The Influence of Early-Life Stressors on the $CD4^+$ T Cell Immunity in Children

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Abstract

The early phase of life, ranging from fetal development during gestation to early infancy, is considered a critical window with increased vulnerability to stressors for the developing child and its immune system. Stressors are risk factors with the potential to exert an adverse and potentially persistent influence on the immune system. Early-life stressors highlighted in the literature include, for instance, maternal chronic diseases, maternal obesity, maternal psychological stress, maternal infections, pregnancy complications, and the use of medications during pregnancy, as well as severe infant infections and the use of antibiotics in the first year of the child's life. Recent studies in mice have suggested that $CD4^+$ T cells in the offspring are affected by these risk factors. Specifically, murine offspring exposed to stressors displayed alterations in $CD4^+$ T cell frequencies, accompanied by altered inflammatory responses. $CD4^+$ T cells play a fundamental role in adaptive immunity against pathogens, however, when dysregulated, $CD4^+$ T cells also play a decisive role in the development of allergies and autoimmune diseases. Epidemiological studies of pregnant women and their children demonstrate an association between stressors and the onset of immune-related disorders in their children. These observations support the hypothesis that there is a link between early-life stressors and the development of altered $CD4^+$ T-cell responses that may contribute to the onset of immune-related diseases later in life. As there is still a paucity of human studies examining the potential long-term consequences of risk factors beyond infancy, the objective of the present study was to investigate the association between early-life stressors and the $CD4^+$ T cell immunity in 5-year-old children.

First, the association between early-life stressors and the frequencies of $CD4^+$ T cell subsets in 5-year-old children was investigated. For this, univariable and multivariable linear regression analyses were conducted using the flow cytometric and demographic data of 118 children participating in the Prenatal Identification of Children's Health (PRINCE) study. In univariable analyses, single stressors were not significantly associated with $CD4^+$ T cell subsets. However when combining stressors, exposure to maternal medication, particularly L-thyroxine, during pregnancy or systemic antibiotic treatment in the first year of life, were each significantly associated with increased frequencies of Th17-like cells in 5-year-old children who experienced abdominal pain, nausea or headaches (abdominal symptoms/headaches). Subsequent functional analyses of $CD4^+$ T cells from children with these exposures showed a strong trend towards increased frequencies of IL-17A-producing $CD4^+$ T cells upon T-cell receptor (TCR)-specific stimulation. Moreover, the mothers of these children exhibited significantly elevated levels of the non-specific inflammatory marker C-reactive protein (CRP) and significantly reduced levels of interleukin (IL)-4 in the serum during the third trimester, indicating altered maternal immune homeostasis during pregnancy.

Taken together, these findings suggest that exposure to maternal thyroid hormone dysregulation, as indicated by the need for the use of L-thyroxine, during pregnancy and a severe infection requiring systemic antibiotic treatment in the first year of life, alter the $CD4^+$ T cell development during the early phase of life, resulting in an increased differentiation towards Th17 cells in 5-year-old children. Furthermore, an aberrant maternal cytokine profile may have played a pivotal role in the long-term enhanced Th17 cells and the onset of abdominal symptoms/headaches in these children at the age of 5. In sum, early-life stressors are associated with long-term changes in $CD4^+$ T cells in children and a clinical phenotype potentially indicating pediatric irritable bowel syndrome. Further studies are needed to investigate the direct link with maternal thyroid hormone dysregulation or severe infection requiring systemic antibiotic treatment during infancy, immune development and the consequences for the child's health.

Zusammenfassung

Die frühe Lebensphase, einschließlich der fötalen Entwicklung während der Schwangerschaft und des ersten Lebensjahres, gilt als kritisches Zeitfenster mit erhöhter Empfindlichkeit des sich entwickelnden Kindes und seines Immunsystems gegenüber Stressoren. Als Stressoren werden Risikofaktoren bezeichnet, die das Potenzial haben, einen nachteiligen und potentiell anhaltenden Einfluss auf das Immunsystem auszuüben. In der wissenschaftlichen Literatur werden insbesondere das Auftreten bzw. das Vorhandensein von z.B. chronischen Erkrankungen der Mutter, mütterlicher Adipositas, mütterlichem psychologischem Stress, mütterlichen Infektionen, Schwangerschaftskomplikationen sowie die Einnahme von Medikamenten während der Schwangerschaft als auch schwere Infektionen und eine systemische Behandlung mit Antibiotika im ersten Lebensjahr des Kindes, als mögliche Stressoren genannt. Jüngste Studien an Mäusen deuten darauf hin, dass $CD4^+$ T-Zellen von diesen Risikofaktoren betroffen sind. So zeigten Mäusenachkommen, die Stressoren ausgesetzt waren, Veränderungen in der Häufigkeit von $CD4^+$ T-Zellen, begleitet von veränderten pro-inflammatorischen Reaktionen. $CD4^+$ T-Zellen sind von fundamentaler Bedeutung für die adaptive Immunität gegen Pathogene, jedoch kann eine Dysregulation dieser Zellen auch eine entscheidende Rolle bei der Entwicklung von Allergien, und Autoimmunkrankheiten spielen. Epidemiologische Studien an schwangeren Frauen und ihren Kindern haben einen Zusammenhang zwischen Stressoren und dem Auftreten von Immunkrankheiten bei den Kindern aufgezeigt. Diese Beobachtungen stützen die Hypothese, dass es einen Zusammenhang zwischen Stressoren in der frühen Lebensphase und der Entwicklung veränderter $CD4^+$ T-Zell-Reaktionen gibt, die zum Auftreten immunologischer Erkrankungen im späteren Leben beitragen können. Da es bisher nur eine geringe Anzahl an Humanstudien gibt, in denen die möglichen Langzeitfolgen von immunologischen

Risikofaktoren über das Säuglingsalter hinaus untersucht wurden, war es das Ziel dieser Studie, den Zusammenhang zwischen Stressoren und der $CD4^+$ T-Zell-Immunität bei 5-jährigen Kindern zu untersuchen.

Als Erstes wurde der Zusammenhang zwischen Stressoren und der Häufigkeiten spezifischer $CD4^+$ T-Zell-Untergruppen bei 5-Jährigen untersucht. Dafür wurden univariable und multivariable lineare Regressionsanalysen mithilfe der durchflusszytometrischen und demografischen Daten von 118 Kindern durchgeführt, die an der PRINCE-Studie (Prenatal Identification of Children's Health) teilnahmen. In univariablen Analysen wurde keine signifikante Verbindung zwischen einzelnen Stressfaktoren und $CD4^+$ T-Zell-Untergruppen festgestellt. Multivariable Analysen zeigten jedoch jeweils einen signifikanten Zusammenhang zwischen der Exposition gegenüber mütterlichen Medikamenten, insbesondere L-Thyroxin, während der Schwangerschaft beziehungsweise gegenüber der Einnahme von Antibiotika im ersten Lebensjahr aufgrund von schweren Infektionen, und einer erhöhten Häufigkeit von Th17-ähnlichen Zellen bei 5-Jährigen, die häufiger über Bauchschmerzen, Übelkeit oder Kopfschmerzen berichteten. Anschließende funktionelle Analysen von $CD4^+$ T-Zellen von Kindern mit diesen Expositionen zeigten einen starken Trend zu erhöhten Häufigkeiten von IL-17A-exprimierenden $CD4^+$ T-Zellen bei T-Zell-Rezeptor-spezifischer Stimulation. Darüber hinaus wiesen die Mütter dieser Kinder im dritten Trimester signifikant erhöhte Werte des unspezifischen Entzündungsmarkers C-reaktives Protein (CRP) und signifikant reduzierte Werte des Zytokins Interleukin (IL)-4 im Serum auf, was auf eine veränderte mütterliche Immunhomöostase während der Schwangerschaft hindeutet.

Insgesamt deuten die Ergebnisse dieser Studie darauf hin, dass eine mütterliche Schilddrüsenhormon-Dysregulation während der Schwangerschaft, wie sie durch die Notwendigkeit der Einnahme von L-Thyroxin angezeigt wird, und eine Infektion, die eine systemische Antibiotikabehandlung während des ersten Lebensjahres erfordert, die Entwicklung von $CD4^+$ T-Zellen in der frühen Lebensphase verändern, was zu einer verstärkten Differenzierung von Th17-Zellen bei 5-jährigen Kindern führt. Darüber hinaus könnte ein abweichendes mütterliches Zytokinprofil während der Schwangerschaft eine entscheidende Rolle bei der langfristigen Erhöhung der Th17-Zellen und dem Auftreten von Bauch- und Kopfbeschwerden bei diesen Kindern im Alter von 5 Jahren gespielt haben. Zusammenfassend lässt sich festhalten, dass frühe Stressfaktoren im Leben mit langfristigen Veränderungen der $CD4^+$ T-Zellen bei Kindern und einem klinischen Phänotyp assoziert sind, der möglicherweise auf ein pädiatrisches Reizdarmsyndrom hinweist. Weitere Studien sind erforderlich, um den direkten Zusammenhang mit einer mütterlichen Schilddrüsenhormon-Dysregulation oder einer schweren Infektion, die eine systemische Antibiotikabehandlung im ersten Lebensjahr erfordert, mit der Immunentwicklung und den Folgen für die Gesundheit des Kindes zu untersuchen.

List of Abbreviations

°C	Celsius
AHR	Aryl hydrocarbon receptor
APCs	Antigen-presenting cells
ART	Assisted reproductive technologies
Bcl6	B cell lymphoma 6
BH correction	Benjamini-Hochberg correction
BMI	Body mass index
C regions	Constant regions
$CD4_{conv}$ T cells	Conventional $CD4^+$ T cells
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CRTh2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DCs	Dendritic cells
DN cells	Double negative cells
DOHaD	Developmental origins of health and disease
DP cells	Double positive cells
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FACS	Flow cytometry
FAPDs	Functional abdominal pain disorders
FAP-NOS	Functional abdominal pain not otherwise specified
FDR	False discovery rate
FLTR	From left to right
FOXP3	Forkhead box protein P3
GA	Gestational age
GATA3	GATA-binding protein 3
GC	Germinal center
GDM	Gestational diabetes mellitus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
GW	Gestational week

HCC	Hair cortisol concentrations
HCHH	Healthy Cohort Hansestadt Hamburg
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA axis	Hypothalamic-pituitary-adrenal axis
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICOS	Inducible T-cell costimulator
ICOSL	inducible costimulator ligand
IFN	Interferon
IgA	Immunoglobulin A antibodies
IgE	Immunoglobulin E antibodies
IgG	Immunoglobulin G antibodies
IL	Interleukin
IL-2Rα	IL-2 receptor
IL-4R	IL-4 receptor
IL-6Rα	Interleukin 6 receptor alpha
ITMAs	Immunoreceptor tyrosine-based activation motifs
IUGR	Intrauterine growth retardation
LCK	Lymphocyte-specific protein tyrosine kinase
LIV	Leibniz Institute of Virology
LPS	Lipopolysaccharide
L-thyroxine	Levothyroxine
MCP-1	Monocyte chemotactic protein1
MHC	Major histocompatibility complex
MIA	Maternal immune activation
MNSC	Mothers of non-symptomatic children
mRNA	Messenger ribonucleic acid
MSC	Mothers of symptomatic children
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSC	Non-symptomatic children
NK cells	Natural killer cells
NP	Non-pregnant group of women
OTC medication	Over-the-counter medication
PBMCs	Peripheral blood mononuclear cells

PMA	Phorbol 12-myristate 13-acetate
poly(I:C)	Polyinosinic-polycytidylic acid
PPIs	Proton-pump inhibitors
PPRs	Pathogen recognition receptors
PRINCE study	Prenatal Identification of Children's Health study
PSS-14	Perceived stress scale 14
pTregs	Peripherally induced Tregs
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
Runx3	Runt-related transcription factor 3
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
S. aureus	*Staphylococcus aureus*
SC	Symptomatic children
SCFAs	short-chain fatty acids
SFB	Segmented filamentous bacteria
sgp130	Soluble form of glycoprotein130
sIL-6Rα	Soluble form of the IL-6 receptor
SIRS	Systemic inflammatory response syndrome
SP cells	Single positive cells
STAT	Signaling of the transcription factor signal transducers and activators of transcription
T3	Triiodothyronine
T4	Thyroxine
T-bet	Transcription factor T-box expressed in T cells
T-B border	T cell-B cell border
TCF-1	T cell factor 1
TCR	T-cell receptor
T_{FH} cells	Follicular T helper cells
TGF-β	Transforming growth factor beta
Th cells	T helper cells
Thpok	T-helper-inducing POZ-Kruppel Factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TR	Thyroid receptor
Tregs	Regulatory T cells
tTregs	Thymus-derived Tregs

UKE	University Medical Centre Hamburg-Eppendorf
V regions	Variable regions
ZO-1	Zonula occludens-1

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1 Introduction

1.1 The role of CD4$^+$ T cells in the human immune system

The human immune system is comprised of a complex network of specialized cells, tissues, and molecules that serve to protect the organism from harmful influences, including malignant cells and infections by pathogens (Abbas, Lichtman, & Pillai, 2014). The immune system is generally divided into the innate and the adaptive immune system (Abbas et al., 2014; Marshall, Warrington, Watson, & Kim, 2018). The innate immune system functions as the first line of host defense and provides a rapid response against various pathogens. It encompasses epithelial barriers, the production of acute phase proteins, the complement system and several types of innate immune cells, including granulocytes, monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells (Abbas et al., 2014; Gordon, 2016b, 2016a; Marshall et al., 2018). NK cells are a subset of innate lymphocytes that possess the capacity to identify and destroy virus-infected or neoplastic host cells through three distinct mechanisms: antibody-dependent cell-mediated cytotoxicity, death receptor-mediated apoptosis, and the release of cytotoxic granules (Prager & Watzl, 2019). Phagocytic myeloid cells, including macrophages, neutrophils, and DCs, are capable of internalizing and subsequently eradicating pathogens (Abbas et al., 2014; Marshall et al., 2018). Furthermore, these cells have the ability to present pathogen-derived peptides on their cell surface, thereby functioning as antigen-presenting cells (APCs) to activate adaptive immune responses. In comparison to the pathogen unspecific effects of the innate immune system, the adaptive immune system mediates a more targeted defense against infections and is capable of establishing an immunological memory through the generation of memory cells (Künzli & Masopust, 2023). Thus, upon reinfection by the same pathogens, memory cells can elicit a more expeditious and stronger immune response than the initial one (Abbas et al., 2014; Künzli & Masopust, 2023). Adaptive immunity is comprised of two principal types: cell-mediated immunity, which encompasses B and T lymphocytes, and humoral immunity, which is constituted by antibodies produced by B lymphocytes (Chi, Pepper, & Thomas, 2024; Cooper, 2015; J. Miller, 2020).

T cells were initially discovered in mice by Jacques Miller and his student Graham Mitchell in the 1960s as a distinct class of lymphocytes that derive from the thymus (J. Miller, 1961, 2020; G. F. Mitchell & Miller, 1968; Shi et al., 2024). Miller and Mitchell demonstrated that these lymphocytes are crucial for the function of the adaptive immune system. Specifically, they observed that thymectomy performed shortly after birth in mice resulted in profound immunodeficiency, allowing the acceptance of foreign skin grafts, including those from rats, but leading to high mortality rates due to infections (J. Miller, 1961; Shi et al., 2024). Subsequent research showed that T cells are required to collaborate with bone marrow-derived B cells to enable the generation of robust antibody immune responses (Cooper, 2010;

J. Miller, 2011; G. F. Mitchell & Miller, 1968; Shi et al., 2024). This T cell "help" in initiating B cell antibody production was their first established function and was later attributed to the subset of T helper cells (G. F. Mitchell & Miller, 1968). Subsequently, the cytotoxic effect of T cells was demonstrated *in vitro*, which were also shown to depend on the presence of T helper cells (Cerottini, Nordin, & Brunner, 1970; Crater, Dunn, Nixon, & Furler O'Brien, 2023; Thomas et al., 1980). The "helper" and "cytotoxic" function of the T cells was then linked to the expression of antigens on the cell surface using monoclonal antibodies, thereby enabling their identification (Crater et al., 2023). Specifically, the OKT4 antibody demonstrated the ability to bind to helper T cells, while the OKT8 antibody exhibited a binding capacity to cytotoxic T cells and both T cell subsets were recognized by the OKT3 antibody (Crater et al., 2023; Kung, Goldstein, Reinherz, & Schlossman, 1979; Phan-Dinh-Tuy, Niaudet, & Bach, 1982; Reinherz, Kung, Goldstein, & Schlossman, 1979). At a later stage, the respective cell surface antigens that bind to OKT4, OKT8 and OKT3 were designated as the clusters of differentiation (CD) 4, CD8, and CD3 (Bernard & Boumsell, 1984). Accordingly, this formed the basis of the nomenclature for the $CD4^+$ and $CD8^+$ T cells.

1.1.1 T cell development in the thymus

T cells arise from self-renewing hematopoietic stem cells and downstream lymphoid progenitor cells that reside in the bone marrow (Bhandoola & Sambandam, 2006; Mold et al., 2010; Timmermans et al., 2009). In the thymus, T cell maturation proceeds through a series of developmental stages, including the formation of double negative ($CD4^-CD8^-$, DN) cells, double positive ($CD4^+CD8^+$, DP) cells and single positive ($CD4^-CD8^+$ or $CD4^+CD8^-$, SP) cells (Hosokawa & Rothenberg, 2021, 2018; L. Sun, Su, Jiao, Wang, & Zhang, 2023; Yui & Rothenberg, 2014; Figure 1). During the DN phase (DN2b to DN3a), thymocytes rearrange their T cell receptor (TCR) components and those thymocytes who express TCR γ and δ chains develop into γδ T lineage, while those thymocytes who express a pre-TCR, consisting of a TCRβ chain together with a pre-TCRα and CD3 molecules, differentiate into αβ T cells (L. Sun et al., 2023; Takahama, 2006). The development of αβ T cells is further facilitated by both pre-TCR and Notch signaling, which promote the survival, proliferation, and β-selection of αβ thymocytes, as well as the transition of DN to DP cells (Dutta, Zhao, & Love, 2021; L. Sun et al., 2023). The differentiation of $CD8^+$ SP cells from DP cells is driven by the recognition of major histocompatibility complex class I (MHC-I)-peptide complexes from thymic antigen-presenting cells (APCs) and the expression of runt-related transcription factor 3 (Runx3; L. Sun et al., 2023; Woolf et al., 2003). Conversely, the differentiation of $CD4^+$ SP cells from DP cells is induced by the recognition of MHC-II-peptide complexes and the expression of the transcription factor T-helper-inducing POZ-Kruppel Factor (Thpok; Dave, Allman, Keefe, Hardy, & Kappes, 1998; Kappes, 2010; Kappes, He, & He, 2006; Keefe, Dave, Allman, Wiest, & Kappes, 1999). Only T cells with moderate binding affinity to MHC complexes survive this

positive selection process. DP cells who fail to or too strongly recognize MHC-peptide complexes undergo apoptosis (Abbas et al., 2014; L. Sun et al., 2023). This process is also referred to as negative selection (Abbas et al., 2014; L. Sun et al., 2023).

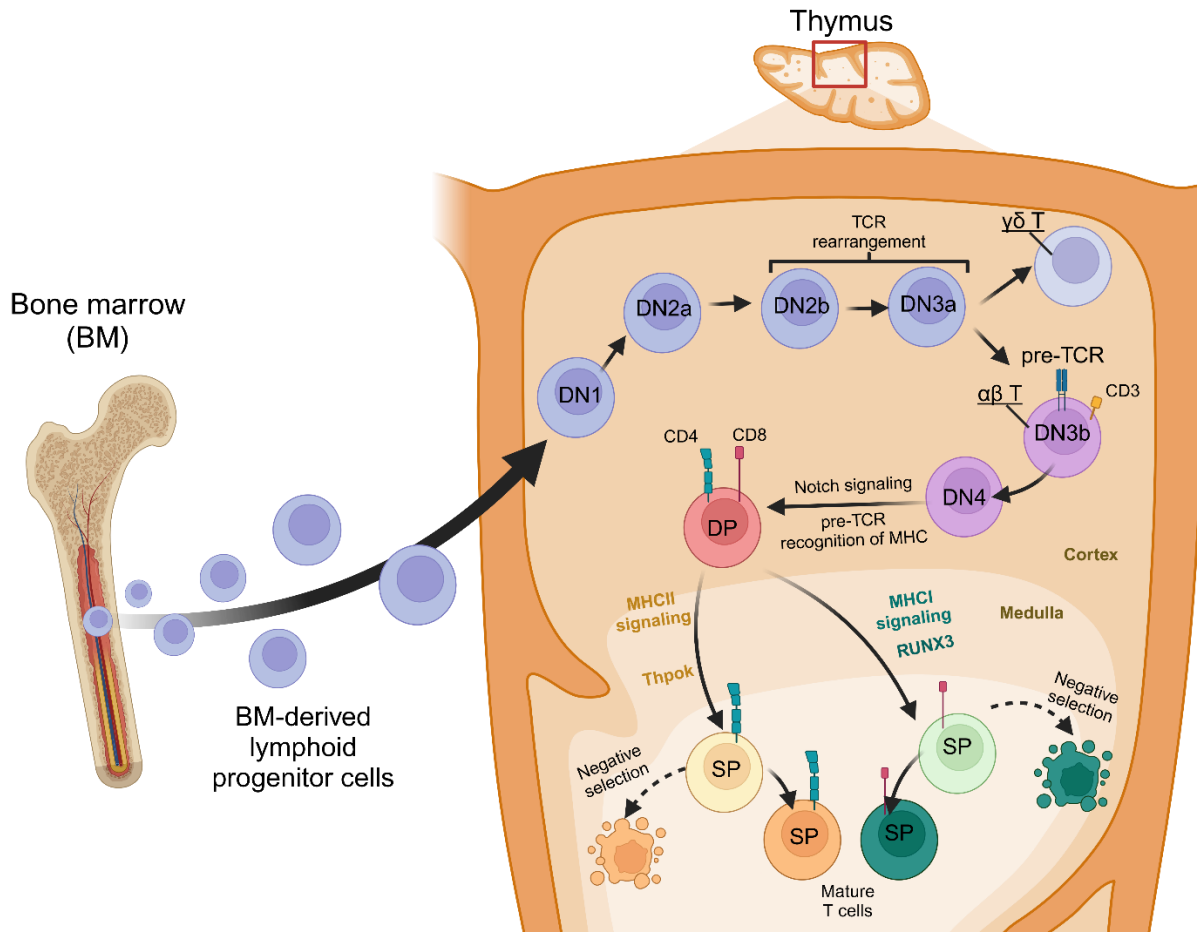


Figure 1: Overview of T cell development in the thymus. T cells originate from bone-marrow (BM)-derived lymphoid progenitor cells. Within the thymus, T cell maturation progresses through a series of developmental stages, encompassing the formation of double negative ($CD4^-CD8^-$, DN) cells, double positive ($CD4^+CD8^+$, DP) cells, and single positive ($CD4^-CD8^+$ or $CD4^+CD8^-$, SP) cells. During the DN2b to DN3a transition, thymocytes undergo a significant rearrangement of their T cell receptor (TCR) components. The expression of TCR γ and δ chains by certain thymocytes leads to their development into the γδ T lineage, while those thymocytes expressing a pre-TCR, comprising a TCRβ chain in conjunction with a pre-TCRα and CD3 molecules, subsequently differentiate into αβ T cells. The progression of αβ T cells is further facilitated by both pre-TCR and Notch signaling, which promote the survival, proliferation, and β-selection of αβ thymocytes, as well as the transition of DN to DP cells. The differentiation of $CD8^+$ SP cells from DP cells is driven by the recognition of major histocompatibility complex class I (MHC-I)-peptide complexes from thymic antigen-presenting cells (APCs) and the expression of runt-related transcription factor 3. In contrast, the differentiation of $CD4^+$ SP cells from DP cells is induced by the recognition of MHC-II-peptide complexes and the expression of the transcription factor T-helper-inducing POZ-Kruppel Factor (Thpok). DP cells that either fail to or too strongly recognize MHC-peptide complexes undergo apoptosis, a process also referred to as negative selection. Generated with BioRender.

