.3.).

6.2.6. Statistical analyses

Statistical analyses were performed using the GraphPad Prism software (version 7). All data were assessed for Gaussian distribution. Statistically significant differences were calculated as indicated in the figure legends. Two groups were analyzed by using Student's *t*-test (parametric) or Mann-Whitney *U* test (non-parametric). Multiple comparisons were performed by one-way ANOVA (parametric) or Kruskal-Wallis test with Dunn's correction (non-parametric). For correlation analyses the Pearson correlational coefficient was used. *P* values ≤ 0.05 were considered to be statistically significant.

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8. Scientific contributions

Publications written in the course of this work:

Bock CN, Babu S, Breloer M, Rajamanickam A, Boothra Y, Brunn ML, Kühl AA, Merle R, Löhning M, Hartmann S, Rausch S. (2017) Th2/1 Hybrid Cells Occurring in Murine and Human Strongyloidiasis Share Effector Functions of Th1 Cells. Front Cell Infect Microbiol 20;7:261. DOI:10.3389/fcimb.2017.00261.

Bock CN, Hedtrich S, Worm M, Heine G, Michaelidou M, Jevtic M, Verheyen L, Merle R, Hartmann S, Rausch S. The majority of circulating human Th2 and Th22 cells is enriched within the skin-homing CLA subset in severe atopic dermatitis, while human Th2/1 are not prone to migrate to skin (manuscript in preparation)

Others:

Müller S, Krüger B, Lange F, **Bock CN**, Nizze H, Glass A, Ibrahim SM, Jaster R (2014). The mtDNA nt7778 G/T Polymorphism Augments Formation of Lymphocytic Foci but Does Not Aggravate Cerulein-Induced Acute Pancreatitis in Mice. PLoS ONE 9(7): e102266.
DOI: 10.1371/journal.pone.0102266.

Müller S, Kaiser H, Krüger B, Fitzner B, Lange F, **Bock CN**, Nizze H, Ibrahim SM, Fuellen G, Wolkenhauer O, Jaster R. (2014) Age-Dependent Effects of UCP2 Deficiency on Experimental Acute Pancreatitis in Mice. PLoS ONE 9(4):e94494. DOI: 10.1371/journal.pone.0094494.

Conference abstracts:

Bock CN, Rausch S, Breloer M, Babu S, Hartmann S. Th2/1 hybrid cells: A multifunctional subset with different characteristics in nematode infected humans and mice. 04.-09.09.2016. 10th International Conference Molecular and Cellular Biology of Helminth Parasites, Hydra, Greece. (Talk)

Bock CN, Rausch S, Babu S, Breloer M, Hartmann S. Th2/Th1 hybrid cells: A multifunctional subset in nematode infected patients. 06.04.2016. Robert-Koch-Symposium “Functional Molecular Infection Epidemiology", Robert Koch Institute, Berlin, Germany. (Talk)

Bock CN, Rausch S, Breloer M, Babu S, Hartmann S. Th2/Th1 hybrid cells: A multifunctional subset in nematode infected patients. 09.-12.03.2016, 27th Annual Meeting of the German Society for Parasitology, Göttingen, Germany. (Talk)

Bock CN, Rausch S, Breloer M, Babu S, Hartmann S. Th2/1 hybrid T helper cells: a regulatory population in nematode infected patients? 19.-22.04.2015. Annual Woods Hole Immunoparasitology Meeting. Woods Hole, MA, USA. (Poster)

Bock CN, Rausch S, Hartmann S. Functional characterization of human Th2/1 hybrid cells in nematode infected patients. 23.-27.02.2015. Hyderabad Winter School on “Pathogen Biology and Genomics”, Hyderabad, India. (Poster)

Bock CN, Rausch S, Hartmann S. Hybrid T helper cells: a regulatory population in nematode infected and allergic patients? 16.07.-19.07.2014. Paratrop 2014, Joint Society Meeting on Parasitology and Tropical Medicine, Zurich, Switzerland. (Poster)

9. Statutory declaration

I hereby declare that the thesis has been written by myself without any external unauthorized help and that I have not used other than the declared sources.

Berlin, October 30, 2018

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