1.1.2 Activation of CD4⁺ T cells

CD4⁺ T cells perform a multitude of functions within the immune system, acting as pivotal effectors, killers and mediators of immune responses (Künzli & Masopust, 2023; Luckheeram, Zhou, Verma, & Xia, 2012). Conventionally, activation and differentiation of naive CD4⁺ T cells into effector CD4⁺ T helper cells follows antigen-specific TCR stimulation via APCs (Abbas et al., 2014; Itano & Jenkins, 2003; H. G. Lee, Cho, & Choi, 2020; Figure 2). In the course of an infection, APCs, such as DCs, macrophages and B cells, internalize antigens and pathogens and migrate to the lymph nodes, where they present peptides from the pathogen via MHC-II molecules to naive CD4⁺ T cells (Inaba et al., 2000; Itano & Jenkins, 2003; Künzli & Masopust, 2023; Minihane et al., 2015; Rastogi et al., 2022). Subsequently, naive CD4⁺ T cells recognize the peptide–MHC-II complex through the TCR (Davis et al., 1998; Furuta, Ishido, & Roche, 2012; Marrack, Scott-Browne, Dai, Gapin, & Kappler, 2008; Tubo et al., 2013). The TCR is comprised of two chains: an α chain and a β chain, both of which contain highly variable (V) and constant (C) regions (Dong et al., 2019; Figure 2). The V regions are responsible for forming the antigen-binding site (Davis & Bjorkman, 1988; Richman et al., 2009). The co-receptor CD4 is associated with the TCR and binds to the MHC-II molecules simultaneously with the TCR binding to the peptide-MHC-II-complex on APCs (Janeway et al., 1987; J. H. Wang et al., 2001). The intracellular domain of CD4 is non-covalently linked to the lymphocyte-specific protein tyrosine kinase (LCK; Chu & Littman, 1994). Upon co-receptor binding, the LCK becomes activated and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the α and β chains of the TCR (Chu & Littman, 1994; Shah, Al-Haidari, Sun, & Kazi, 2021). In addition, LCK phosphorylates the ITAMs of the intracellular domains of the CD3 molecules (CD3εγ, CD3εδ, CD3ζζ) that are noncovalently attached to the TCR (Exley, Varticovski, Peter, Sancho, & Terhorst, 1994; Irving & Weiss, 1991; Letourneur & Klausner, 1992). These events trigger a signaling cascade and thereby initiate the first signal of T cell activation. However, full activation of naive CD4⁺ T cells requires the interaction of costimulatory molecules. The binding of the receptor CD28 on CD4⁺ T cells to B7 molecules, B7-1 (CD80) or B7-2 (CD86), on APCs enhances TCR signaling by inducing the same signaling pathways as upon TCR activation (McAdam, Schweitzer, & Sharpe, 1998; Thebeau & Morrison, 2002; Vasilevko, Ghochikyan, Holterman, & Agadjanyan, 2002; P. Zhang, Martin, Yang, Michalek, & Katz, 2004). Consequently, the first signal generated upon antigen recognition via TCR and the second signal produced upon CD28-B7 binding converge and reinforce one another, thereby ultimately initiating full T cell activation (Alegre, Frauwirth, & Thompson, 2001; Iezzi, Karjalainen, & Lanzavecchia, 1998; Viola & Lanzavecchia, 1996). Beside the conventional CD4⁺ T cell activation, bystander T cell activation represents another mechanism to induce T cell activation independently of the TCR (H. G. Lee et al., 2020). In this context, naive or effector/memory CD4⁺ T cells are stimulated by specific molecules,

including lipopolysaccharide (LPS) or cytokines, such as interleukin (IL)-1β, IL-18, and IL-23 (Caramalho et al., 2003; Di Genova, Savelyeva, Suchacki, Thirdborough, & Stevenson, 2010; Guo et al., 2009; Imanishi et al., 2007; H. G. Lee et al., 2020; Y. K. Lee et al., 2017; Reynolds & Dong, 2013; Unutmaz, Pileri, & Abrignani, 1994). These mediators bind to specific receptors on CD4⁺ T cells, resulting in the subsequent activation and differentiation of CD4⁺ T cells, in the absence of antigen-specific TCR stimulation (H. G. Lee et al., 2020).

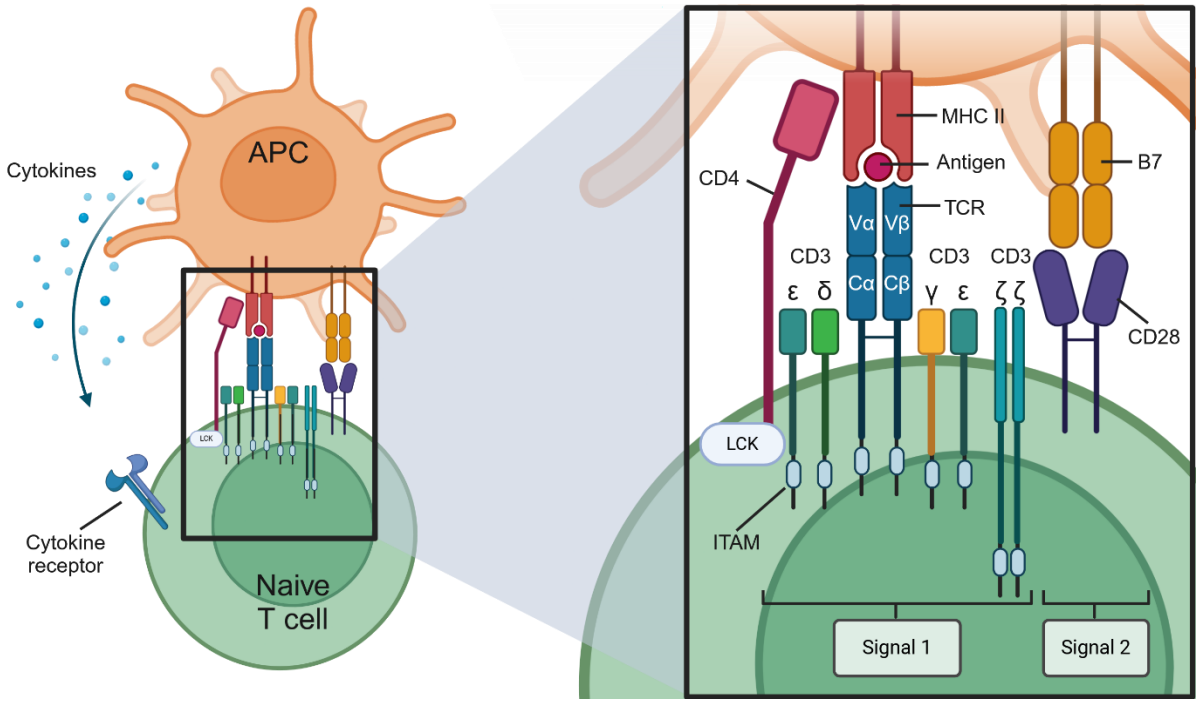


Figure 2: Antigen-specific CD4⁺ T cell activation. Engagement of the T cell receptor (TCR) with the antigen presented by major histocompatibility complex (MHC) class-II molecules and simultaneous binding of the co-receptor CD4 to the MHC-II molecules induces the first signal. Binding of the receptor CD28 on CD4⁺ T cells to B7 molecules on APCs triggers the second signal. Both signals are necessary to induce full CD4⁺ T cell activation. After activation CD4⁺ T cells undergo extensive clonal expansion and differentiation into effector CD4⁺ T cells. In addition to the nature of the antigen, the cytokine milieu and further environmental factors, such as the commensal microbiota, to which naive CD4+ T cells are exposed, are crucial for driving specific CD4+ T cell differentiation. LCK, lymphocyte-specific protein tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motifs. Generated with BioRender.

The activated CD4⁺ T cells undergo clonal expansion and differentiation into various effector CD4⁺ T cells (Künzli & Masopust, 2023; L. Sun et al., 2023). Subsequently, they migrate to the site of infection, where they promote and perform a range of specialized immune responses to support the clearance of pathogens (Künzli & Masopust, 2023; L. Sun et al., 2023). After resolution of an infection, the majority of effector CD4⁺ T cells undergo apoptosis (McKinstry, Strutt, & Swain, 2010). Nevertheless, approximately 5-10% of antigen-specific CD4⁺ T cells survive and become memory cells (Künzli & Masopust, 2023; McKinstry et al., 2010). These memory CD4⁺ T cells are quiescent and persist for months or years, circulating throughout the body in the peripheral blood, lymphatics, and secondary lymphoid organs, such as lymph

nodes and Peyer's patches (Hammarlund et al., 2003; Jokinen, Österlund, Julkunen, & Davidkin, 2007; Künzli & Masopust, 2023; Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999; Ugur, Schulz, Menon, Krueger, & Pabst, 2014). A considerable number of memory $CD4^+$ T cells, predominantly tissue-resident $CD4^+$ T cells, are also present in non-lymphoid tissues, such as the skin, liver, brain, intestine, lung, and adipose tissue (Bartolomé-Casado et al., 2021; Gebhardt et al., 2011; Kabat et al., 2022; Künzli & Masopust, 2023; Pallett et al., 2020; Smolders et al., 2018; Teijaro et al., 2011). Repeated exposure to the same antigens results in the reactivation of memory $CD4^+$ T cells, which then elicit a rapid and heightened immune response (Künzli & Masopust, 2023).

1.1.3 $CD4^+$ T cell subsets

Within the immune system, $CD4^+$ T cells play a crucial role in promoting and regulating immune responses. The numerous functions of these cells include the activation and differentiation of B cells and $CD8^+$ T cells, as well as the stimulation of innate immune cells, such as macrophages and granulocytes (Garside et al., 1998; Kawabe, Matsushima, Hashimoto, Imaizumi, & Hasegawa, 2011; Okada et al., 2005; Phares et al., 2012). Moreover, $CD4^+$ T cells possess the capacity to control excessive immune responses and support tissue repair (D'Alessio, Kurzhagen, & Rabb, 2019; Zaiss et al., 2013; Zeng et al., 2012). In this way, they are enabling the maintenance of immune and tissue homeostasis (D'Alessio et al., 2019; Zaiss et al., 2013; Zeng et al., 2012). The diverse range of functions are performed by different subsets of $CD4^+$ T cells. The differential expression profile of specific hallmark molecules, their pattern of effector cytokine production and immunological function can define distinct $CD4^+$ T cell phenotypes (Figure 3). According to this concept, $CD4^+$ T cells are typically classified into the following major subsets: Th1, Th2, Th17, Th22, follicular T helper (T_{FH}) cells, and regulatory T cells (Tregs; L. Sun et al., 2023). Furthermore, the term "conventional $CD4^+$ T cells" ($CD4_{conv}$ T cells) is often used to collectively refer to all $CD4^+$ T cell subpopulations, with the exception of Tregs (Reinhardt et al., 2022). Differentiation into distinct $CD4^+$ T cell subsets depends on various factors, including the nature of the pathogen whose antigenic peptides are presented to the $CD4^+$ T cells by APCs, the cytokine milieu and additional environmental factors such as the commensal microbiota to which the $CD4^+$ T cells are exposed (H. G. Lee et al., 2020). The subsequent activation of specific transcription factors in the $CD4^+$ T cells induces the production of specific cytokines associated with the $CD4^+$ T cell type. These cytokines, in turn, strengthen the differentiation of the specific $CD4^+$ T cell subsets in an autocrine manner (Abbas et al., 2014).

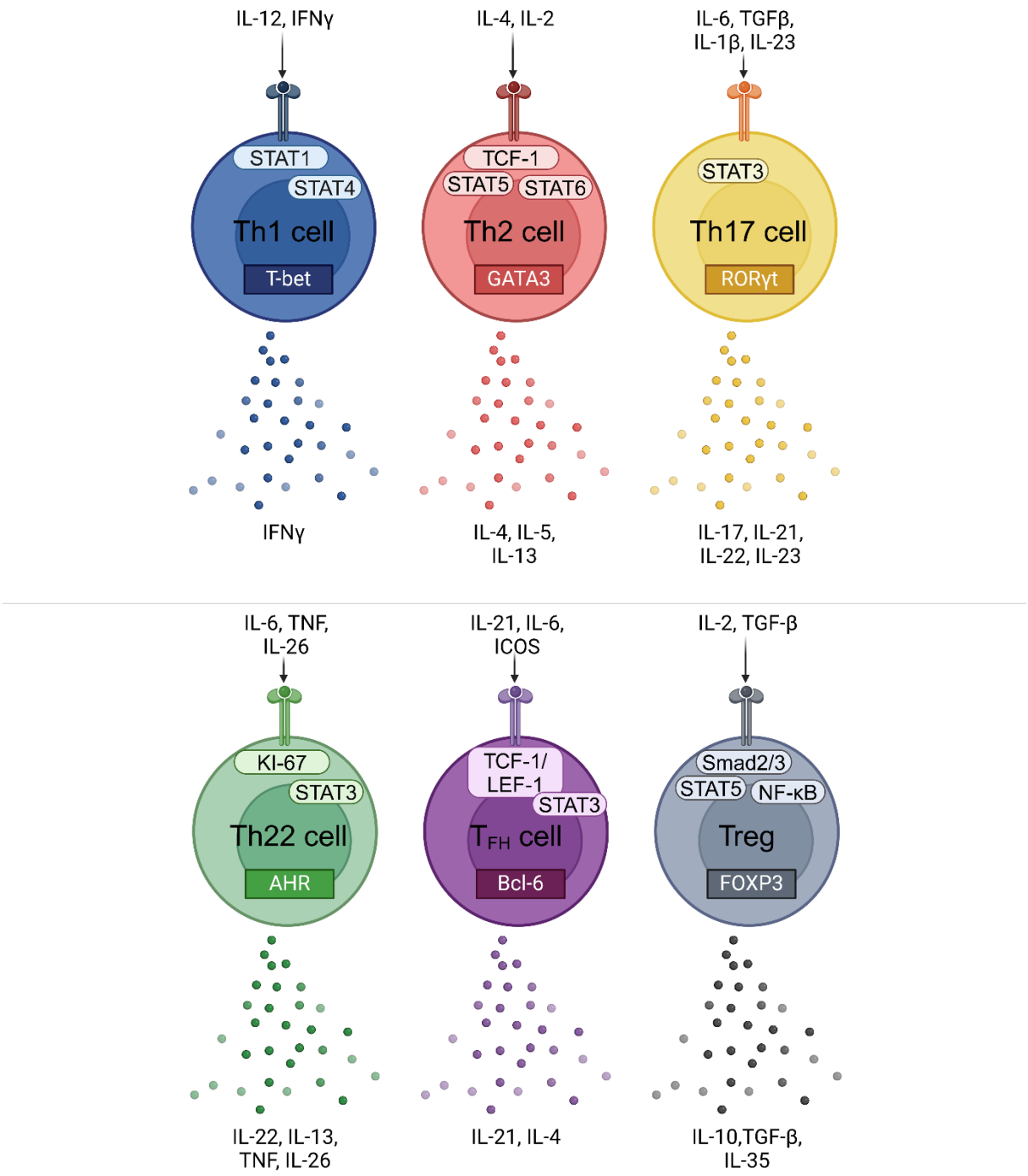


Figure 3: CD4⁺ T helper cell subsets. Specific cytokines and effector molecules induce the activation of intracellular signaling molecules (oval boxes) and signature transcription factors (rectangular boxes), which trigger the differentiation into specialized CD4⁺ T cell subsets: Th1, Th2, Th17, Th22, follicular T helper (T_{FH}) cells, and regulatory T cells (Tregs). Generated with BioRender.

Despite the typically defined CD4⁺ T cell subpopulations, CD4⁺ T cells display a spectrum of plasticity (Sallusto, 2016). Consequently, CD4⁺ T cells have the potential to take on certain phenotypically and functionally characteristics by expressing specific hallmark molecules and cytokines that are primarily associated with other CD4⁺ T cell subsets (Annunziato et al., 2001; Gagliani et al., 2015; Messi et al., 2003; Sallusto, 2016). Depending on the degree of plasticity, some CD4⁺ T cell subtypes can completely transdifferentiate from one subset into another

(Gagliani et al., 2015; Messi et al., 2003). This capacity for plasticity allows $CD4^+$ T cells to adapt and redirect their immune responses with greater precision to specific environmental stimuli and to combat particular infections (Dupage & Bluestone, 2016).

1.1.3.1 Th1 cells

The primary function of Th1 cells in host defense is to support the elimination of intracellular pathogens, particularly bacteria and viruses (Luckheeram et al., 2012; L. Sun et al., 2023). In this capacity, they are instrumental in combating a range of pulmonary pathogens, such as the influenza virus, severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), and mycobacterium tuberculosis (Grifoni et al., 2020; S. M. Miller et al., 2020; Salgame, 2005; L. Sun et al., 2023). In the course of an infection, macrophages and DCs uptake pathogens and in response secrete IL-12 (Abbas et al., 2014). In addition, NK cells become activated upon recognition of pathogens and release interferon (IFN)γ (Abbas et al., 2014). Both IL-12 and IFNγ are indispensable cytokines that, in conjunction with antigen recognition via TCR, drive Th1 cell differentiation (Mountford et al., 1999; Mullen et al., 2001). Specifically, TCR and IFNγ stimulation induces the signaling of the transcription factor signal transducers and activators of transcription (STAT) 1, which, in turn, activates the expression of the transcription factor T-box expressed in T cells (T-bet), the major transcription factor that initiates Th1 cell differentiation (Afkarian et al., 2002; Lighvani et al., 2001; Szabo et al., 2000). In addition, IL-12 promotes the maintenance of T-bet expression via STAT4 signaling and T-bet enhances the IFNγ production by binding to the *Ifng* gene (Chang & Aune, 2005; L. Sun et al., 2023; Ylikoski et al., 2005; F. Zhang & Boothby, 2006). IFNγ promotes Th1 development in an autocrine manner and simultaneously inhibits Th2 and Th17 cell development (Abbas et al., 2014; Macatonia, Hsleh, Murphy, & O'garra, 1993; Seder, Gazzinelli, Sher, & Paul, 1993; L. Sun et al., 2023). Th1 cell-induced IFNγ production is critical for phagocyte uptake and clearance of intracellular pathogens. Specifically, IFNγ stimulates B cells to produce antibodies, such as immunoglobulin G (IgG), which facilitate the recognition of pathogens by phagocytes (Abbas et al., 2014; Clifford M. Snapper & William E. Paul, 1987; Leibson, Gefter, Zlotnik, Marrack, & Kappler, 1984). Furthermore, in response to antigen-recognition on macrophages, Th1 cells express the CD40 ligand (CD40L), which binds to the CD40 receptor (CD40R) on macrophages (Abbas et al., 2014; Luheshi et al., 2014; L. Sun et al., 2023). IFNγ stimulation and CD40L/CD40R binding activates macrophages to produce lysosomal enzymes, nitric oxides and reactive oxygen species (ROS) that in turn enhance intracellular killing of pathogens in the phagolysosomes (Abbas et al., 2014). In addition to T-bet and IFNγ, the C-X-C motif chemokine receptor (CXCR)3 is a hallmark surface molecule for Th1 cells (Bonecchi et al., 1998; Gosselin et al., 2010; Sallusto, Lenig, Mackay, & Lanzavecchia, 1998; Silveira-Mattos et al., 2019). Binding of CXCR3 to its ligands, the chemokines C-X-C motif ligand (CXCL) 9, CXCL10 or CXCL11, activates the migration of Th1 cells into the site of

infection and inflammation (Campanella, Medoff, Manice, Colvin, & Luster, 2008; Groom & Luster, 2011; I. A. Khan et al., 2000; Loetscher, Loetscher, Brass, Meese, & Moser, 1998). Besides the important role of Th1 cells in host defense, persistent Th1 cell responses and enhanced IFNγ production are also associated with the pathogenesis of auto-inflammatory diseases, such as multiples sclerosis (Arellano et al., 2017; L. Sun et al., 2023; Voskuhl et al., 1993).

1.1.3.2 Th2 cells

Th2 cells play a pivotal role for the human organism in mediating protection against extracellular helminthic parasites and promoting tissue repair (L. Sun et al., 2023; Walker & McKenzie, 2018). IL-4 is the key cytokine that drive Th2 cell development (Abbas et al., 2014). Binding of IL-4 to the IL-4 receptor (IL-4R) induces the upregulation of GATA-binding protein 3 (GATA3), the signature transcription factor of Th2 cells (L. Sun et al., 2023; D. H. Zhang, Cohn, Ray, Bottomly, & Ray, 1997; W. Zheng & Flavell, 1997). In addition, TCR stimulation activates T cell factor 1 (TCF-1) signaling, which also promotes GATA3 expression (Q. Yu et al., 2009). GATA3 induces the production of the Th2-related cytokines, in particular IL-4, but also IL-5, IL-10 and IL-13 (Coffman, Seymour, Hudak, Jackson, & Rennick, 1989; Gordon, 2003). In addition, IL-2 stimulation supports the autocrine production of IL-4 via STAT5 signaling and thus enhance the development of Th2 cells (Cote-Sierra et al., 2004; Le Gros, Ben-Sasson, Seder, Finkelman, & E. Paul, 1990). Specifically, activation of STAT5 signaling increases the accessibility of *Il4* chromatin, and maintains the expression of the IL-4R gene *Il4ra* (Walker & McKenzie, 2018; J. Zhu, 2015). In addition to IL-4 and GATA-3, Th2 cells are typically identified by the expression of CCR4 and CRTh2, an acronym for chemoattractant receptor-homologous molecule expressed on Th2 cells (Bonecchi et al., 1998; Nagata et al., 1999; Sallusto et al., 1998). IL-4 together with IL-13 produced by Th2 cells have the ability to stimulate the secretion of mucus by goblet cells, thereby facilitating the expulsion of parasites from mucosal organs, such as the intestine (Abbas et al., 2014). Th2 cells also drive B cell proliferation and immunoglobulin class-switching to immunoglobulin E antibodies (IgE) via IL-4 (Abbas et al., 2014; Deo, Mistry, Kakade, & Niphadkar, 2010). IgE antibodies form complexes with the Fcε receptor (FcεR) on the surface of mast cells and basophils, which subsequently activates and sensitizes these cells (Stone, Prussin, & Metcalfe, 2010). Upon parasitic infection, IgE antibodies bound to mast cells and basophils have the capacity to bind helminthic antigens which results in the degranulation of these granulocytes (Abbas et al., 2014; Stone et al., 2010). The subsequent release of granule components, such as histamine, then facilitates the activation and the recruitment of eosinophils to the site of infection (Klion & Nutman, 2004; Sereda, Hartmann, & Lucius, 2008). Binding of eosinophils to IgE antibodies that are bound to antigens on the surface of parasites leads to the release of granule enzymes that kill the parasite (Abbas et al., 2014; Gazzinelli-Guimaraes, Jones, Voehringer, Mayer-

Barber, & Samarasinghe, 2024). In this context, Th2 cells release IL-5, which further promotes the activation of eosinophils (Coffman et al., 1989). Despite the protective function of Th2 cells, Th2 induced immune responses can also lead to allergic inflammation and tissue damage that can contribute to chronic inflammation such as asthma and chronic allergic diseases (Deo et al., 2010; L. Sun et al., 2023; Walker & McKenzie, 2018). To combat tissue injury, Th2 cells are able to stimulate alternative activation of macrophages via IL-4, IL-10 and IL-13 (Gordon, 2003). Subsequently, macrophages enhance the expression of extracellular matrix proteins, including growth factors that induce fibroblasts to enhance collagen synthesis (E. Song et al., 2000). However, in patients with chronic parasitic infections or allergic diseases, this may also lead to fibrosis or in turn to an exacerbation of tissue damage (Morimoto et al., 2018).

1.1.3.3 Th17 cells

Th17 cells represent a functionally diverse $CD4^+$ T cell subset that provides immunity against extracellular bacteria and fungi, particularly within mucosal tissue, but also contribute to the pathogenesis of several inflammatory diseases (Amezcua Vesely et al., 2019; Bacher et al., 2019; Conti et al., 2009; Eastaff-Leung, Mabarrack, Barbour, Cummins, & Barry, 2010; L. Han et al., 2015; Ishigame et al., 2009; S. C. Liang et al., 2006; Schnell, Littman, & Kuchroo, 2023). Th17 cells are characterized by the expression of a $CCR4^+CXCR3^-CCR6^+CD161^+$ phenotype (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Cosmi et al., 2008; Kleinschek et al., 2009). Several cytokines are involved in the differentiation of Th17 cells, whereby IL-6 in combination with transforming growth factor beta (TGF-β) are essential to drive Th17 cell differentiation (Schnell et al., 2023; L. Yang et al., 2009; L. Zhou et al., 2007). Binding of IL-6 to the IL-6 receptor (IL-6R) induces STAT3 activation and the expression of the retinoic acid related orphan receptor γt (RORγt), the hallmark transcription factor of Th17 cells (Harbour, DiToro, et al., 2020). In addition, IL-6 signaling has been demonstrated to suppress the expression of forkhead box protein 3 (FOXP3), which is a crucial transcription factor for the development of Tregs (Bettelli et al., 2006). TGF-β, IL-21 and IL-23 further stabilize Th17 development by enhancing and maintaining STAT3 activation (Z. Chen et al., 2006; Martinez et al., 2010; Spolski & Leonard, 2014; L. Sun et al., 2023). The STAT3-induced expression of RORγt results in the expression of the signature cytokines IL-17A, IL-17F, and IL-22 (Durant et al., 2010; Gaffen, Jain, Garg, & Cua, 2014). Upon infection with extracellular microbes, Th17 cells release IL-17A for the recruitment of neutrophils to the site of inflammation (X. Fan, Shu, Wang, Ji, & Zhang, 2023; Huber, Gagliani, & Flavell, 2012; S. C. Liang et al., 2006; Pelletier et al., 2010). Precisely, IL-17A stimulates the production of specific chemokines and cytokines, such as CXCL1, CXCL6 and CXCL8, from a variety of non-immune cells, including epithelial cells, endothelial cells and fibroblasts (X. Fan et al., 2023; C. E. Jones & Chan, 2002; Kolls & Lindén, 2004; Laan et al., 1999; Prause, Laan, Lötvall, & Lindén, 2003). These chemokines bind to the CXCR1 on neutrophils, thereby inducing their migration to the site of antigen

recognition where they perform phagocytosis, degranulation of antimicrobial proteins and the formation of neutrophil extracellular traps (NETs) to directly kill or capture pathogens for other phagocytes to kill them (Brinkmann et al., 2004; Godaly, Hang, Frendéus, & Svanborg, 2000; Klebanoff, Kettle, Rosen, Winterbourn, & Nauseef, 2013; Lacy, 2006; Nordenfelt & Tapper, 2011; Ulfig & Leichert, 2021). Beside neutrophil recruitment, IL-17A, IL-17F, and IL-22 can also directly stimulate the expression of antimicrobial peptides, such as β-defensin, by epithelial cells and promote the destruction of pathogens (Huber et al., 2012; S. C. Liang et al., 2006; Pelletier et al., 2010). Moreover, Th17-related cytokines have been demonstrated to exert beneficial effects on the maintenance of epithelial barrier integrity. In particular, they have been shown to promote epithelial cell proliferation, facilitate tissue repair, and enhance the expression of tight junction components (J. S. Lee et al., 2015; Patterson et al., 2021; Pickert et al., 2009; Schnell et al., 2023; Sugimoto et al., 2008). In performing the aforementioned functions, Th17 cell contribute to the maintenance of mucosal tissue homeostasis, particularly within the intestine. To avoid overactivity, Th17 cells are regulated by Tregs through the production of anti-inflammatory cytokines, such as IL-10 (Diefenhardt et al., 2018; Eisenstein & Williams, 2009). In a healthy state, there is a balance between Th17 cells and Tregs, whereby the balance is influenced by various factor, such as the surrounding cytokine milieu and the microbial environment (Ivanov et al., 2009, 2008; LiLi Xu, Kitani, Fuss, & Strober, 2007; L. Zhou et al., 2008). These factors regulate the cell plasticity of Th17 cells and Tregs. Consequently, Th17 cells can undergo transdifferentiation into Tregs and vice versa, with IL-10-producing Th17 cells and IL-17A-producing Tregs representing intermediates during the process of Treg/Th17 cell transformation (Beriou et al., 2009; Cui et al., 2024; Gagliani et al., 2015; Hanna et al., 2023; Koenen et al., 2008; Omenetti & Pizarro, 2015). However, a perturbed Th17 cell/Tregs balance with a dysregulated Th17 cell development, including an increased prevalence of Th17 cells and elevated IL-17 levels, are also strongly implicated in the induction and progression of autoimmune diseases, such as psoriasis, asthma, and inflammatory bowel diseases (IBD; Eastaff-Leung et al., 2010; Schnell et al., 2023). An increasing body of evidence indicates that Th17 cells may adopt a pathogenic Th17 cell state, which is responsible for auto-inflammatory processes (Harbour, Maynard, Zindl, Schoeb, & Weaver, 2015; Nizzoli et al., 2018; Schnell et al., 2023; Wen, Wang, Tian, & Wang, 2024). IL-23 has been proposed as the primary driver of pathogenicity in Th17 cells. (Annunziato et al., 2007; Ghoreschi et al., 2010; Y. Lee et al., 2012; Nizzoli et al., 2018). The presence of IL-23, in combination with either IL-6 and IL-1β or IL-6 and TGFβ, results in the differentiation of $CXCR6^+$ pathogenic Th17 cells (Ghoreschi et al., 2010; Y. Lee et al., 2012; Nizzoli et al., 2018). In addition to IL-17A, these cells produce the pro-inflammatory cytokines IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF), which exacerbate intestinal tissue inflammation (Hirota et al., 2011; Langrish et al., 2005; Y. Lee et al., 2012; Schnell et

al., 2021). Specifically, IFNγ secreted by IL-17$^+$ T cells enhance epithelial barrier permeability by affecting the expression of the tight junction Zonula Occludens-1 (ZO-1) and reducing the expression of the anti-inflammatory cytokine IL-10 by intestinal CD4$^+$ T cells (Nizzoli et al., 2018). GM-CSF can activate phagocytic myeloid cells, such as neutrophils, who in turn can promote tissue damage by the release of pro-inflammatory cytokines and ROS (Becher, Tugues, & Greter, 2016; Subramaniam et al., 2014; Zhan et al., 2012).

1.1.3.4 Th22 cells

In 2009, two independent research groups first described Th22 cells as a skin-homing memory T helper cell population (Duhen, Geiger, Jarrossay, Lanzavecchia, & Sallusto, 2009; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). In the human immune system, Th22 cells have been demonstrated to perform both anti-inflammatory and pro-inflammatory functions, and exhibit certain phenotypic and functional similarities with Th17 cells (K. Zhang, Chen, Zhu, Zhang, & Liang, 2023). Specifically, Th22 and Th17 cells share the expression of the chemokine receptors CCR4 and CCR6 and the non-expression of CXCR3 (Nayrac et al., 2021; Ramirez et al., 2010; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). However, Th22 cells additionally express CCR10 and do not express CD161 in comparison to Th17 cells (Eyerich et al., 2009; Nayrac et al., 2021; Trifari et al., 2009). Furthermore, Th22 cells are characterized by an enhanced expression of the transcription factor aryl hydrocarbon receptor (AHR), and an elevated production of IL-22 (Trifari et al., 2009). Multiple factors contribute to the differentiation of Th22 cells and the production of IL-22. *In vitro* studies have shown that DCs and Langerhans cells, are able to induce the differentiation into Th22 cells by stimulating naive CD4$^+$ T cells with IL-6 in conjunction with tumor necrosis factor (TNF; Duhen et al., 2009; Fujita et al., 2009; K. Zhang et al., 2023). Furthermore, IL-26, via Ki-67 signaling, and IL-21, via STAT3 signaling, have the capacity to enhance AHR activation, thus promoting IL-22 secretion, and Th22 formation (Niu et al., 2021; Yeste et al., 2014; K. Zhang et al., 2023). In addition to the major cytokine IL-22, Th22 cells are also capable of producing IL-13, TNF, IL-26, and granzyme B (Eyerich et al., 2009; Y. Pan et al., 2022; Plank et al., 2017; Trifari et al., 2009). In the course of viral or bacterial infections, Th22 cells locally mediate innate immunity by stimulating epithelial cells with IL-22 to secrete antimicrobial proteins, such as β-defensin 2, psoriasin (S100A7) , calgranulin A (S100A8) and the specific antibacterial protein sRegIIIβ and RegIIIγ (Wolk et al., 2004, 2006; K. Zhang et al., 2023; Y. Zheng et al., 2008). In addition, IL-22 can enhance mucus secretion by goblet cells, thereby promoting the clearance of pathogens. (A. Singh et al., 2024; Turner, Stockinger, & Helmby, 2013; K. Zhang et al., 2023). Th22 cells also have the potential to reinforce the mucosal epithelial barrier. Specifically, IL-22 has been demonstrated to inhibit apoptosis of epithelial cells, promote their growth, stimulate the production of tight junction components, and facilitate tissue repair in the intestinal mucosa (Hebert et al., 2020; Pickert et al., 2009; A. Singh et al., 2024; K. Zhang et al., 2023). Beside

their protective functions, an increased presence of Th22 cells and IL-22 is also associated with the development of autoimmune diseases, such as MS, rheumatoid arthritis, psoriasis, and autoimmune thyroid diseases (Luan, Ding, Han, Zhang, & Liu, 2014; Perriard et al., 2015; L. Sun et al., 2023; Vitales-Noyola et al., 2017; Lei Zhang et al., 2011).

1.1.3.5 T_{FH} cells

T_{FH} cells constitute a heterogeneous group of $CD4^+$ T helper cells that are essential for humoral responses by facilitating the maturation of B cells, isotype switching and antibody production. The differentiation of T_{FH} cells follows a stepwise process (Choi et al., 2011; Crotty, 2011; Künzli & Masopust, 2023). The first step includes the differentiation to a pre-T_{FH} cell phenotype upon TCR stimulation with the presence of both IL-6 and IL-21 by DCs (Eto et al., 2011; K. T. Lu et al., 2011; Nurieva et al., 2008, 2009). The major transcription factor involved in T_{FH} cell development is B cell lymphoma 6 (Bcl6; Nurieva et al., 2009). Both, stimulation via TCR and via IL-6/IL-21 promote the activation of Bcl6 by inducing TCF-1/LEF-1 and STAT3 signaling, respectively (Ballesteros-Tato et al., 2016; Choi et al., 2015; Eto et al., 2011; L. Sun et al., 2023; Lifan Xu et al., 2015). Furthermore, binding of the inducible costimulator ligand (ICOSL), expressed on activated monocytes and DCs, to its receptor ICOS on T cells facilitates the maintenance of T_{FH} cells via Bcl6 (Choi et al., 2011; L. Sun et al., 2023; Weber et al., 2015). Bcl6 upregulates the expression of CXCR5, which enables pre-T_{FH} cells binding to CXCL13 and the migration to the T cell-B cell border (T-B border) of secondary lymphoid organs (Nurieva et al., 2009; D. Yu et al., 2009; D. Yu & Vinuesa, 2010). At the T-B border, pre-T_{FH} cells encounter cognate B cells, which express ICOSL on their surface (Garside et al., 1998; N. M. Haynes et al., 2007; Künzli & Masopust, 2023; MacLennan et al., 1997; Okada et al., 2005). TCR and ICOSL/ICOS stimulation further strengthen the differentiation of germinal center (GC)-T_{FH} cells and their subsequent migration into the GC (Künzli & Masopust, 2023; Suan et al., 2015). In return, T_{FH} cells facilitate the activation, differentiation, and selection of B cells by releasing IL-21 and by directly binding to CD40 on B cells via CD40L (Choi et al., 2011; Eto et al., 2011; Künzli & Masopust, 2023). In this way, they are enabling the production of high-affinity antibodies within the GC (Crotty, 2014; Nurieva et al., 2008; Sallusto, 2016; Vogelzang et al., 2008; D. Yu & Vinuesa, 2010). Besides, T_{FH} cells are crucial for the differentiation into memory B cells and plasma cells (Bryant et al., 2021). Upon infection or vaccination, this process ensures a rapid humoral response following reinfection (Allen, Okada, & Cyster, 2007). Depending on the type of pathogenic infection, T_{FH} cells can take on specific characteristics of Th1, Th2 and Th17 cells by expressing low levels of T-bet, GATA3 or RORγt (Eisenbarth et al., 2021; K. T. Lu et al., 2011). The additional expression of these transcription factors can, in turn, result in the secretion of IFNγ, IL-4, or IL-17, the lineage-specific cytokines of Th1, Th2, and Th17 cells, respectively (Eisenbarth et al., 2021). These cytokines have been suggested to trigger specific isotype switching of B cells. Specifically,

studies in mice indicated that T_{FH} cells that additionally produce IFNγ specifically promote the development of an IgG isotype, whereas T_{FH} cells that secrete IL-4 promote IgE and T_{FH} cells that secrete IL-17 promote an IgA isotype (Bentebibel et al., 2016; Eisenbarth et al., 2021; Gowthaman et al., 2019; Hirota et al., 2013; R. Morita et al., 2011; Nakayamada et al., 2011; Oestreich, Huang, & Weinmann, 2011; Powell, Read, Sreekumar, Jones, & Oestreich, 2019). In addition, the differential expression of CXCR5 has also been linked to play a role in this process. In particular, studies showed the presence of intermediate CXCR5-expressing T_{FH} cells that express high levels of IFNγ, IL-4, and IL-17 (Ma et al., 2009; D. Yu et al., 2009; D. Yu & Vinuesa, 2010). These cells have also been suggested to be precursors of GC T_{FH} cells (Vinuesa & Cook, 2011; D. Yu & Vinuesa, 2010). Beside their important role in supporting and regulating humoral responses in the course of infections, T_{FH} cells have also been associated with the progression of autoimmune diseases. For instance, in systemic lupus erythematosus, an increased presence of circulating T_{FH} cells enhances the proliferation of auto-reactive B cells and the production of autoantibodies (Gensous et al., 2018; Saggau et al., 2024; L. Sun et al., 2023; X. Zhang et al., 2015).

1.1.3.6 Regulatory T cells

Regulatory T cells (Tregs) represent a specialized $CD4^+$ T cell population with the ability to suppress the activity of other T cells and immune cells (Dieckmann, Plottner, Berchtold, Berger, & Schuler, 2001; Schmidt, Oberle, & Krammer, 2012; L. Sun et al., 2023). In this way, they facilitate immunological tolerance to self-antigens, commensal microbes and exogenous antigens, such as those derived from food (J. M. Kim, Rasmussen, & Rudensky, 2007; L. Sun et al., 2023). Tregs can be classified according to their origin into two main categories: thymus-derived Tregs (tTregs) and peripherally induced Tregs (pTregs) (Abbas et al., 2013; Z. Li et al., 2023). tTregs originate from $CD4^+CD25^+$ T cells in the thymus and pTregs derive from naive $CD4^+$ T cells in the periphery, in particular in mucosal tissues (Q. Gu et al., 2023; Z. Li et al., 2023; S. G. Zheng, Wang, Wang, Gray, & Horwitz, 2007). A major factor for differentiation to Tregs is the recognition of self-reactive antigens via TCR (Jordan et al., 2001; Josefowicz, Lu, & Rudensky, 2012; Kanamori, Nakatsukasa, Okada, Lu, & Yoshimura, 2016; L. Sun et al., 2023). By this, TCR and CD28 stimulation induces a signaling cascade, which in turn leads to the expression of FOXP3, the key transcription factor that drives Treg differentiation (Fontenot, Gavin, & Rudensky, 2017; Hori, Nomura, & Sakaguchi, 2017; Khattri, Cox, Yasayko, & Ramsdell, 2017). In addition, TGF-β and IL-2 signaling promote FOXP3 expression, which is particularly essential for the development of pTregs (Q. Chen, Kim, Laurence, Punkosdy, & Shevach, 2011; W. J. Chen et al., 2003; Coombes et al., 2007). FOXP3 induces the characteristic high expression of CD25, the α-chain of the IL-2 receptor (IL-2Rα), on the surface of Tregs (Camperio et al., 2012). Upregulation of CD25 further increases the susceptibility of Tregs to bind IL-2 and reinforce the maintenance of Tregs (Camperio et al.,

2012). Besides the expression of CD25, Tregs are characterized by a high production of the cytokines IL-10, TGF-β, and IL-35 (Groux et al., 1997; Hori et al., 2017; Maynard et al., 2007; L. Sun et al., 2023; Uhlig et al., 2012; J. Wang, Zhao, & Wan, 2023; Wei et al., 2017). These cytokines directly target the activation of effector immune cells and their capacity to proliferate. For instance, studies have been shown that IL-10 can inhibit full activation of naive T cells and their differentiation into effector T cells by inhibiting costimulatory CD28 signaling (Akdis, Joss, Akdis, Faith, & Blaser, 2000; Joss, Akdis, Faith, Blaser, & Akdis, 2000; Taylor, Verhagen, Blaser, Akdis, & Akdis, 2006). IL-35 can suppress T cell proliferation by inducing cell cycle arrest in the G1 phase of cell division and by inhibiting the expression of transcription factors and cytokines that are essential for the development of $CD4_{conv}$ T cell subsets (Casella et al., 2017; Collison et al., 2007; A. Huang et al., 2017). Beside the secretion of inhibitory cytokines, Tregs express a number of co-inhibitory surface molecules, such as CTLA-4, Lag-3, and TIGIT (P. Gu et al., 2012; D. Kim et al., 2024; Kurtulus et al., 2015; Qureshi et al., 2011; L. Sun et al., 2023; Tekguc, Wing, Osaki, Long, & Sakaguchi, 2021). Binding of CTLA-4 to B7 molecules (CD80 and CD86) and Lag-3 to MHC-II-molecules on APCs like DCs, blocks the antigen-presenting and costimulatory function, and subsequently T cell activation (Huard, Tournier, Hercend, Triebel, & Faure, 1994; D. Kim et al., 2024; B. Liang et al., 2008; Tekguc et al., 2021). Moreover, CTLA-4 has the capacity to deplete B7 molecules through transendocytosis and trogocytosis, thereby making APCs unable to provide co-stimulation via CD28 (P. Gu et al., 2012; Qureshi et al., 2011; L. Sun et al., 2023; Tekguc et al., 2021). These mechanisms serve to prevent the overactivation of immune responses and the potential for self-harm during the course of infection (Cardona & Cardona, 2019; L. Sun et al., 2023; Wan et al., 2020; White, McManus, & Maizels, 2020; Z. Xu, Jiang, Dai, & Li, 2022). Given the pivotal function of Tregs in maintaining immunological self-tolerance and homeostasis, it is evident that these cells play a vital role in preventing the onset and progression of autoimmune diseases (M. A. Khan, 2020). A distinctive feature of Tregs is their adaptability under inflammatory conditions. In response to inflammatory stimuli, Tregs are recruited to the site of inflammation, where they can take on specific phenotypic and transcriptional characteristics of the leading effector $CD4_{conv}$ T cells responsible for the inflammatory process (L. Sun et al., 2023; Wing & Sakaguchi, 2012). As a result of this plasticity, they are capable of exerting a more specialized and an enhanced suppression of the corresponding immune responses (L. Sun et al., 2023; Wing & Sakaguchi, 2012).

1.1.4 $CD4^+$ T cell development in early life

The development of the human immune system commences during the early stages of gestation, with the presence of innate and adaptive immune cells already observed in the first trimester (Gollwitzer & Marsland, 2015; J. E. Park, Jardine, Gottgens, Teichmann, & Haniffa, 2020; Popescu et al., 2019; Ygberg & Nilsson, 2012). Analysis of the human embryonic yolk

sac revealed the presence of hematopoietic stem cell (HSC)-like progenitor cells and innate immune cell types, including monocytes and macrophages, natural killer (NK) cell progenitors and innate lymphoid cells as early as by gestational week (GW) 4. (Davies et al., 1992; Ginhoux & Guilliams, 2016; Gollwitzer & Marsland, 2015; Guilmot, Hermann, Braud, Carlier, & Truyens, 2011; Omar, Salhadar, Wooliever, & Alsgaard, 2000; J. E. Park, Jardine, et al., 2020; Popescu et al., 2019). The earliest lymphoid progenitors, which originate from the fetal liver, can be detected from GW 8 (B. F. Haynes & Heinly, 1995; J. E. Park, Jardine, et al., 2020). At this point they migrate into the fetal thymus, where they undergo transition from early thymic progenitors into naive T cells (B. F. Haynes & Heinly, 1995; J. E. Park, Jardine, et al., 2020). A single-cell transcriptome analysis of the developing human thymus has revealed that the development of the thymus occurs as a result of considerable intercellular communication between a number of cell types (J. E. Park, Botting, et al., 2020; J. E. Park, Jardine, et al., 2020). These include thymic epithelial cells, mesenchymal cells, early thymic progenitors, developing and mature T cells, and other immune cells (J. E. Park, Botting, et al., 2020; J. E. Park, Jardine, et al., 2020). At GW 10 to 11, circulating T cells are present in the body's peripheral blood and tissues, following emigration from the thymus and migration to other tissues (B. E. Haynes, Martin, Kay, & Kurtzberg, 1988; J. E. Park, Jardine, et al., 2020). Studies on the fetal intestine identified the presence of memory $CD4^+$ T cells and Tregs (N. Li, van Unen, Guo, et al., 2019; J. E. Park, Jardine, et al., 2020; Schreurs et al., 2019; Stras et al., 2019; Xiaoming Zhang et al., 2014). These studies underscore the potential of fetal $CD4^+$ T cells to respond to foreign antigens and to establish a balance between activation and suppression of T cell immune responses (N. Li, van Unen, Abdelaal, et al., 2019; N. Li, van Unen, Guo, et al., 2019; J. E. Park, Jardine, et al., 2020; Schreurs et al., 2019; Stras et al., 2019; Xiaoming Zhang et al., 2014). In addition, fetal memory $CD4^+$ T cells have also been shown to promote tissue development (Schreurs et al., 2019). A study by Schreurs and colleagues (2019) revealed the presence of fetal intestinal $CD4^+$ effector memory T cells that promote intestinal epithelial development by secreting moderate levels of TNF (Schreurs et al., 2019). Besides these specific effector functions, the fetal immune system furthermore exhibits tolerogenic responses, enabling the fetus to tolerate self and maternal antigens while avoiding the exaggeration of immune responses that could compromise fetal viability (Bronevetsky, Burt, & McCune, 2016; Burt, 2013; Michaëlsson, Mold, McCune, & Nixon, 2006; Mold et al., 2008, 2010; M. S. F. Ng, Roth, Mendoza, Marson, & Burt, 2019). Therefore, the effector function of fetal immune cells is compartmentalized (Gollwitzer & Marsland, 2015; Ygberg & Nilsson, 2012; Xiaoming Zhang et al., 2014). Furthermore, the presence of fetal Tregs also plays a crucial role in maintaining tolerogenic immunity. In line with this, *in vitro* stimulation of isolated naive $CD4^+$ T cells from cord blood and mesenteric lymph node preferentially give rise

to Tregs in comparison to adult naive $CD4^+$ T cells (Burt, 2013; Gollwitzer & Marsland, 2015; Mold et al., 2008, 2010; J. E. Park, Jardine, et al., 2020).

Following birth, neonatal peripheral $CD4^+$ T cells exhibit non cytotoxic responses, with the majority of $CD4^+$ T cells being Tregs and Th2 cells, while Th1 responses, including IFNγ production, are underrepresented (Crofts & Alexander-Miller, 2020; Debock & Flamand, 2014; Gollwitzer & Marsland, 2015; Hebel et al., 2014; Mercy PrabhuDas et al., 2011; Vekemans et al., 2001; G. Wang et al., 2010; Wilson et al., 1986). This state is thought to prevent postnatal autoimmunity, as infants are exposed to high levels of microbes from their mother and the surrounding environment at the time of birth (Gollwitzer & Marsland, 2015). However, as a consequence of the reduced Th1 cell functionality, newborns are more susceptible to bacterial and viral infections (Debock & Flamand, 2014; Gollwitzer & Marsland, 2015; Steiner, Diesner, & Voitl, 2019; Wilson et al., 1986). In the following months, the number of peripheral Th1 cells increases, with the full potential for Th1 cell immune reactions being reached at approximately 1 year of age (Debock & Flamand, 2014; Gollwitzer & Marsland, 2015). This illustrates the transition from a previously predominant tolerogenic immune system *in utero* to an increasing postnatal protective immune system.

Although the number of T cells increases from year to year, it is not until approximately 6 years of age that the number of peripheral T cells reaches the levels observed in adults (Gollwitzer & Marsland, 2015; van Gent et al., 2009). During the first 5 years of age, maturation of the T cell immune system is driven by a variety of cell-intrinsic and extrinsic factors that are crucial to shape and direct the immune system to acquire protective functions (Gollwitzer & Marsland, 2015; Olin et al., 2018). Nevertheless, certain factors may also exert detrimental effects on the immune system, particularly during critical initial developmental stages and predispose to dysregulated immune responses (Connors et al., 2023; Olin et al., 2022, 2018).

1.2 Early-life stressors

The early phase of life, particularly the gestational period and the first year after birth, is often described as an "immunological window of opportunity," a period during which the developing child's immune system is particularly susceptible to risk factors (Acevedo et al., 2015, 2021; Barker, 2007; Olin et al., 2022; Renz & Skevaki, 2021). The heightened vulnerability of the fetus is attributed to the rapid development and plasticity that are indispensable for the formation of a complex organism, including its immune system, during gestation (Barker, 2007; Bauman & Van de Water, 2020). Although the infant's immune system undergoes substantial changes throughout the initial year of life, its functional capabilities are still limited, leading to a heightened vulnerability to risk factors, such as infections (Hill et al., 2020). The existing literature highlights a number of early-life stressors that have been associated with alterations in the offspring's immune system (Gollwitzer & Marsland, 2015). Stressors are considered as

risk factors with the potential to exert an adverse and persistent influence on the immune system. Early-life stressors include pre-existing conditions, such as obesity or chronic diseases of the pregnant woman (Denizli, Capitano, & Kua, 2022; Kersten et al., 2014b). Moreover, the literature underscores the influence of prenatal exposure to elevated maternal stress levels, maternal infections, maternal medications and pregnancy complications (Atladóttir et al., 2010; Boulanger et al., 2024; Kumar, Saadaoui, & Al Khodor, 2022; Louchet et al., 2024; Pascal et al., 2023). In addition, severe infections and the necessity for systemic antibiotics in the first year of life, have been also linked to aberrant immune functions in the developing child (Shekhar & Petersen, 2020). These aforementioned stressors will be subsequently elaborated in greater detail in sections 1.2.2 to 1.2.8. A pivotal defining feature of a stressor is the level of intensity (V. X. Han, Patel, Jones, & Dale, 2021). Thus, a potent stressor may act as a strong immune insult and cause severe impairments in the offspring, while a less potent stressor may not cause any or only minor alterations (V. X. Han, Patel, Jones, & Dale, 2021). For instance, in the event of a maternal mild upper respiratory tract infection, it is assumed that the maternal immune system is capable of returning to immune homeostasis, with minimal impact on the child's immune system. Conversely, a severe maternal infection, such as influenza, that causes substantial morbidity in the mother during pregnancy may result in a more strongly altered maternal immune response, which in turn may exert a profound influence on the developing fetal immune system. In this context, the activation of the maternal immune system is regarded as a crucial mechanism through which stressors, particularly during pregnancy, can induce alterations in the offspring's immune system (Estes & McAllister, 2016; Shimizu, Sakata-Haga, Saikawa, & Hatta, 2023).

1.2.1 The concept of maternal immune activation

In response to stressors, the maternal immune system can be activated during pregnancy, resulting in an increased production of pro-inflammatory mediators, including cytokines and chemokines (Andersson, Li, Mills, Ly, Nomura, Hospital, et al., 2016; Antonson et al., 2020; H. J. Chen et al., 2020; Jain et al., 2021; Lim et al., 2021; Shimizu et al., 2023, 2021; Figure 4). These elevated levels have the potential to impact not only the maternal immune system but also that of the fetus. (Estes & McAllister, 2016; Ye Li et al., 2023). Specifically, cytokines have the potential to cross the placenta and enter the fetal circulation (Brynge et al., 2022; Paraschivescu, Barbosa, Lorivel, Glaichenhaus, & Davidovic, 2020; S. E. P. Smith, Li, Garbett, Mirnics, & Patterson, 2007; Urakubo, Jarskog, Lieberman, & Gilmore, 2001; Zaretsky, Alexander, Byrd, & Bawdon, 2004). In this way, they can affect the placental environment and the development of the fetus. A multitude of pro-inflammatory cytokines and in particular acute phase cytokines are linked to maternal immune activation (MIA), such as IL-1β, IL-17A, IFNγ, monocyte chemotactic protein1 (MCP-1), and TNF (Andersson, Li, Mills, Ly, Nomura, Hospital, et al., 2016; Antonson et al., 2020; Garay, Hsiao, Patterson, & McAllister, 2013; Gilmore,

Jarskog, Vadlamudi, & Lauder, 2004; Ye Li et al., 2023; Meyer et al., 2006, 2008; Shimizu et al., 2021). One of the earliest recognized and most frequently cytokine associated with MIA is IL-6 (Buka et al., 2001; Ye Li et al., 2023; Spann, Monk, Scheinost, & Peterson, 2018; Zaretsky et al., 2004). IL-6 has been the focus of considerable research in the context of MIA (Ye Li et al., 2023; Zawadzka, Cieślik, & Adamczyk, 2021).

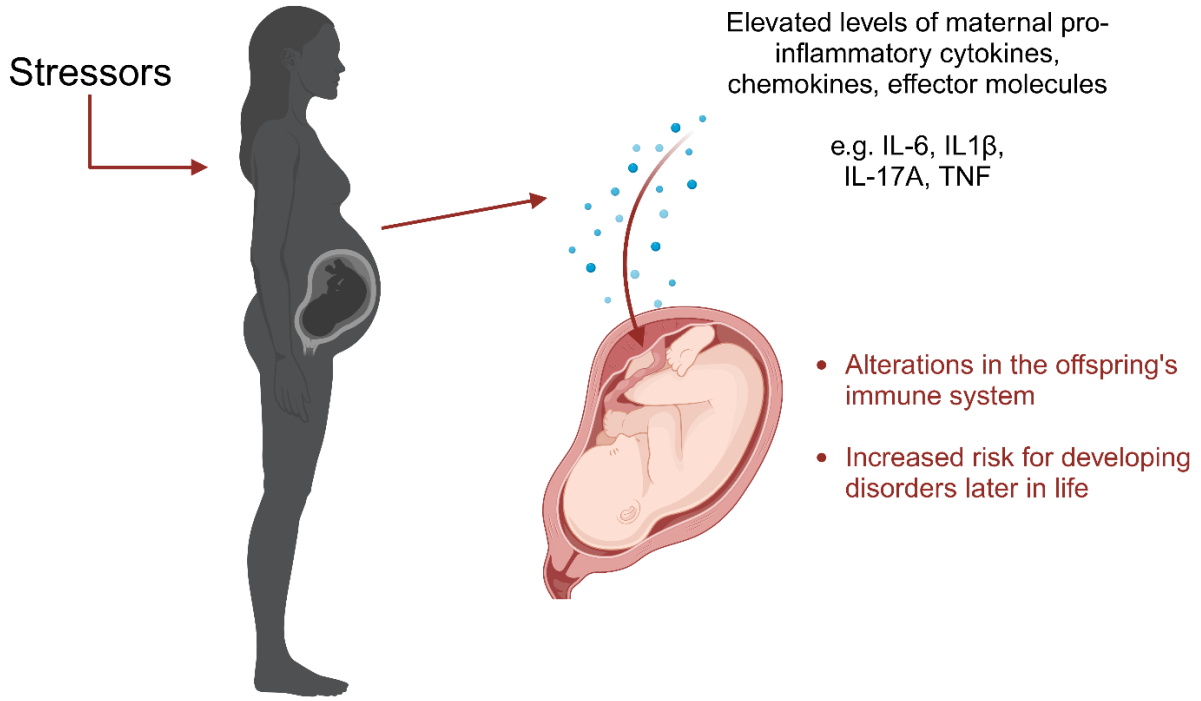


Figure 4: Maternal immune activation (MIA). Stressors trigger the activation of the maternal immune system, resulting in an increased production of pro-inflammatory mediators, including cytokines, chemokines and effector molecules, such as IL-6, IL-1β, IL-17A and TNF. Cytokines have the potential to cross the placenta and enter the fetal circulation, thereby affecting the fetal immune system. MIA is associated with an altered development of the offspring's immune system and an increased risk for developing immunological disorders later in life. Generated with BioRender.

The most common used and well-established animal model to investigate the impact of MIA is the maternal polyinosinic-polycytidylic acid (poly(I:C)) model (Reisinger et al., 2015; Shimizu et al., 2023). Poly(I:C) is a synthetic analogue of double-stranded RNA that is used to mimic the effects of a viral infection (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Matsumoto, Kikkawa, Kohase, Miyake, & Seya, 2002). Activation of the Poly(I:C)/TLR3 pathway induces the production of inflammatory cytokines and chemokines such as IL-6, TNF and CXCL10 (Hameete et al., 2021). In a murine MIA model, elevated IL-6 levels were observed in the placenta that were derived from the mother and were associated with an altered fetal development (Hsiao & Patterson, 2011; Zawadzka et al., 2021). Particularly in the field of neurological research, MIA and elevated IL-6 levels have been long implicated as a risk factor for the development of neurodevelopmental and neuropsychiatric disorders in offspring, such as autism spectrum disorder or schizophrenia (Brown et al., 2004; Brown & Derkits, 2010;

Estes & McAllister, 2016; Massarali, Adhya, Srivastava, Baron-Cohen, & Kotter, 2022). Furthermore, emerging data also strongly suggest an association between MIA and aberrant immune function in offspring. This includes both exaggerated immune responses and immune deficiency, as well as the emergence of immunological disorders (Atladóttir et al., 2010; Brown, 2012; Hodyl, Krivanek, Lawrence, Clifton, & Hodgson, 2007; McKeever, Lewis, Smith, & Hubbard, 2002; Onore, Schwartzer, Careaga, Berman, & Ashwood, 2014; Shimizu et al., 2023; Yue et al., 2018). Additionally, epidemiological studies indicated that MIA may contribute to the onset of wheezing, allergic diseases and obesity in childhood (Denizli et al., 2022; Englich et al., 2017; J. H. Kim, Kim, Woo, & Shim, 2008; Rothers et al., 2018). Recent studies in rodents also suggest that MIA not only affect the child's development during pregnancy, but also may affect the child postnatally during the lactation period. Specifically, MIA was associated with an altered profile of breast milk constituents, including elevated milk corticosterone concentrations and reduced microbial diversity, accompanied by long-term physiological and behavioral alterations in offspring (Browne et al., 2019; DeRosa, Caradonna, Tran, Marrocco, & Kentner, 2022). DeRosa and colleagues (2022) also showed that MIA did not affect IL-6 concentration in the milk (DeRosa et al., 2022). Nevertheless, the potential involvement of additional inflammatory cytokines in milk and their subsequent impact on offspring development, including their immune system, cannot be entirely ruled out and requires further research (DeRosa et al., 2022).

1.2.2 Maternal chronic diseases during pregnancy

Estimates from population-based studies suggest that the prevalence of pregnant women with a chronic disease varies from 15% to 27% (S. Chatterjee, Kotelchuck, & Sambamoorthi, 2008; Jølving et al., 2016; Kersten et al., 2014b). Among these women, allergies, thyroid diseases and chronic lung diseases, such as asthma or chronic obstructive pulmonary disease, are some of the most frequent diseases (S. Chatterjee et al., 2008; Jølving et al., 2016; Kersten et al., 2014b). Epidemiological studies indicate that maternal chronic diseases may affect the development of the child during pregnancy, leading to adverse birth outcomes and long-term alterations, including an increased risk of intrauterine growth retardation (IUGR), preterm birth, and the emergence of asthma, obesity and neurodevelopmental disorders later in life (Ajslev, Sorensen, Sci, Jess, & Sci, 2012; Jølving et al., 2016; Kersten et al., 2014b; Nørgard, Hundborg, Jacobsen, Nielsen, & Fonager, 2007; Nosarti et al., 2012). In particular, autoimmune diseases and chronic inflammatory conditions can affect the immune state during pregnancy and contribute to MIA (Furman et al., 2019; V. X. Han, Patel, Jones, Nielsen, et al., 2021). Specifically, the presence of autoantibodies and elevated concentrations of pro-inflammatory markers play a key role in this process (Björkander et al., 2012; Gardner, Brynge, Sjöqvist, Dalman, & Karlsson, 2024; V. X. Han, Patel, Jones, & Dale, 2021; Ishihara & Hirano, 2002; Saggau et al., 2024). For instance, increased maternal levels of IL-6 are observed in

rheumatoid arthritis, psoriasis, IBD, asthma, and hyperthyroidism (Alhendi & Naser, 2023; Ishihara & Hirano, 2002; Nikolaus et al., 2018; R. Pan et al., 2023; Salvi et al., 1996). It is likely that the altered prenatal immune status resulting from the chronic conditions may also exert an influence on the development of the fetal immune system, which could potentially give rise to long-term consequences. In this context, a number of studies have indicated that children born to mothers with allergies are more prone to developing allergies and asthma than children born to fathers with allergies (Barrett, 2008; Litonjua, Carey, Burge, Weiss, & Gold, 1998; Omran & Russell, 1996). Similarly, infants of mothers with atopic dermatitis have an almost fivefold higher probability of developing this condition in comparison with children born to fathers with atopic dermatitis (Barrett, 2008; G. G. Ruiz, Kemeny, & Price, 1992). In addition to genetic factors, these findings may also indicate an increased susceptibility for disease priming *in utero*.

1.2.3 Maternal obesity during pregnancy

Obesity is a complex disease, defined by an excessive accumulation of adipose tissue and a body mass index (BMI) equal to or greater than 30 kg/m² (BMI ≥ 30; World Health Organization, 2000, 2024). A nationwide data survey carried out in 2021 showed that almost one quarter of pregnant women in Germany had a BMI of ≥ 30 (IQTIG – Institut für Qualitätssicherung und Transparenz im Gesundheitswesen, 2022). Maternal obesity is associated with the onset of chronic, systemic low-grade inflammation, including elevated levels of IL-6 and C-reactive protein (CRP; Subhabrata Basu, Leahy, et al., 2011; Denizli et al., 2022; Ingvorsen, Brix, Ozanne, & Hellgren, 2015; Jarmund et al., 2021; Parisi, Milazzo, Savasi, & Cetin, 2021). In human studies, children born to mothers with obesity have elevated circulating concentrations of CRP and an increased risk of developing a range of inflammatory and metabolic diseases, including type 2 diabetes, asthma, and obesity itself (Castro-Rodriguez et al., 2020; Catalano, Presely, Minium, & Mouzon, 2009; Forno & Young, 2014; Lecoutre, Maqdasy, & Breton, 2021; Leibowitz et al., 2012; Lieb et al., 2009; Voerman et al., 2019). Additionally, they appear to be more susceptible to developing cardiovascular complications and neuropsychiatric disorders (Getz, Anderka, Werler, & Jick, 2016; Hebsgaard, Per, Thomsen, & Aagaard, 2017; Razaz, Villamor, Muraca, Bonamy, & Cnattingius, 2020). Obesity is frequently concomitant with overnutrition based on a high-fat, high-sugar and low-fiber "Western diet" (Gollwitzer & Marsland, 2015). Various animal models on rodents, and non-human primates have demonstrated an elevated inflammatory status, including local inflammatory processes in various tissues, in offspring from mothers with obesity or high-fat diet in comparison to those from lean mothers (L. Fan et al., 2013; Ingvorsen et al., 2015; Latouche et al., 2014; Nash et al., 2023). For example, studies conducted on mice demonstrated that offspring exposed to mothers with a high-fat diet exhibited enhanced intestinal inflammation and impaired intestinal epithelial barrier function (Myles et al., 2013; Xue, Wang, Du, & Zhu, 2014). In this context,

isolated intestinal lymphocytes from murine offspring exposed to a maternal high-fat diet showed an augmented inflammatory response, as evidenced by an increased production of the pro-inflammatory cytokines IL-6, IL-1β and IL-17A (Myles et al., 2013). Moreover, the researchers noted a markedly diminished frequency of Tregs within the colon (Myles et al., 2013).

1.2.4 Prenatal psychological stress

The term "psychological stress" is used to describe an emotional reaction exhibited by an individual in response to a stimulus that is perceived as overwhelming (Goodnite & Goodnite, 2014; Pascal et al., 2023). The experience of psychological stress during pregnancy is not uncommon. Recent reports have suggested that about 23-25% of pregnant women experience high levels of stress and anxiety throughout their pregnancy (Pascal et al., 2023; Wu, De Asis-Cruz, & Limperopoulos, 2024; Wu et al., 2020). Various factors may contribute to the development of maternal stress during the gestational period. These include a low socioeconomic status, work-related pressures, family conflicts, trauma, the loss of a close friend or relative, and physiological changes that accompany pregnancy, including hormones associated with mood alterations, as well as the onset of pregnancy complications (Daalderop, Lagendijk, Steegers, El Marroun, & Posthumus, 2023; Rothenberger, Moehler, Reck, & Resch, 2011; Wu et al., 2024). Psychological stress can have a detrimental impact on maternal and fetal health. Specifically, prenatal psychological stress is associated with several adverse pregnancy outcomes, including preeclampsia, gestational diabetes, preterm birth, lower birthweight, and neurodevelopmental problems in the offspring (Cai et al., 2017; Madigan et al., 2018; Pascal et al., 2023; Rondo et al., 2003; Y. Yu et al., 2013; P. Zhu, Tao, Hao, Sun, & Jiang, 2010). Furthermore, epidemiological studies have demonstrated that children exposed to chronic stress during pregnancy have an elevated risk of developing immune-related diseases, including asthma, eczema, and wheezing (El-Heis et al., 2017; Ye Li et al., 2023; O'Connor et al., 2013; Pape et al., 2020). In particular, high levels of chronic stress, e.g. caused by maternal bereavement, are associated with immune alterations in the mother and offspring, leading to an increased probability of neurodevelopmental disorders in childhood, such as attention deficit hyperactivity disorder (Ye Li et al., 2023; O'Connor, 2019; Persson & Rossin-Slater, 2018; A. C. Phillips et al., 2006; Su et al., 2021). In this regard, high maternal stress has been shown to induce MIA, including upregulated expression of pro-inflammatory cytokines, along with reduced immunity and increased susceptibility to infectious diseases (Andersson, Li, Mills, Ly, Nomura, & Chen, 2016; Bronson & Bale, 2014; H. J. Chen et al., 2020; Dhabhar, 2014; Gumusoglu, Maurer, & Stevens, 2022; Ye Li et al., 2023; A. C. Phillips et al., 2006). These finding have been identified along with the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which ultimately leads to an augmented secretion of glucocorticoids into the blood stream (Garcia-Flores et al., 2020; S. M. Smith & Vale, 2006).

These maternal physiological mechanisms have been thought to play a major role in determining offspring outcomes (Antonson et al., 2020). Glucocorticoids are known to have immunomodulatory functions and placental transfer of these stress hormones may affect the development of the fetal immune system (Coutinho & Chapman, 2011; Gollwitzer & Marsland, 2015). Specifically, glucocorticoids exert pleiotropic effects on different T cell subsets, such as the suppression of Th1 cell responses and the promotion and permission of Th17 cell immune responses (de Castro Kroner, Knoke, Kofler, Steiger, & Fabri, 2018; Taves & Ashwell, 2021). In human studies, prenatal maternal stress was associated with higher concentrations of IL-6 in childhood (Clayborne, Gilman, Khandaker, & Colman, 2024). Moreover, a recent study in rats reported elevated serum levels of IL-6 and TNF in the offspring exposed to chronic unpredictable mild stress during pregnancy (Ye Li et al., 2023). The study further observed alterations in the $CD4^+$ T cell compartment, demonstrated by a markedly higher proportion of Th17 cells, and a lower proportion of Tregs in the spleen (Ye Li et al., 2023).

1.2.5 Medication use during pregnancy

Medications are defined as substances or combinations of substances used to treat, cure, prevent or diagnose a disease or disorder, to alleviate symptoms, or to restore or correct physiological functions by exerting a pharmacological, immunological or metabolic action (European Medicines Agency (EMA), 2025; Federal Institute for Drugs and Medical Devices (BfArM), 2025). According to the United States Food and Drug Administration (FDA), medications can be classified into 40 general categories based on their therapeutic effects, ranging from A for analgesics to V for vitamins (United States Food and Drug Administration (US FDA), 2015). The utilization of medications during the gestational period is not uncommon, with a notable increase observed over the past decade (Bjørn et al., 2011; Dathe & Schaefer, 2019; A. A. Mitchell et al., 2011; Østergaard Thunbo, Vendelbo, Witte, Larsen, & Pedersen, 2024; Smolina, Hanley, Mintzes, Oberlander, & Morgan, 2015; Subramanian et al., 2023). According to estimates from pregnancy and birth cohort studies of Western European countries, the prevalence of women taking prescription medications at least once during their pregnancy ranges from 44% to 93% (Bérard et al., 2019; Daw, Gillian E. Hanley, Greyson, & Morgan, 2011; A. A. Mitchell et al., 2011; Østergaard Thunbo et al., 2024). Moreover, the overall prevalence of medication use is presumed to be relatively higher when considering over-the-counter (OTC) medications (Bérard et al., 2019; A. A. Mitchell et al., 2011; Østergaard Thunbo et al., 2024). During pregnancy, systemic medications and their metabolites may have the potential to cross the placenta by passive diffusion, facilitated diffusion, active or vesicular transport, thereby exerting a direct effect on the developing fetus and its immune system (Evseenko, Paxton, & Keelan, 2006; Fant, Yeakley, & Harrison, 1983; G. I. Henderson et al., 1993; Syme, Paxton, & Keelan, 2004; Ward, 1995). In particular, lipophilic, non-ionized, small-molecule medications with a molecular weight of less than 500 g/mol can permeate the

placenta via passive diffusion (Fein, Arral, Kim, Newby, & Whitehead, 2023; Mao & Chen, 2022; Syme et al., 2004; Ward, 1995). Drugs with molecular weights higher than 500 g/mol exhibit reduced placental permeability or do not cross the placental membrane (Sachdeva, Patel, & Patel, 2009; Syme et al., 2004). However, these pharmaceuticals may also elicit certain pharmacological, metabolic or immunological drug reactions, including MIA, in the mother or at the maternal-fetal interface that may indirectly affect the fetus. Two of the most commonly used medications during pregnancy are antibiotics and antiemetics. (Haas et al., 2018; Mansour et al., 2024; Palmsten et al., 2015; Thorpe et al., 2013). Antibiotics are prescribed for the treatment of bacterial infections, while antiemetics are used to treat symptoms, including nausea and vomiting (Athavale, Athavale, & Roberts, 2020). In addition, there has been an increase in the use of medications for the treatment of chronic conditions, including levothyroxine (L-thyroxine) and insulin, which are used to treat thyroid hormone dysregulation and diabetes mellitus during pregnancy, respectively (Mansour et al., 2024; Palmsten et al., 2015; Tinker, Broussard, Frey, & Gilboa, 2015). Emerging data indicate an association between maternal medication use during pregnancy and altered immunity in children (Louchet et al., 2024; J. E. Miller et al., 2018; Nakitanda et al., 2023). With regard to antibiotic use, recent observational studies based on national databases have observed that prenatal and perinatal exposure to antibiotics is associated with an increased risk of bacterial or viral infections and higher rates of antimicrobial prescription fills in childhood (Louchet et al., 2024; J. E. Miller et al., 2018; Nakitanda et al., 2023). In addition, studies in humans and mice have shown that pre- and peripartum antibiotics promote microbial dysbiosis in infants, which in turn was associated with dysregulated T-cell function, such as elevated percentages of Th17 cells, and an increased risk of developing immune-mediated diseases later in life (Alhasan et al., 2023; Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014; Arrieta et al., 2015; Laforest-Lapointe & Arrieta, 2017; Miyoshi et al., 2017; Olin et al., 2018). There are various classes of antiemetics targeting diverse pathways to mitigate nausea and vomiting, such as antagonists of the serotonin, dopamine, histamine systems, corticosteroids and benzodiazepines (Athavale et al., 2020). Studies in human and rodents have indicated the potential of specific antiemetics to exert immunomodulatory effects (Bain et al., 2018; Motavallian-Naeini, Minaiyan, Rabbani, & Mahzuni, 2012). For instance, the administration of dexamethasone, a synthetic glucocorticosteroid, has been demonstrated to rapidly and temporarily diminish the gene expression of pro-inflammatory cytokines, such as IL-1β, and to reduce the number of Tregs in peripheral blood (Bain et al., 2018). In addition to the use of medications, an underlying chronic condition or maternal infection may also be a significant contributing factor in the onset of developmental changes, as previously (see 1.2.2) and subsequently discussed (see 1.2.6).

1.2.6 Maternal infections during pregnancy

The maternal immune system undergoes a series of systemic immunological adaptations during pregnancy that are necessary to maintain tolerance to the semi-allogenic fetus while preserving immunity to protect against pathogens (Abu-Raya, Michalski, Sadarangani, & Lavoie, 2020; Aghaeepour et al., 2017; Berry et al., 2021; Mor, Aldo, & Alvero, 2017). However, given the restricted maternal immunity, pregnancy represents a period of increased susceptibility to infections (Godfrey et al., 2017; Kourtis, Read, & Jamieson, 2014; Sappenfield, Jamieson, & Kourtis, 2013; Vermillion & Klein, 2018). Therefore, maternal infections, of a bacterial, viral, parasitic or fungal nature, can give rise to complications at any stage of pregnancy (Kumar et al., 2022). Epidemiological studies on humans have demonstrated that maternal infection during pregnancy can result in a range of disorders associated with abnormal immunity in the offspring. These include type 1 diabetes, asthma, allergies, and neurodevelopmental disorders, such as schizophrenia and autism (Atladóttir et al., 2010; Brown, 2012; Collier, Risnes, Norwitz, Bracken, & Illuzzi, 2013; Fuchs & von Mutius, 2013; McKeever et al., 2002; Shimizu et al., 2023; Yue et al., 2018). In this context, the vertical transmission of infections and the maternal immune response associated with an infection are considered to be two important mechanisms that can lead to pregnancy complications and developmental alterations in children (Kumar et al., 2022). Specifically, infections with TORCH pathogens (*Toxoplasma gondii*, rubella, cytomegalovirus (CMV), herpes simplex virus (HSV), others) or the Zika virus can be vertically transmitted to the child during pregnancy or delivery and can cause a range of pregnancy complications, including congenital infections, miscarriage, IUGR or permanent disabilities (Abou-Bacar et al., 2004; James, Shef, & Kimberlin, 2014; Kumar et al., 2022; Maucourant et al., 2019; Ornelas et al., 2016; Pinninti, 2013; Ling Zhang et al., 2015). Besides, in the event of an infection, the maternal immune system is activated with the objective of combating the infection and eliminating the pathogen. Studies in humans and animals examining the impact of diverse infections, including influenza, SARS-CoV-2, Zika virus, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and other intrauterine infections, have identified the emergence of MIA, characterized by elevated levels of pro-inflammatory cytokines, such as IL-6, IL-1β, TNF, and IFNγ (Adachi, Nielsen-saines, & Klausner, 2016; Kumar et al., 2022; Le Gars, Kay, Bayless, & Aziz, 2016; Ornelas et al., 2016; Vallely et al., 2021; Villar et al., 2021). The poly(I:C) model and the maternal lipopolysaccharide (LPS) model are the most commonly utilized animal models to simulate viral and bacterial infections, respectively, during pregnancy (Hameete et al., 2021; Shimizu et al., 2023, 2021). Furthermore, both models are frequently employed to investigate the impact of MIA in offspring (Shimizu et al., 2023). Thus, offspring of poly(I:C) exposed mothers in particular show increased concentrations of IL-6 (Hameete et al., 2021). Furthermore, a recent study reported that maternal infections including high maternal IL-6 levels can have permanent and tissue-

specific impacts on offspring immunity (Lim et al., 2021). Specifically, Lim and colleagues (2021) used a mouse infection model in which pregnant dams were exposed to *yopM*, an attenuated strain of the foodborne pathogen *Yersinia pseudotuberculosis* (Lim et al., 2021). Adult offspring exposed to *yopM*-infected mothers had elevated frequencies of Th17 cells in the small and large intestinal lamina propria which was associated with increased IL-6 levels in maternal serum (Lim et al., 2021). Furthermore, this study indicated that increased IL-6 levels can directly induce epigenetic changes on fetal intestinal epithelial stem cells, resulting in an enhanced protective immunity and resistance to gut infections (Lim et al., 2021). However, the augmented immune responses may also lead to an increased susceptibility to colitis-related inflammation in the adult offspring (Lim et al., 2021).

1.2.7 Pregnancy complications

Pregnancy complications encompass a variety of health problems that may arise during pregnancy and affect the health of the mother and the fetus (van Esch et al. 2017; Gomez-lopez et al. 2019; Henderson, Carson, and Redshaw 2016; Hernández-Díaz, Toh, and Cnattingius 2009; Jensen et al. 2003; McIntyre et al. 2019; Miller et al. 2020; Tanner et al. 2022). Common pregnancy complications include gestational hypertension, pre-eclampsia, gestational diabetes mellitus (GDM), placental abruption, and spontaneous preterm birth (McNestry, Killeen, Crowley, & McAuliffe, 2023). The underlying factors leading to the emergence of pregnancy complications are divers. Emerging data build on pregnancy cohorts and animal studies suggest that maternal-fetal human leukocyte antigen (HLA) incompatibility, intra- or extra-uterine infections, microbial dysbiosis, and in particular aberrant immune system function may play a pivotal role in the onset of pregnancy complications (Jehan et al., 2020; Kumar et al., 2021, 2022; Macintyre et al., 2015; Pansieri, Pandolfini, Clavenna, Choonara, & Bonati, 2020; Piler et al., 2017; Stefańska et al., 2023; van ‘t Hof et al., 2021; Waken, Fuentes, & Rao, 2017). In particular, the pathophysiology of various pregnancy complications involves a strong activation of the maternal immune system. In this regard, pregnant women with pre-eclampsia, a condition defined by hypertension and proteinuria or hypertension accompanied by end organ dysfunction after 20 weeks of gestation, experience exaggerated and sustained activation of the immune system (Boulanger et al., 2024). This is evidenced by an increased number of cytotoxic $CD8^+$ T cells, in addition to Th17 cells, along with augmented levels of pro-inflammatory cytokines and a diminished number of Tregs (Boulanger et al., 2024; Darmochwal-kolarz et al., 2012; K. Morita, Tsuda, Kobayashi, Hamana, & Tsuda, 2020; Perez-Sepulveda, Torres, Khoury, & Illanes, 2014; Saito, Tsuda, & Nakashima, 2023). Furthermore, it has been demonstrated that the occurrence of GDM can have an impact upon both the placental immune system and the neonatal immune system (Atègbo et al., 2006; Mrizak et al., 2014; Yanai et al., 2016). In particular, Mrizak and colleagues discovered enhanced levels of IL-6 and TNF in the serum and elevated mRNA expression of IL-6, TLR4, and TGF-β in the

placental tissue in comparison to women without GDM (Mrizak et al., 2014). Furthermore, Atègbo and colleagues determined augmented serum concentrations of the Th1-associated cytokines, IL-2 and IFNγ, and reduced serum concentration of IL-10 in neonates born to mothers with GDM (Atègbo et al., 2006). Accordingly, it is likely that the dysregulated maternal and neonate immune system due to pregnancy complications may also induce long-term effects in the immune system of children. In this regard, children who have been exposed to preeclampsia showed an increased risk of developing immune-mediated diseases, such as allergies and asthma later in life (Koulouraki et al., 2023; Liu et al., 2015; Pinheiro, Brunetto, Ramos, Bernardi, & Goldani, 2016).

1.2.8 Severe infections and systemic antibiotic use in infancy

Despite the considerable progress of the infant's immune system during the first year of life, its functional capabilities are still limited, resulting in an elevated susceptibility to infections, as previously delineated in sections 1.1.4 (Hill et al., 2020; Kollmann et al., 2009; Raymond, Rincon, Wynn, Moldawer, & Larson, 2017; Raymond, Stortz, et al., 2017). In particular, within the first 28 days after birth, neonates exhibit an elevated risk of experiencing severe infections when exposed to pathogens (Bergin et al., 2015; Stoll et al., 2011; World Health Organization, 2024a). Neonatal infections are predominantly of bacterial origin, encompassing conditions such as sepsis, pneumonia and meningitis (Bergin et al., 2015; Stoll et al., 2011; World Health Organization, 2024a, 2024c). The most frequent pathogens involved in neonatal bacterial sepsis are the Gram-negative bacteria *Escherichia coli* (*E. coli*) and the Gram-positive bacteria *Streptococcus agalactiae*, also known as group B *Streptococcus* (Stoll et al., 2011; Witt, Greenfield, & Knoop, 2024). *E. coli* has the potential to translocate from the neonatal gastrointestinal tract due to reduced intestinal epithelial barrier defense mechanisms and subsequently disseminate systemically in the neonatal body (S. Basu, 2015; Witt et al., 2024). Conversely, Group B *Streptococcus* can be passed on from the mother to the child through vertical transmission during birth (Heath & Jardine, 2014; Witt et al., 2024). Sepsis is a clinical syndrome characterized by systemic inflammation, also known as systemic inflammatory response syndrome (SIRS) (S. Basu, 2015). Following the activation of pathogen recognition receptors (PPRs), such as TLR2 for Gram-positive bacteria or TLR4 for Gram-negative bacteria, neonates produce increased levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, IL-18, IFNγ, and tumor necrosis factor TNF, during sepsis (P. C. Ng, 2004; Wynn & Wong, 2017). These cytokines, up-regulate the expression of cell adhesion molecules by endothelial cells, which in turn promotes leukocyte recruitment (Celik, Hanna, Canpolat, & Pammi, 2022; Cornell, Wynn, Shanley, Wheeler, & Wong, 2010; Figueras-Aloy et al., 2007). Furthermore, Paneth cells and intestinal lymphoid cells have been shown to produce IL-17, a crucial factor in the development of SIRS (Celik et al., 2022; Deshmukh et al., 2014; Takahashi et al., 2008). Nevertheless, neonates encounter challenges in overcoming the infection due to

deficiencies in multiple innate and adaptive immunological defense mechanisms. These include a reduced expression of complement proteins, antimicrobial peptides, and adhesion molecules, impaired neutrophil function, a Th2-dominated response over Th1, and reduced antibody responses compared to those of adults (Buhrer, Graulich, Stibenz, Dudenhausen, & Obladen, 1994; Celik et al., 2022; Kollmann, Kampmann, Mazmanian, Marchant, & Levy, 2017; Raymond, Stortz, et al., 2017). Following the resolution of severe infections, infants are at an increased risk for recurrent infections. In the context of severe sepsis, the immune system of the child can undergo a transition to a persistent anti-inflammatory immune state, also termed immunoparalysis (Frazier & Hall, 2008; Schrijver et al., 2023; Wynn & Wong, 2017). This state encompasses a reduced MHC-II expression and TNF production (Hotchkiss et al., 2001; Wynn & Wong, 2017). In addition, neonates and infants experience an increased apoptosis of $CD4^+$ T and B cells, which results in a compromised generation of pathogen-specific memory T cells (Felmet, Hall, Clark, Jaffe, & Carcillo, 2005; Glavina-Durdov et al., 2003; Hotchkiss et al., 2001; Welsh, Bahl, & Wang, 2004; Zens, Connors, & Farber, 2017). Infections during the neonatal and infant periods may also have long-term consequences on the child's health. Data from epidemiological studies suggest that infections in infancy predispose children to a range of disorders associated with abnormal immunity in childhood, including wheezing, asthma, type 1 diabetes and functional abdominal pain disorders (FAPDs; Bønnelykke, Vissing, Sevelsted, Johnston, & Bisgaard, 2015; Illi et al., 2001; Kordonouri et al., 2022; Kusel et al., 2007; Mercy PrabhuDas et al., 2011; Restori, Srinivasa, Ward, & Fixman, 2018).

The administration of antibiotics is critical for the treatment of bacterial infections (Aminov, Otto, & Sommer, 2010; Gould, 2016; Shekhar & Petersen, 2020). Antibiotics are among the most frequently prescribed medications for infants and children globally (Adriaenssens et al., 2011; Chai et al., 2012; Clavenna & Bonati, 2011; Gerber et al., 2010; Meesters, Chappell, & Demirjian, 2024; Miyara et al., 2009; Stam et al., 2012; Versporten et al., 2016). In Germany, it is estimated that approximately one-third of children receive at least one course of systemic antibiotics during their first year of life (Stam et al., 2012). In addition to their benefits, antibiotics have also been demonstrated to exert negative collateral effect on the host microbiota, inducing microbiota perturbations or dysbiosis (Shekhar & Petersen, 2020). In infants, these effects are more pronounced than in adults, given that microbial diversity is still very low and therefore more susceptible to disruptions (Roswall, Olsson, & Kovatcheva-datchary, 2021; Stewart et al., 2018; Wernroth et al., 2022). It is only by the end of the first 3 to 5 years of life that the microbiota reaches an adult-like state (Roswall et al., 2021). Moreover, recent reports have indicated that antibiotic-induced alterations in the infant microbiota can result in a long-term reduction in microbiota diversity (Fallani et al., 2010; Kabat, Srinivasan, & Maloy, 2014; Kozyrskyj, Bahreinian, & Azad, 2011; Mangin, Suau, Gotteland, Brunser, & Pochart, 2010;

Shekhar & Petersen, 2020; Stam et al., 2012; Vangay, Ward, Gerber, & Knights, 2015). Commensal gut bacteria fulfill crucial functions in the host organism. In addition to the absorption and synthesis of various nutrients and metabolites, they play a crucial role in promoting and regulating host immunity (Brestoff & Artis, 2013; Shekhar & Petersen, 2020; S. Wang et al., 2018b). For instance, specific bacterial antigens and metabolites, such as *segmented filamentous bacteria* (*SFB*) protein P3340 and short-chain fatty acids (SCFAs), have been shown to promote the differentiation of homeostatic Th17 cells and IL-10-secreting Tregs, thereby supporting the maintenance of gut homeostasis (C. H. Kim, 2023; Ney et al., 2023; J. Park et al., 2015; Y. Wang et al., 2019; Y. Yang et al., 2014). In contrast, microbial dysbiosis is associated with an altered induction of $CD4^+$ T cells (Yuan Li et al., 2021; López et al., 2016; Paik et al., 2022; C. Y. Sun, Yang, Zheng, Liu, & Xu, 2023). Studies in mice have reported that offspring treated with antibiotics shortly after birth exhibited altered T cell frequencies, such as a diminished frequency of intestinal Tregs (Atarashi et al., 2011; Ozkul et al., 2020; V. E. Ruiz et al., 2017; S. L. Russell et al., 2012). Furthermore, systemic antibiotic use and microbial dysbiosis are associated with an altered immune response to vaccines and an increased risk of developing chronic inflammatory disorders, including asthma, allergies, and IBD (Arrieta et al., 2015; Brestoff & Artis, 2013; Fouhy et al., 2012; Korpela et al., 2016; Penders, Kummeling, & Thijs, 2011; Shekhar & Petersen, 2020; Tanaka, Narazaki, & Kishimoto, 2014; S. Wang et al., 2018a).

1.2.9 Effects of exposure to multiple stressors on the immune system of children

Considering the effects of stressors, a single potent stressor might have the capacity to adversely alter the child's immune system at a critically sensitive developmental period (V. X. Han, Patel, Jones, & Dale, 2021). However, a growing body of evidence suggests that the emergence of developmental alterations may be caused following the exposure to two or more stressors (Feigenson, Kusnecov, & Silverstein, 2014; Hsueh et al., 2018; Korzeniewski et al., 2014; Leviton et al., 2013; Shimizu et al., 2021; Verstraeten, McCreary, Weyers, Metz, & Olson, 2019). This "*two-hit*" model, which is also referred to as the "*two-hit hypothesis*" or "*multiple-hit*" model, is based on the hypothesis that exposure to an initial stressor primes for alterations and then subsequent exposure to a second or more stressors ultimately triggers the actual onset of a phenotypic alteration or disease (Bayer, Falkai, & Maier, 1999; Gundacker et al., 2023; X. Li, Zhang, Pan, Xu, & Sun, 2017). In this context, MIA has been put forth as an underlying prenatal mechanism and disease primer (V. X. Han, Patel, Jones, & Dale, 2021; Shimizu et al., 2023). A study conducted by Shimizu and colleagues (2021) demonstrated that murine offspring exposed to poly(I:C) both prenatally and postnatally exhibited significantly elevated serum levels of IL-6, IL-17, and TNF in comparison to offspring exposed solely to poly(I:C) during either the prenatal or postnatal period (Shimizu et al., 2023, 2021). Similarly, a study by Hsueh and colleagues, published in 2018, examined the immune response in

offspring exposed to LPS-induced MIA and a second LPS stimulus in the postnatal period (Hsueh et al., 2018). Offspring exposed to LPS during the gestational and postnatal period had markedly elevated concentrations of IL-1β, IL-6, IL-10, IL-12, IL-17, TNF and IFNγ in their serum compared to offspring only exposed to LPS postnatally (Hsueh et al., 2018). Consequently, these observed elevated serum levels of predominantly pro-inflammatory cytokines suggest an altered immune status in the offspring. In the field of neuroimmunology, the two-hit model has been the subject of considerable research in relation to the etiology of neurological disorders. For instance, a study by Leviton *et al*. in 2013 discovered an association of small for gestational age and postnatal systemic inflammation with the development of brain damage in preterm newborns (Leviton et al., 2013). Other studies showed that double hit of inflammation prenatal and later in life, also in combination with postnatal hypoxia, can lead to the onset of neurological disorders like schizophrenia and autism-like behavior (Feigenson et al., 2014; van Tilborg et al., 2018). Nevertheless, there is still a paucity of animal and, in particular, human studies examining the association between exposure to two or multiple stressors and developmental alterations in the immune system or the onset of immune mediated diseases in offspring.

1.2.10 Consequences of exposure to early-life stressors on children's health

The potential consequences of exposure to a single or multiple early-life stressors as well as subsequent alterations in the $CD4^+$ T cell immunity may be far-reaching, potentially leading to the manifestation of immune-mediated diseases. As pathogens primarily gain entry into the body at mucosal sites, such as the gastrointestinal tract or respiratory system, the abundant presence of immune cells, especially $CD4^+$ T cells, at these sites is imperative for effective defense against pathogens (X. Zhou et al., 2025). In addition to their role in host defense, $CD4^+$ T cells fulfill an essential function in the maintenance of tissue homeostasis within these tissues (D'Alessio et al., 2019; Oja et al., 2018; Zaiss et al., 2013; Zeng et al., 2012). It is therefore likely that stressor-induced dysregulation of $CD4^+$ T cells may be particularly detrimental to tissue homeostasis and may trigger the onset of immune-mediated diseases in the respiratory or gastrointestinal tracts, such as asthma or FAPDs.

A multitude of early-life stressors have been associated with the development of asthma, including maternal chronic diseases, maternal obesity, maternal psychological stress, maternal infections, pregnancy complications, and medication use during pregnancy, as well as systemic antibiotic use in the first year of life, as previously mentioned in sections 1.2.1 to 1.2.8 (Castro-Rodriguez et al., 2020; Collier et al., 2013; Illi et al., 2001; Litonjua et al., 1998; Liu et al., 2015; Pape et al., 2020; Penders et al., 2011). Asthma is a chronic inflammatory disease of the lungs that is characterized by symptoms such as shortness of breath, chest tightness, coughing, and wheezing during exhalation (Hammad & Lambrecht, 2021). Dysregulated Th2-cell-induced immune responses are considered the predominant contributor to chronic

inflammation, and the development and progression of allergic asthma, which often manifests during childhood (Deo et al., 2010; Hammad & Lambrecht, 2021; L. Sun et al., 2023; Walker & McKenzie, 2018). Specifically, elevated production of the Th2-related cytokines IL-4, IL-5, IL-9, IL-10 and IL-13, has been observed to trigger several immune responses within the lungs (Boonpiyathad, Sözener, Satitsuksanoa, & Akdis, 2019; Hammad & Lambrecht, 2021; Russkamp et al., 2019). These responses include heightened eosinophil recruitment and accumulation in the airway wall, augmented mucus production, and increased IgE synthesis by allergen-specific B cells (Boonpiyathad et al., 2019; Hammad & Lambrecht, 2021; Heeringa, 2018; Ohtomo et al., 2010). Furthermore, emerging data also indicate that Th17 cell/Tregs imbalance with an increased prevalence of Th17 cells and elevated IL-17 levels, are also strongly implicated in the induction and progression of asthma in childhood (Hu et al., 2020; R. Zheng et al., 2021).

FAPDs are among the most prevalent pediatric ailments, affecting up to 25% of children globally (Gieteling, Lisman-van Leeuwen, van der Wouden, Schellevis, & Berger, 2011; Krause, Sarganas, Thamm, & Neuhauser, 2019; Robin et al., 2018; Thapar et al., 2020). According to the Rome IV criteria, an international symptom-based guideline for the diagnosis of functional gastrointestinal disorders (FGID), pediatric FAPDs are divided into several clinically distinct categories, namely irritable bowel syndrome (IBS), functional dyspepsia, abdominal migraine, and functional abdominal pain not otherwise specified (FAP-NOS) (Hyams et al., 2016; Thapar et al., 2020). Symptoms of FADPs include recurrent episodes of abdominal pain that, depending on the subtype, may be accompanied by other symptoms such as headache, nausea, abnormal bowel movements, altered defecation and postprandial fullness (Hyams et al., 2016; Thapar et al., 2020). Epidemiological studies identified a number of postnatal early-life stressors that increase the risk of FAPDs development later in life, such as bacterial gastrointestinal infections, antibiotic use and immune-mediated conditions like Henoch-Schönlein purpura (Cremon et al., 2014; Saps, Dhroove, & Chogle, 2011; Thabane et al., 2010; Thapar et al., 2020; Uusijärvi et al., 2014). Emerging data indicate that the disruption of the microbiota–gut–brain axis plays a crucial part in the etiopathogenesis and pathophysiology of FAPDs (Thapar et al., 2020). The microbiota–gut–brain axis is a bidirectional communication system between the gut microbiota, the gut and the central nervous system that involves a network of immunological, neuronal and endocrine signaling pathways (Ahmed et al., 2022; Thapar et al., 2020). Growing evidence indicates that microbial dysbiosis and inflammation, induced by antibiotics, food or infections, may promote dysregulated immune responses of innate and adaptive immune cells, including mast cells and $CD4^+$ T cells, and may alter the physiology and functionality of the enteric nervous system (Yuan Li et al., 2021; López et al., 2016; Major et al., 2017; Miquel et al., 2013; Paik et al., 2022; C. Y. Sun et al., 2023; J. Yang et al., 2014; S. Y. Zhou et al., 2018). This, in turn, may

cause visceral hypersensitivity and central hypervigilance and the onset of abdominal pain (Di Lorenzo et al., 2001; Thapar et al., 2020).

2 Hypothesis and Aims

The development of the human immune system undergoes dynamic changes early in life (Acevedo et al., 2015, 2021; Olin et al., 2022; J. E. Park, Jardine, et al., 2020). Emerging data suggest that the gestational period and the first year after birth together represent a critical window of increased vulnerability to stressors for the developing child and its immune system (Barker, 2007; Fouhy et al., 2019; Olin et al., 2022). In particular, recent studies in humans and mice have reported that the occurrence of early-life stressors are associated with an altered immunity in offspring (Ye Li et al., 2023; V. E. Ruiz et al., 2017; Shimizu et al., 2023, 2021). In adition, findings build on data from murine models have suggested that $CD4^+$ T cells in the offspring are affected by stressors (Hsiao, McBride, Chow, Mazmanian, & Patterson, 2012; Mandal, Marzouk, Donnelly, & Ponzio, 2010). Moreover, epidemiological studies of pregnant women and their children have indicated a link between exposure to stressors and an increased risk for immune-mediated diseases in offspring later in life (Collier et al., 2013; Fuchs & von Mutius, 2013; McKeever et al., 2002; Olin et al., 2018; Yue et al., 2018). Nevertheless, there is still a paucity of longitudinal human studies examining the potential long-term consequences of early-life stressors on $CD4^+$ T cell immunity and health in children beyond infancy.

Therefore, I hypothesize that exposure to stressors affects the development of fetal and infant $CD4^+$ T cells during the early phase of life, resulting in long-term alterations of $CD4^+$ T cells, including aberrant frequencies and dysregulated immune responses, in childhood. In addition, MIA serves as a stressor-responsive mechanism that induces modifications to fetal $CD4^+$ T cells during the gestational period.

To investigate this hypothesis, the following aims have been set out in this thesis:

1. Determine the association between early-life stressors and the frequencies of $CD4^+$ T cell subpopulations of children at 5 years of age based on the flow cytometric and demographic data from a prospective, longitudinal cohort of mothers and their children.
2. Assess the functionality of $CD4^+$ T cells from 5-year-old children exposed to stressors by determining the cytokine production upon TCR-specific stimulation.
3. Examine the immune profile in prenatal serum of mothers of children exposed to early-life stressor to assess MIA.

3 Materials and Methods

3.1 Materials

3.1.1 Flow cytometry antibodies

Antibody	Fluorochrome	Clone	Manufacturer	Catalogue No.	Dilution
CCR7	BV510	G043H7	Biolegend, Inc., San Diego, California, USA	353232	1:50
CD14	Spark Blue 550	63D3	Biolegend, Inc., San Diego, California, USA	367148	1:400
CD19	Spark NIR 685	HIB19	Biolegend, Inc., San Diego, California, USA	302270	1:400
CD4	BUV737	SK3	BD (Becton, Dickinson & Company), Franklin Lakes, New Jersey, USA	621748	1:50
CD45RA	PB/BV421	HI100	BD (Becton, Dickinson & Company), Franklin Lakes, New Jersey, USA	562885	1:50
CD8a	Qdot800/BV785	RPA-T8	Biolegend, Inc., San Diego, California, USA	301046	1:250
FOXP3	APC	PCH101	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	17-4776-42	1:50
IFNγ	BUV805	4S.B3	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	368-7319-41	1:50
IL-17A	BV711	BL168	Biolegend, Inc., San Diego, California, USA	512328	1:40
IL-22	PE	22URTI	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	12-7229-42	1:40
IL-4	Qdot605/BV605	MP4-25D2	Biolegend, Inc., San Diego, California, USA	500828	1:40
IL-6	AF700	MQ2-13A5	eBioscience/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	56-7069-42	1:25
IL-6Ra	PE-Cy7	UV4	Biolegend, Inc., San Diego, California, USA	352810	1:50
IL-8	PerCP-eFluor 710	8CH	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	46-8088-42	1:40
TNF	BV650	MAb11	Biolegend, Inc., San Diego, California, USA	502938	1:100
TRα1/β1 (TRα2,TRβ2)	Alexa Fluor 790	C4	Santa Cruz Biotechnology	sc-740 Af790	1:50

3.1.2 Buffer media and solutions

Buffer/medium	Manufacturer	Catalogue No.
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma Aldrich, St. Louis, Missouri, USA	D8537-500ml
RPMI-1640 medium	Gibco by Life Technologies/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	21875-034-500ml

3.1.3 Media reagents

Medium reagent	Manufacturer	Catalogue No.
Fetal bovine serum (FBS)	Capricorn scientific	FBS-11A
GlutaMAX™ (100X)	Gibco by Life Technologies/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	35050-061
HEPES Buffer Solution (1M)	Gibco by Life Technologies/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	15630-056
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA; 100X)	Gibco by Life Technologies/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	11140-035
Penicillin-Streptomycin	Sigma Aldrich, St. Louis, Missouri, USA	P4333-100ml
Sodium pyruvate solution	Sigma Aldrich, St. Louis, Missouri, USA	S8636-100ML

3.1.4 Cultivation medium and supplements

Solution	Media	Supplement
R10	RPMI – 1640 (1X)	10 % (v/v) FBS 1% GlutaMAX 1% MEM NEAA 1% Penicillin-Streptomycin 1% Sodium pyruvate solution
FACS buffer	DPBS	2% FBS 0.05% NaN_3

3.1.5 Cell culture stimulants

Reagent	Working concentration	Duration	Manufacturer	Catalogue No.
Brefeldin A solution (1.000X)	7 µg/ml	17.5 hours	Sigma Aldrich, St. Louis, Missouri, USA	B7651-5MG
CD28	2 µg/ml	18 hours	Sanquin. Amsterdam, Netherlands	M1650
CD3	2 µg/ml	18 hours	Sanquin. Amsterdam, Netherlands	M1654

Ionomycin calcium salt	1 µg/ml	18 hours	Sigma Aldrich, St. Louis, Missouri, USA	I0634-1MG
Phorbol 12-myristate 12-acetate (PMA)	0.05 µg/ml	18 hours	Sigma Aldrich, St. Louis, Missouri, USA	P1585-1MG

3.1.6 Chemicals and consumables

Reagent	**Manufacturer**	**Catalogue No.**
LIVE/DEAD Fixable Blue Dead Cell Stain Kit	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	L23105
Brilliant stain buffer	BD, Franklin Lakes, New Jersey, USA	566349
eBioscience™ Fixation/ Perm diluent	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	00-5223-56
Fixation/Permeabilization Concentrate	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	00-5123-43
Human True Stain Fcx	Biolegend, Inc., San Diego,	422302
Sodium azide (NaN3)	Sigma Aldrich, St. Louis, Missouri, USA	71289-5G
Permeabilization Buffer 10X	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	00-8333-56
Trypan blue solution, 0.4 %	Sigma Aldrich, St. Louis, Missouri, USA	T8154-100ml
Trypsin-EDTA solution	Sigma Aldrich	T3924-100ML
UltraComp eBeads Plus Compensation Beads	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	01-3333-42

3.1.7 Preparation kits

Kit name	**Manufacturer**	**Catalogue No.**
Luminex Human Discovery Assay (12-Plex) Human Premixed Multi-Analyte Kit (customized)	Sigma Aldrich, St. Louis, Missouri, USA	LXSAHM-12
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen/ Thermo Fisher Scientific, Waltham, Massachusetts, USA	00-5523-00

3.1.8 Equipment

Equipment	**Manufacturer**
Biological safety cabinet HERASAFE KS 12	Biological safety cabinet KS 12 Thermo Fisher Scientific, Waltham, Massachusetts, USA
Centrifuge Heraeus Fresco 21	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Centrifuge Megastar 1.6R	VWR International, Radnor, Pennsylvania, USA

CO2-Incubator Heracell vios 160i	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Cytek Aurora 5 Laser Spectral Cytometer	Cytek Biosciences, Fremont, California, USA
Eppendorf Research Plus Pipette	Eppendorf, Hamburg, Germany
GFL Gesellschaft Fuer Labortec™ Stainless Steel Waterbath	GFL/ LAUDA Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany
Grant Instruments™ Multi-Spin PVC 6000 Centrifuge/Vortex Mixer	Grant Instruments, Shepreth, Cambridgeshire, United Kingdom
Multichannel pipetter, 12-channels, 30-300 µl	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Pipet Boy	INTEGRA Biosciences AG, Zizers, Switzerland
TC20 Automated cell counter	Bio-Rad Laboratories, Inc., Hercules, California, USA

3.1.9 Plasticware

Product	Manufacturer	Catalogue No.
15 ml tubes	Greiner Holding, Kremsmünster, Austria	188271
50 ml tubes	Greiner Holding, Kremsmünster, Austria	227261
96-well Polypropylene Cluster Tubes	Corning, Corning, New York, USA	4401
ClipTip™ Filtered Pipette Tips 300 µl	Thermo Fisher Scientific, Waltham, Massachusetts, USA	13296269
Counting slides	Bio-Rad Laboratories, Inc., Hercules, California, USA	145.0011
FACS tubes	Sarstedt, Nümbrecht/Rommelsdorf, Germany	55.1579
Filter tip 10 µl	Sarstedt, Nümbrecht/Rommelsdorf, Germany	70.1130.210
Filter tip 100 µl	Sarstedt, Nümbrecht/Rommelsdorf, Germany	70.760.212
Filter tip 1000 µl	Sarstedt, Nümbrecht/Rommelsdorf, Germany	70.762.211
Micro tube 1.5 ml SafeSeal	Sarstedt, Nümbrecht/Rommelsdorf, Germany	72.706.400
Pipetting reservoirs	VWR International, Radnor, Pennsylvania, USA	89094-680
Serological pipettes 5 ml	Sarstedt, Nümbrecht/Rommelsdorf, Germany	86.1253.001
Serological pipettes 10 ml	Sarstedt, Nümbrecht/Rommelsdorf, Germany	86.1254.001
Serological pipettes 25 ml	Sarstedt, Nümbrecht/Rommelsdorf, Germany	86.1685.001

TC plate 96 Well, Standard, R	Sarstedt, Nümbrecht/Rommelsdorf, Germany	83.3925

3.1.10 Software

Software	Manufacturer
BioRender	BioRender, Toronto, Ontario, Canada
FlowJo_v10.8.1	BD, Franklin Lakes, New Jersey, USA
GraphPad Prism, Version 10	GraphPad Software Inc., California, USA
Mendeley Desktop, version 1.19.8	Mendeley Desktop, version 1.19.1, London, United Kingdom
Microsoft Office 2016	Microsoft, Redmond, Washington, USA
SpectroFlo	Cytek Biosciences, Fremont, California, USA
Stata/MP 18 for Windows	StataCorp LLC, Texas, USA

3.2 Methods

3.2.1 Study population

The influence of early-life stressors on $CD4^+$ T cells in children was examined using the demographic and flow cytometric data from blood-derived lymphocytes of children at 5 years of age who participated in the Prenatal Identification of Children's Health (PRINCE) study. The PRINCE study is a prospective longitudinal pregnancy cohort study initiated in 2011 at the Department of Obstetrics and Prenatal Medicine of the University Medical Centre Hamburg-Eppendorf (UKE), Germany. The study included women with a maternal age of 18 or above and a viable singleton pregnancy of 12-14 weeks of gestation. Women were excluded from the study if they had infections, such as Human immunodeficiency virus (HIV) or hepatitis B/C, a history of drug or alcohol abuse, or if they had multiple pregnancies or pregnancies conceived through assisted reproductive technologies (ART). At the end of each trimester and annually after birth, in the month of birth, data was documented on the anthropometry and health status of the mother and child. To date (April 2024), the PRINCE study involves 749 study participants. At the time of the analysis (May 2021), 233 children participated in the 5-year examination of the PRINCE study. Out of these 233 children, the first 118 participating children were examined for flow cytometry and could be included in the presented study here. The subsequent analysis included the flow cytometric data from blood-derived lymphocytes taken at the age of 5, as well as the demographic and anthropometric data of the 118 children and their mothers. Moreover, peripheral blood mononuclear cells (PBMCs) from 19 children

enrolled in the PRINCE study, aged 5 years, along with the serum samples of their mothers in the third trimester, were subjected to cytokine measurement analyses (see sections 3.2.7.1 and 3.2.8.1). An overview of the study population is presented in Figure 5.

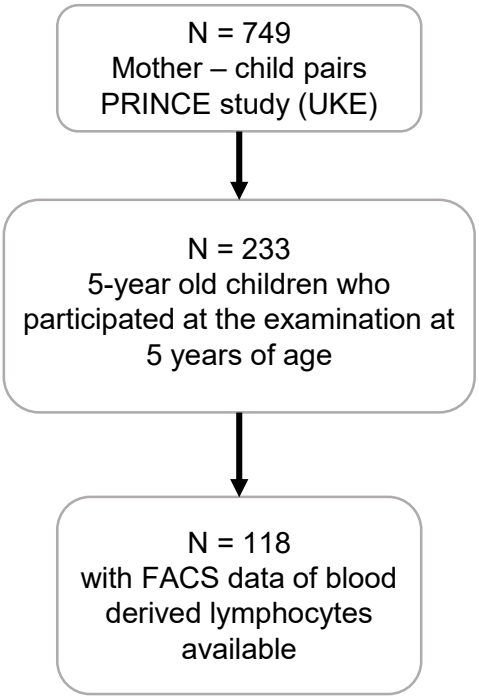


Figure 5: Study population. Abbreviations used: UKE, University Medical Centre Hamburg-Eppendorf, Germany; FACS, flow cytometric.

3.2.2 Ethical approval

The PRINCE study protocol was approved by the ethics committee of the Hamburg Chamber of Physicians under the registration number PV3694 and was conducted in accordance with the Declaration of Helsinki for Medical Research involving Human Subjects. All study participants have signed the informed consent forms.

The Healthy Cohort Hansestadt Hamburg (HCHH) was approved by the ethics committee of the Medical Association of Freie Hansestadt Hamburg (Ärztekammer Hamburg). All study participants have provided written informed consent.

3.2.3 Assessment of early-life stressors

The PRINCE study employed a series of standardized questionnaires to collect and assess a range of maternal demographic characteristics, anthropometric data, and health status indicators, including psychometric measures, at each trimester. In a comparable manner, the children underwent an annual examination after birth to gather information pertaining to their anthropometric characteristics as well as their general health status.

In this study, the following stressors were selected for the present analyses based on literature: maternal chronic diseases, maternal obesity (BMI ≥ 30) at first trimester (GW 12-14), maternal stress (Perceived Stress Scale (PSS-14) score >26) at first trimester (GW 12-14), second trimester (GW 24), and third trimester (GW 34-36), maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and systemic antibiotic use in the infant's first year of life. Maternal smoking during pregnancy was not included as a stressor due to the small number of women who reported smoking among the 118 participants in this study (n = 9; data not shown). Besides, preliminary linear regression models assessing the association between maternal smoking and $CD4^+$ T cells of the 5-year old children indicated no statistical association (data not shown). For each of the aforementioned stressors, a binary categorical variable was calculated based on whether the stressor occurred during pregnancy (Yes/No) or in terms of infant antibiotic use, in the first year of life (Yes/No), respectively. The stressor variables were used in the statistical analysis of this study, as detailed below in section 3.2.6.

3.2.3.1 Assessment of maternal chronic diseases

At the initial study visit, which occurred during the first trimester (GW 12-14), pregnant women were queried regarding the presence and type of chronic diseases. All types of chronic diseases that were reported by the women were included in the analysis. Based on this information, the binary categorical variable “chronic diseases” was generated, encompassing all women who reported the presence and type of a chronic disease (Yes) and all women who stated that they did not have a chronic disease (No). Women who did not answer the question regarding chronic diseases were not included in the variable and treated as missing data.

3.2.3.2 Assessment of maternal obesity

The BMI was calculated from the mothers' measured height and weight at the end of the first trimester (GW 12-14). Maternal obesity was defined according to the World Health Organization (WHO) as the presence of a BMI value equal to or greater than 30 kg/m² (BMI ≥ 30; WHO, 2000). Given the inability to record the BMI of women prior to pregnancy, the first trimester represents the earliest possible time to record existing maternal obesity. All women with a BMI of less than 30 kg/m² (BMI < 30) were classified as non-obese for subsequent analysis.

3.2.3.3 Assessment of maternal perceived stress

In the PRINCE study, maternal perceived stress was measured using a German version of the Perceived Stress Scale (PSS)-14 at each trimester (GW 12-14, GW 24, and GW 34-36; Diemert et al., 2017). The PSS-14 is a 14-item questionnaire, developed in 1983 by Cohen, Kamarck, and Mermelstein, which enables participants to appraise their stress perception. Each item is scored from 0 to 4. A total score is calculated by reversing the scores on the

seven positively stated items (0=4, 1=3, 2=2, 3=1, 4=0) and then totaling all the scores for the 14 items (Cohen et al., 1983). Consequently, the total score ranges from 0 to 56, with higher values indicating a greater level of self-appraised stress. It is important to note that the PSS-14 questionnaire only assesses the participant's subjective perception of stress and not the actual level of stress experienced. For the present analyses, a categorical variable was computed from the original continuous PSS-14 score variable in order to investigate the influence of high self-perceived stress. In the literature, there is no defined threshold characterizing a PSS-14 score as high stress. In line with Polinski *et al.* (2020), perceived stress in the present study was classified into two distinct categories: low to moderate perceived stress (PSS-14 score 0–26) and high perceived stress (PSS-14 score >26). For each trimester, a categorical variable was generated to assess high perceived stress during pregnancy.

3.2.3.4 Assessment of maternal medication use

As described in Bremer *et al.*, (2017), the medication intake of each pregnant women included in the PRINCE study was determined at each trimester (GW 12-14, GW 24, and GW 34-36) through a questionnaire-assisted interview conducted by the study gynecologists. At each study visit, women were requested to provide detailed information on the medications they had been taking since the beginning of their pregnancy or since the previous study visit (Bremer et al., 2017). However, not all women provided information on their medication use for each trimester. Therefore, medication use was summarized for the entire pregnancy in this study. For the present analyses, a categorical variable was generated, whereby the participants were divided into two groups: those who took medication during their pregnancy (Yes) and those who reported not taking medication during pregnancy (No). The group of women who took medication during pregnancy also includes women who took different types of medication during pregnancy. As only a limited number of participants provided data on the duration and dosage of the medication, this information was not included in the subsequent analysis. Regarding the type of medication, all reported medications were included in the analysis, with the exception of topical medications, natural remedies and analgesics. In the initial stages of this study, analgesics were assessed separately from the other medications. A previous study by Bremer *et al.*, (2017) using the PRINCE dataset indicated an association of Paracetamol intake during the third trimester with a reduced relative numbers of hematopoietic stem cells in cord blood. In this study, preliminary linear regression analyses revealed no association with maternal analgesic intake and $CD4^+$ T cell subsets of 5-year old children. Therefore, women who only took analgesics during their pregnancy were not included in the analyses. The types of medication women reported taking during pregnancy and included in this study belong to the following categories: antibiotics, anticoagulants, antihypertensives, diabetes medications,

thyroid medications, antiallergics, dietary supplements, antiemetics, and proton-pump inhibitors (PPIs).

3.2.3.5 Assessment of maternal infections

At all three study visits during pregnancy (GW 12-14, GW 24, and GW 34-36) and after birth, participants completed questionnaires about infections that had occurred during their pregnancy. The women were asked to provide information on the type of infection (e.g. gastrointestinal infection, etc.) and pathogens, if known. In addition to these questions about general infections, the women were also specifically asked whether they had experienced a cold/flu infection (with cough, cold, fever, joint pain) since the last survey or beginning of the pregnancy, respectively. It is generally assumed that in many cases women have received a diagnosis for their infection if they have consulted a physician during the course of their infection. The PRINCE study itself did not perform any polymerase chain reaction (PCR) or other microbial tests to confirm the infection and the pathogen, nor does the study have any information on whether these tests were performed during any visits to the participants' physicians. Regarding the severity of infection, none of the 118 women included in this study had to be hospitalized due to an infection during their pregnancy (data not shown). Other possible indicators for categorizing the severity of an infection, such as the type of pathogen, symptoms during the infection, medication used to treat the infection and the duration of the infection, were only partially reported or not reported at all by the women. Consequently, the severity of each individual reported infection could not be clearly determined and was therefore not included in this study. For the present analyses of this study, women were divided into two groups: women who reported having an infection, independently of the type and number of infection during pregnancy (Yes), and women who reported not having any infections during pregnancy (No). Based on this, the binary category variable “maternal infections” (Yes/No) was computed.

3.2.3.6 Assessment of pregnancy complications

Information on the occurrence of pregnancy complications (pregnancy-related hypertension, preeclampsia, HELLP- syndrome, gestational diabetes, preterm labor, miscarriage, preterm birth, etc.) were obtained from a pre-stamped postcard, which was given to the pregnant women at the second study visit (as described in Diemert et al., 2017). Infections during pregnancy could also be marked as a pregnancy complication. However, as there was no precise information on the severity of these reported infections and the resulting complications for the mother and the fetus/unborn child, these reported infections were excluded from the list of pregnancy complications and included in the “maternal infection” variable as described in section 2.3.5. Apart from infections, all reported pregnancy complications were included in the analyses of this study. Based on this, a categorical variable was generated which categorizes

women into two groups: women who reported that they had experienced a complication during pregnancy (Yes) and women who reported not having a complication during pregnancy (No). Women who did not provide information on whether they experienced a pregnancy complication were not included in the analyses and treated as missing data.

3.2.3.7 Assessment of systemic infant antibiotic use within the first year of life

One year after birth, mothers were asked to provide information about whether their child had received systemic antibiotics in the first year of life. Based on this question, which could be answered with either "Yes" or "No", a categorical variable was created for subsequent analyses. Accordingly, the variable categorized children in two groups: those who received systemic antibiotic during their first year of life (Yes) and those children with mothers reporting that they had not received antibiotics during their first year of life (No). Furthermore, information on the type of antibiotic, the number of different antibiotics used, the intake duration and the dosage of the antibiotics was only provided in a few cases and was therefore not included in the present analyses.

3.2.4 Assessment of abdominal pain, nausea or headaches in children at 5 years of age

During the annual PRINCE examination, mothers were asked to provide information about whether their 5-year-old child had frequently complained about abdominal pain, nausea or headaches (abdominal symptoms/headaches) within the last 6 months. Mothers were given the opportunity to respond to this statement with “not applicable”, “partially applicable” or “clearly applicable”. Based on this information, a binary categorical variable was computed, dividing children in two categories: 5-year-old children who “partially” and “clearly” complained about abdominal symptoms/headaches (Yes), and 5-year-old children who did not complained about abdominal symptoms/headaches (No). Children of mothers who did not provide information on whether their child experienced abdominal symptoms/headaches were not included in the analyses and treated as missing data. For the present analyses, the variable was used to assess the association between the reported abdominal symptoms/headaches, exposure to stressors, and the frequencies of $CD4^+$ T cell groups in 5-year-old children (see section 3.2.6).

3.2.5 Determination of $CD4^+$ T cell subsets in flow cytometric data from blood-derived lymphocytes of children at 5 years of age included in the PRINCE study

In order to assess the association between early-life stressors and T cell subsets of children at the age of 5 years, the subsequent step was to determine the frequencies of $CD4^+$ T cell subpopulations in the children's flow cytometric data from blood-derived lymphocytes.

As part of the annual examination, blood samples were obtained from the 5-year-old participants in the PRINCE study at the UKE. The blood samples were subsequently processed and flow cytometry analyses (FACS) were performed in the Department of Obstetrics and Prenatal Medicine in collaboration with the research group "Immune regulation" of Prof. Eva Tolosa (UKE). In brief, 50 µL of fresh ethylenediaminetetraacetic acid (EDTA) blood was combined with 50 µL of the antibody-staining-mixture. All anti-human antibodies and conjugated fluorochromes used for the staining are listed in table 1. Following a 30-minute incubation period, 1 mL lysis buffer (1:10 BD FACS Lysing Solution) were added to the cells for a further 6 minutes. Subsequently, the cells were washed twice with 1 mL of PBS and fixed in FACS buffer containing 1% paraformaldehyde. Data was acquired on a FACS LSR Fortessa flow cytometer using the FACS-Diva software (BD Biosciences).

Table 1: Antibodies and conjugated fluorochromes panel used for flow cytometry analyses to assess peripheral blood lymphocytes in children at 5 years of age included in the PRINCE study.

Antibody	Fluorochrome
CCR10	APC
CCR4	PE-Cy7
CCR6	PerCP-cy5.5
CD127	BV650
CD161	BV605
CD25	BV785
CD3	V500
CD4	AF700
CD45RA	BV711
CRTH2	BV421
CXCR3	FITC
CXCR5	PE-Texas Red
TCRγδ	PE

For this study, the flow cytometric data of the first 118 children who participated in the PRINCE examination at the age of 5 were made available for analysis. All FACS data files were provided anonymized to ensure that the data could not be linked to the study participants. The provided FACS data was analyzed using FlowJo software (version 10.8.1; TriStar/BD). Specifically, a gating strategy developed in collaboration with Prof. Eva Tolosa (Immune Regulation Group, Department of Immunology, UKE) was applied to identify $CD4^+$ T cell subsets based on their chemokine receptor expression (Figure 6). In order to ensure consistency between the samples, the compensation for each FACS sample was initially verified and if needed revised in order to account for any acquisition differences between the samples, such as the device and compensation settings of the day. Figure 6 depicts the representative flow cytometric plots of the gating strategy used in this study. In some samples, a diagonal artefact was observed

when using the marker CCR10-APC against the marker CCR4-PE-Cy7. In order to avoid any potential interference on the subsequent T cell subsets, this diagonal was excluded after gating for $CD3^+$ cells. Subsequently, $CD4^+$ T cells were determined as high expression of CD4 and low expression of TCRγδ within the $CD3^+$ compartment. $CD4^+$ Tregs were determined by high expression of CD25 and low expression of CD127 and excluded from the conventional $CD4^+$ T cells ($CD4^+_{conv}$ T cells; Hartigan-O'Connor, Poon, Sinclair, & McCune, 2007; Seddiki et al., 2006). The expression of the surface proteins CD45RA and CCR4 on $CD25^+CD127^+$ Tregs were used to identify naive Tregs ($CD25^+CCR4^-CD45RA^+$ cells) and activated/effector Tregs ($CD25^+CCR4^+CD45RA^-$ cells; Halim et al., 2017; Miyara et al., 2009). In conventional $CD4^+$ T cells, naïve and memory $CD4^+$ T cells were subsequently identified by the expression of CD45RA and CD127. $CD45RA^{hi}CD127^{hi}$ cells were determined as naïve $CD4^+_{conv}$ T cells (naive $CD4^+CD25^-$ cells) and non-$CD45RA^{hi}CD127^{hi}$ cells as memory $CD4^+_{conv}$ T cells ($CD4^+CD25^-$ memory cells; Dunham et al., 2008; Sallusto, Geginat, & Lanzavecchia, 2004; Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999; Seddiki et al., 2006; Tian et al., 2017). Within the $CD4^+CD25^-$ memory cell compartment, six $CD4^+$ memory T cell subsets were determined based on their chemokine receptor expression. $CD4^+CD25^-$ memory cells expressing CXCR3 and no CCR6 ($CXCR3^+CCR6^-$ cells; Bonecchi et al., 1998; Gosselin et al., 2010; Sallusto, Lenig, Mackay, & Lanzavecchia, 1998; Silveira-Mattos et al., 2019) and $CD4^+CD25^-$ memory cells expressing CRTh2 and high levels of CCR4 ($CRTh2^+CCR4^{hi}$ cells; Bonecchi et al., 1998; Nagata et al., 1999; Sallusto et al., 1998) were identified, and can be classified as Th1-like cells and Th2-like cells, respectively. Furthermore, intermediate and high CXCR5 expressing $CD4^+CD25^-$ memory cells were determined ($CXCR5^{int}$ cells; $CXCR5^{hi}$ cells). Based on their CXCR5 expression, these cells can also be described as intermediate CXCR5 expressing follicular helper CD4+ T (T_{FH})-like cells, and high CXCR5 expressing T_{FH}-like cells (Breitfeld et al., 2000; Schaerli et al., 2000). Moreover, $CD4^+CD25^-$ memory cells expressing CXCR3, CCR4, CCR6, and CD161 ($CCR6^+CCR4^+CD161^+$ cells) and $CD4^+CD25^-$ memory cells expressing CXCR3, CCR4, CCR6, but not CD161 ($CCR6^+CCR4^+CD161^-$ cells) were included in subsequent analyses. Based on their chemokine receptor expression, $CCR6^+CCR4^+CD161^+$ cells can be classified as Th17-like cells (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Cosmi et al., 2008; Kleinschek et al., 2009) and $CCR6^+CCR4^+CD161^-$ cells can be classified as Th22-like cells (Nayrac et al., 2021; Ramirez et al., 2010; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). To identify the $CD4^+$ memory T helper cell subsets, chemokine receptor expression on naive $CD4^+CD25^-$ cells were used as gating control (Figure 6). Following the analysis of the 118 FACS data, a quality control of the determined frequencies of the T cell populations was conducted to re-check any identified outliers. Hereinafter, the exported T cell counts and frequencies were transmitted to the bioinformatician responsible for the PRINCE study. The data was then integrated into the

PRINCE study database and assigned to the respective study participants. Subsequently, the frequencies of the T-cell populations, along with the assigned demographic data of the 118 participants from the PRINCE study, were made available for subsequent analyses.

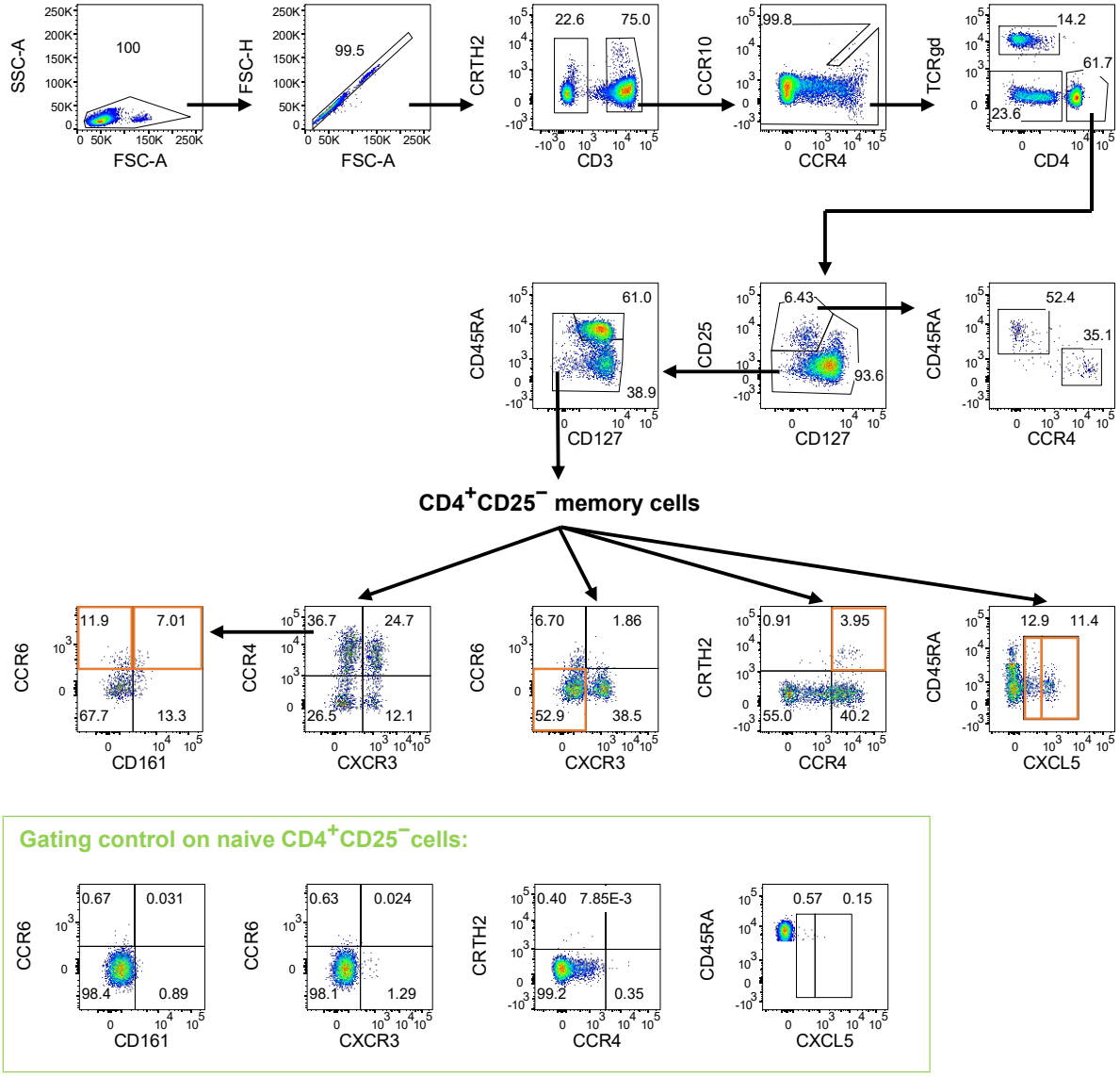


Figure 6: Flow cytometry gating strategy to identify $CD4^+$ T cell subsets in flow cytometric data from blood-derived lymphocytes of children at 5 years of age included in the PRINCE study. $CD4^+$ T cell subsets were determined based on their chemokine receptor expression and include $CD25^+CCR4^-CD45RA^+$ cells, $CD25^+CCR4^+CD45RA^-$ cells, naive $CD4^+CD25^-$ cells and $CD4^+CD25^-$ memory cells. Among the $CD4^+CD25^-$ memory cell compartment, the following $CD4^+$ memory T cell subsets were determined (FLTR): $CCR6^+CCR4^+CD161^-$ cells, $CCR6^+CCR4^+CD161^+$ cells, $CXCR3^+CCR6^-$ cells, $CRTh2^+CCR4^{hi}$ cells, $CXCR5^{int}$ cells and $CXCR5^{hi}$ cells (orange boxes). Figure shows plots of a representative donor.

3.2.6 Statistical analysis: Descriptive statistics and linear regression analyses

All descriptive statistics and linear regression analyses were carried out using Stata/MP 18 (StataCorp LLC, Texas, USA). Descriptive analyses were performed on the anthropometric and demographic characteristics of the 118 mothers and their 5-year-old children who participated in the PRINCE study and were examined in this study. Mean (M), standard

deviations (SD) and range were presented for continuous variables, and total numbers (N) and percentages (%) for categorical variables.

Linear regression models were fitted to examine the associations between the frequencies of $CD4^+$ T cell populations and early-life stressors. Thereby, T cell subsets were assessed as the dependent variables and stressors as the independent variables. All univariable and multivariable linear regression models were adjusted for mode of delivery and sex of the child (Klein & Flanagan, 2016; Schlinzig et al., 2017). Furthermore, linear regression models were only performed if the number of participants for whom the independent variable or variables applied, was $N > 4$. To account for multiple testing, *P*-values of the associations were adjusted by Benjamini-Hochberg's false discovery rate (FDR) correction (Benjamini & Hochberg, 1995). *P*-values < 0.05 were considered statistically significant and *P*-values < 0.15 were considered a statistical trend.

In a first step, univariable linear regression analyses were used to assess the association between the frequencies of $CD4^+$ T cell subsets and each of the following stressors: maternal chronic diseases, maternal obesity (BMI ≥ 30) at first trimester (GW 12-14), maternal stress (PSS-14 score >26) at first trimester (GW 12-14), second trimester (GW 24), and third trimester (GW 34-36), maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and systemic antibiotic use in the infant's first year of life. All stressors used in the linear regression models are binary categorical variables. As described above, the stressor variables were calculated based on whether the stressor occurred or not (Yes/No; see section 3.2.3).

The next step was to investigate the association between the exposure to multiple hits of stressors and $CD4^+$ T cell subsets in children at 5 years of age, independently and depending on the combination of specific stressors. First, a cumulative stressor variable was generated to examine exposure to multiple hits, independently of the type of stressors. For this, the following stressors were recorded cumulatively in this variable: maternal obesity, maternal stress (PSS-14 score >26) at third trimester, maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and antibiotic use in the first year of life. Only maternal stress in the third trimester were included in the cumulative stressor variable, as univariable regression analyses did not indicate any associations by a statistical trend for the first and second trimesters. Also, since 75% of the women with a chronic disease were also taking medication during pregnancy in this study (data not shown), the variable “chronic diseases” were not integrated into the cumulative variable due to overlap. Subsequently, univariable linear regression models with $CD4^+$ T cell subpopulations of 5-year-olds and the cumulative stressor variable were conducted. Next, multivariable linear regression models with $CD4^+$ T cell subsets and interaction term between stressors were performed to evaluate the interactions between multiple stressors and $CD4^+$ T cells in 5-year-old children,

dependently of the type of stressors. As with the cumulative variable, the stressors included in these models were maternal obesity, maternal stress (PSS-14 score >26) at third trimester, maternal medication use, maternal infections, pregnancy complications, and systemic antibiotic use in the first year of life. Subsequently, multivariable linear regression models with interactions between two prenatal stressors and interactions between a prenatal stressor and infant antibiotic use in the first year of life were run.

Abdominal pain, often accompanied by nausea or headaches, is one of the most frequent reasons for children to consult a pediatrician (Gieteling et al., 2011; Krause et al., 2019; Léa et al., 2021; A. C. Russell, Stone, & Walker, 2017; Santucci, 2020). At the annual examination of 5-year-old children for the PRINCE study, mothers could report whether their child had complained about abdominal pain, nausea or headaches (abdominal symptoms/headaches) in the previous 6 weeks (see section 3.2.4). In the present study, the association between the reported abdominal symptoms/headaches, exposure to stressors, and the frequencies of $CD4^+$ T cell subsets in 5-year-olds was assessed. In a first step, univariable linear regression models were used to examine whether there was a direct association between abdominal symptoms/headaches and $CD4^+$ T cell subsets in 5-year-old children. Next, logistic regression models were conducted to examine an association between the exposure of individual stressors and abdominal symptoms/headaches in 5-year-olds. The subsequent step was to investigate whether T cells were associated with exposure to individual stressors and the occurrence of abdominal symptoms/headaches in children. For this, multivariable linear regression analyses were fitted with $CD4^+$ T-cell subsets and interaction term between individual stressors and abdominal symptoms/headaches.

In all depicted figures, the frequencies, including the median frequencies and the interquartile ranges (IQR), were presented using GraphPad Prism Version 10 (GraphPad Software).

3.2.7 Functional analysis of $CD4^+$ T cells from 5-year-old children included in the PRINCE study

In this study, multivariable linear regression analyses revealed that exposure to early-life stressors, including the use of medications during pregnancy and the use of systemic antibiotics in the first year of life, were significantly associated with increased frequencies of $CCR6^+CCR4^+CD161^+$ cells in children at the age of 5 who experienced abdominal symptoms/headaches. It was therefore the next objective of this study to determine the cytokine production by $CD4^+$ T cells in these children upon T-cell receptor (TCR)-specific stimulation to assess the functionality of these cells.

3.2.7.1 PBMC samples

PBMC samples from 19 children at the age of 5 years who participated in the PRINCE study were available for analysis. All of these children are part of the 233 children who underwent

the annual PRINCE examinations at 5 years of age. Of the 19 children, 7 children experienced abdominal symptoms/headaches at the age of 5 and were exposed to early-life stressors, including maternal medication during pregnancy or systemic antibiotic use in the first year of life. These children were included in the group of “symptomatic children”. The group “non-symptomatic children” compromised PBMC samples from 5-year-olds who reportedly did not experience abdominal symptoms/headaches and who were not exposed to early-life stressors, including maternal medication during pregnancy or systemic antibiotic use in the first year of life (n = 12). All samples were collected between 2017 and 2019 during the annual PRINCE examinations at the age of 5 years. Healthy adult PBMC samples were obtained from individuals though the HCHH (n = 5). These samples were collected between 2020 and 2022 and included 3 women and 2 men between the ages of 25 and 59 years. All PBMC samples were stored in liquid nitrogen tanks at -160 °C until use.

3.2.7.2 Stimulation assays for cytokine production by $CD4^+$ T cells

To assess the functionality of $CD4^+$ T cells from the aforementioned 5-year-old children (see section 3.2.7.1), intracellular cytokine production was determined following T cell activation using flow cytometry. For this, cells were stimulated with antibodies targeting the molecules CD3 and CD28 to directly induce TCR specific stimulation. The CD3 molecules (CD3εγ, CD3εδ, CD3ζζ) are an important part of the TCR–CD3 complex (De La Hera, Müller, Olsson, Isaaz, & Tunnacliffe, 1991; Punt, Roberts, Kearse, & Singer, 1994; Z. Y. J. Sun, Kim, Wagner, & Reinherz, 2001). Following the recognition of an antigen by the TCR in conjunction with MHC molecules on APCs, the CD3 molecules initiate intracellular signaling for the activation of the T cell (Exley et al., 1994; Irving & Weiss, 1991; Letourneur & Klausner, 1992). However, full activation of T cells requires recognition of costimulators, such as B7 molecules (B7-1 (CD80) and B7-2 (CD86)), which are increasingly expressed on APCs when the APCs encounter pathogens (McAdam et al., 1998; Thebeau & Morrison, 2002; Vasilevko et al., 2002; P. Zhang et al., 2004). B7 is recognized by the CD28 receptor, which is in turn expressed on T cells (Linsley, Clark, & Ledbetter, 1990; Sharpe & Freeman, 2002). The signals generated by the recognition of an antigen via the TCR and the signals generated by the binding of B7 to CD28 converge and ultimately initiate T cell activation and responses (Alegre et al., 2001; Iezzi et al., 1998; Viola & Lanzavecchia, 1996). Besides anti-CD3 and anti-CD28, stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin was used as a TCR-independent activation and positive stimulation control in this study. PMA and ionomycin are potent immune cell activator achieving overall stimulation by activating transcription factors, such as NF-κB and NFAT, which results in the production of cytokines (Brignall et al., 2017).

Due to the limited number of cells (some of the vials contained less than 2 million PBMCs) and to avoid additional stress on the cells, the $CD4^+$ T cells were not isolated from the PBMCS prior to stimulation in this study. Consequently, the entire population of PBMCs were stimulated and

$CD4^+$ T cells were identified in subsequent flow cytometry analyses, allowing also studies of other immune cells (see Figure 7).

First, frozen vials containing cryopreserved PBMCs were thawed in a water bath at 37°C until a small pellet of ice remained. Once the cell pellet had been completely thawed, the cell suspension was immediately transferred into a 50 mL tube containing 9 mL of pre-warmed R10 medium and centrifuged for 5 minutes, at 700*g*, at room temperature (RT). Subsequently, the cell pellet was resuspended in 5 mL of R10 and the cell number was determined using Trypan blue and the Bio-Rad TC20™ Automated Cell Counter. Briefly, for the purpose of cell counting, an aliquot of cells were diluted in a 1:1 ratio with Trypan blue. Thereafter, 10 µL of the mixture was added to cell counting slides, which were then inserted into the TC20™ Automated Cell Counter. After setting the gating to 6 – 17 µm to determine PBMCs, the cell counter provided the cell concentration (cell number per mL). For each donor, the volume of cell suspension was calculated in order to obtain a concentration of 5×10^5 cells per well. The calculated volumes of cell suspensions were then transferred to 1.5 mL or 15 mL tubes and centrifuged 5 minutes, at 700*g*, at RT. Subsequently, 5×10^5 cells were resuspended in 150 µL R10, including the respective stimulation condition. Specifically, PBMCs of each donor were either left unstimulated, stimulated with 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28, or stimulated with 50 ng/ml PMA and 1 µg/ml ionomycin. If the number of cells per donor was sufficient, cells were seeded as duplicates per condition. Otherwise, each condition was seeded as a single unit. For the 18-hour stimulation period, cells were seeded in a 96-well round-bottom plate and incubated at 37 °C and 5% CO_2. After 30 minutes, 7 µg/ml brefeldin A was added to the cells.

3.2.7.3 Flow cytometry analyses of $CD4^+$ T cells to determine cytokine production

Flow cytometry analyses were performed to determine the intracellular cytokine production of stimulated $CD4^+$ T cells from 5-year-old symptomatic and non-symptomatic children as well as from adults upon TCR-specific stimulation (see section 3.2.7.2).

As a first step, live/dead staining was performed using the blue-fluorescent reactive dye of the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit to discriminate between live and dead cells during flow cytometry analysis. For this, cells were centrifuged for 5 minutes, at 700*g*, at RT, to discard the supernatants following 18-hours stimulation. Subsequently, the cell pellet was resuspended in 100 µL PBS and centrifuged for a further 5 minutes, at 700*g*, at RT. A live/dead master mix was prepared in PBS including a 1:1000 dilution of the blue-fluorescent reactive dye and a 1:200 dilution of Human True Stain Fcx. The latter reagent was included to block Fc receptors in parallel to prevent interference with antibody-mediated specific staining. 100 µL of live/dead master mix was added to the cells and incubated for 30 minutes at RT in the dark. Subsequently, cells were washed 2 times with 100 µL FACS buffer by centrifuging for 5 minutes, at 700*g*, at RT.

All flow cytometry antibodies and dilutions used in this assay are listed in section 3.1.1. For surface molecule staining, a master mix with all surface antibodies was prepared in FACS buffer and 50 µL/well brilliant stain buffer. The following antibodies targeting the cell surface markers were included in the master mix: CCR7, CD14, CD19, CD4, CD45RA, CD8a, and IL-6Rα. Cells were stained with 100 µL surface master mix for 40 minutes, at 4 °C in the dark. For intracellular molecule staining, the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set was used. Briefly, surface-stained cells were washed with 200 µL FACS buffer and then fixated with 200 µL Fixation/Permeabilization buffer for 30 minutes, in the dark at RT. The Fixation/Permeabilization buffer was prepared beforehand by 1 part Fixation/Permeabilization Concentrate and 3 parts eBioscience™ Fixation/ Perm diluent. A master mix for intracellular staining was prepared in 1X Permeabilization Buffer with antibodies targeting the following intracellular molecules: FOXP3, IFNγ, IL-17A, IL-22, IL-4, IL-6, IL-8, TNF, and TRα1/β1. Subsequently, cells were washed with 200 µL 1X Permeabilization Buffer and then incubated with 50 µL intracellular cytokine master mix for 45 minutes, at 4 °C in the dark. After the incubation, cells were washed 2 times with 1X Permeabilization Buffer, resuspended in 200 µL FACS buffer, and then transferred into cluster tubes. Stained cells were acquired on a Cytek Aurora 5 Laser Spectral Cytometer using SpectroFlo software within 24 hours after staining.

Data was analyzed using FlowJo software (version 10.8.1; Treestar/BD). Representative plots of the flow cytometry-based gating strategy of PBMCs from a 5-year-old child are shown in Figure 7. Based on the expression of surface molecules, $CD4^+$ T cells were identified as viable $CD19^-CD14^-CD8^-CD4^+$ cells. The transcription factor FOXP3 in $CD4^+$ T cells is important key marker for Tregs (Miyara et al., 2009). Consequently, the expression of FOXP3 were used to discriminate between conventional $CD4^+$ T cells ($CD4^+FOXP3^-$ cells) and Tregs ($CD4^+FOXP3^+$ cells). Within the $CD4^+FOXP3^-$ cell compartment, the expression of the surface molecules CD45RA and CCR7 was used to identify naive and memory $CD4^+$ memory T cells (Sallusto et al., 2004, 1999; Tian et al., 2017). Specifically, $CD45RA^+CCR7^+$ cells were determined as naive $CD4^+$ T cells and non-$CD45RA^+CCR7^+$ cells were determined as $CD4^+FOXP3^-$ memory T cells. As CCR7 is barely expressed on Tregs, only the present or absent expression of CD45RA was used to identify naive Tregs ($CD45RA^+$: naive $CD4^+FOXP3^+$ T cells) and memory Tregs ($CD45RA^-$: $CD4^+FOXP3^+$ memory T cells; Miyara et al., 2009). Viable $CD19^+CD14^-$ cells were determined as $CD19^+$ B cells and viable $CD19^-CD14^+$ cells were determined as $CD14^+$ monocytes that include classical ($CD14^+$), non-classical ($CD14^{dim}$), and intermediate ($CD14^+$) monocytes (Kapellos et al., 2019; Nadler et al., 1983; K. Wang, Wei, & Liu, 2012; Ziegler-Heitbrock, 2014; Ziegler-Heitbrock et al., 2010).

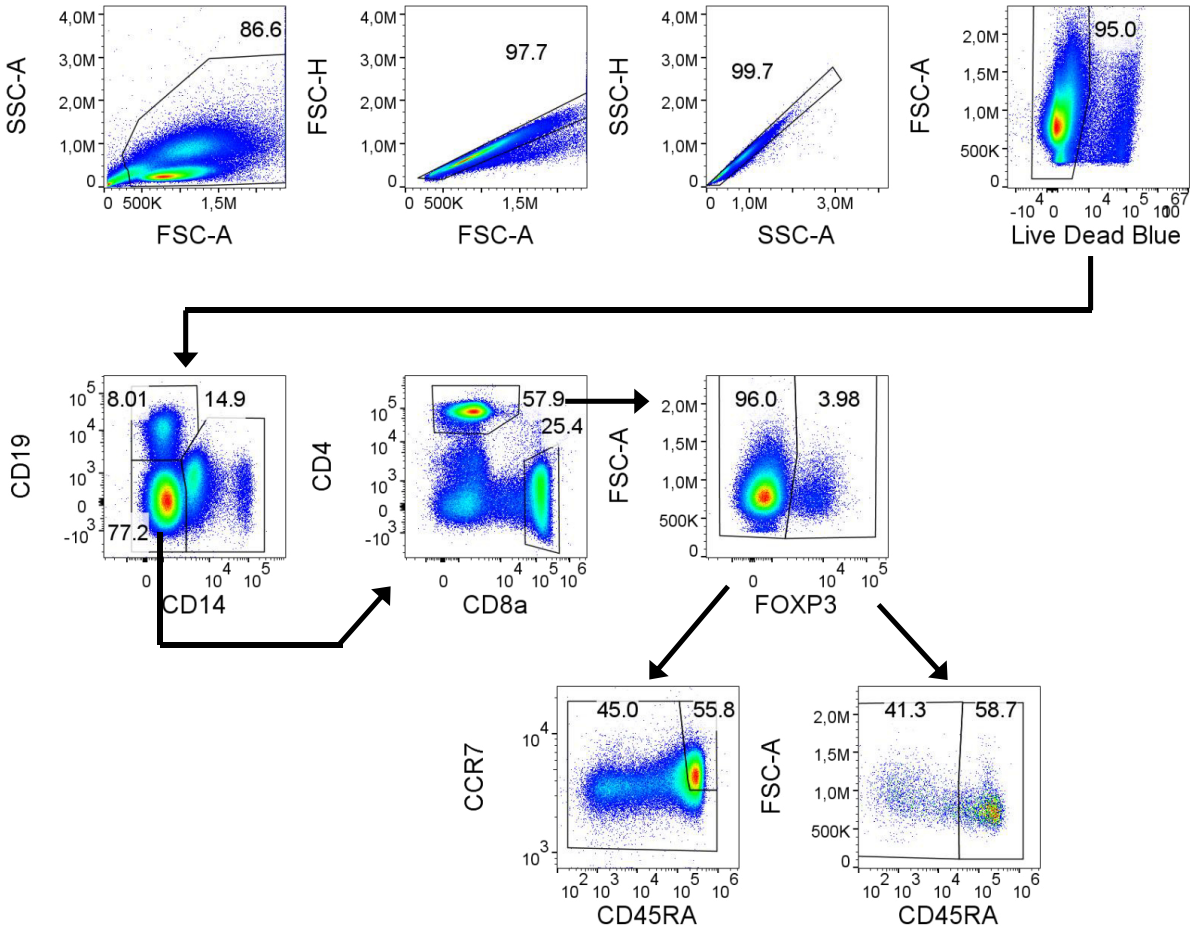


Figure 7: Flow cytometry gating strategy used to identify $CD4^+FOXP3^-$ memory T cells and $CD4^+FOXP3^+$ memory T cells from 5-year-old children and adults. Figure shows plots of a representative donor (5-year-old, unstimulated).

3.2.8 Serum cytokine measurements

The results of the multivariable linear regression analyses indicated that exposure to early-life stressors, including the use of medications during pregnancy and the use of systemic antibiotics in the first year of life, were significantly associated with increased frequencies of $CCR6^+CCR4^+CD161^+$ cells in children at the age of 5 who experienced abdominal symptoms/headaches. In order to assess whether these children had been exposed to MIA (Jain et al., 2021; Shimizu et al., 2023, 2021; Terasaki & Schwarz, 2016) during pregnancy, concentrations of specific cytokines and effector molecules associated with inflammation were determined in the serum samples of their mothers taken at the third trimester.

3.2.8.1 Serum samples

Serum samples of 19 pregnant women in their third trimester (GW 34-36) who participated in the PRINCE study could be analyzed. These women are the biological mothers of the symptomatic and non-symptomatic children whose PBMC samples were examined in the

course of this study (see section 3.2.7.1). For subsequent analyses, serum samples from mothers of symptomatic children (MSC; n = 7) were assessed and compared to serum samples from mothers of non-symptomatic children (MNSC; n = 12). Mothers of symptomatic children were between 27 to 41 years, with an average age of 32 years (data not shown). The age of the mothers of non-symptomatic children ranged from 27 to 36 years, with an average age of 32 years. All maternal serum samples were collected between March 2011 and April 2013. Serum samples from 5 non-pregnant women between the ages of 28 and 36 (average age: 31) who participated in the HCHH were used as a reference non-pregnant group (NP). These samples were collected between 2015 and 2018. All samples were stored at -80 °C until analysis.

3.2.8.2 Multiplex-bead assay for the quantification of soluble factors in serum

A magnetic bead-based multiplex assay was performed to determine the concentrations of specific pro-inflammatory cytokines and effector molecules in the serum samples from the aforementioned described pregnant women in their third trimester (see section 3.2.8.1). Therefore, a customized Luminex 12-plex Human Premixed Multiple-Analyte Kit was used. The multiplex bead assay was performed by Urte Matschl (Department of Virus Immunology at the LIV) in accordance with the manufacturer's protocol. The customized kit included CRP, CXCL5, CXCL10, gp130, IL-1β, IL-2, IL-4, IL-6, IL-17A, IL-18, IL-6R alpha (IL-6Rα) and TNF. Samples were analyzed in duplicates using a Bio-Plex-200 analyzer (Biorad) and Bio-Plex Analysis Software. All serum samples were diluted 1:1 in the assay, except for CRP which was pre-diluted 1:200 according to the manufacturer's protocol.

3.2.9 Statistical analysis of cytokine production by $CD4^+$ T cells of 5-year old children and cytokines in maternal serum

The results of the flow cytometry analyses (section 3.2.7) and the multiplex-bead assays (section 3.2.8) were subjected to statistical analysis using GraphPad Prism (Version 10; GraphPad Software). Differences between groups were evaluated by non-parametric unpaired Mann–Whitney *U* Test, where *P*-values < 0.05 were used to identify a significant result. *P*-values < 0.15 were considered a statistical trend. In figures and text, median frequencies or median concentrations, including interquartile ranges (IQR) are stated.

4 Results

4.1 Associations between early-life stressors and CD4$^+$ T cell subsets in 5-year-old children

Emerging data indicate that exposure to stressors during pregnancy and the first year of life are associated with an altered immunity in offspring (Atègbo et al., 2006; Ing Lim et al., 2021; Leibowitz et al., 2012; Ye Li et al., 2023; Ozkul et al., 2020; V. E. Ruiz et al., 2017; Shimizu et al., 2023, 2021). In studies of murine models, offspring exposed to stressors, such as maternal stress, maternal infections, maternal obesity or early-life antibiotic intake showed altered lymphocyte frequencies, including altered inflammatory responses (Garcia-Flores et al., 2020; Hsiao et al., 2012; Mandal et al., 2010; Myles et al., 2013; S. L. Russell et al., 2012; Shimizu et al., 2021). Especially, CD4$^+$ T cells have been suggested to be affected by stressors, as for instance shown by a diminished frequency of CD4$^+$Foxp3$^+$CD25$^+$ Tregs and a preferential development of Th17 cells, including an increased production of IL-17 (Hsiao et al., 2012; Mandal et al., 2010). In addition, studies of pregnant women and their children have indicated an association between maternal infections during pregnancy and the onset of immune-related-disorders, such as asthma, type 1 diabetes and allergy diseases (Collier et al., 2013; Fuchs & von Mutius, 2013; McKeever et al., 2002; Yue et al., 2018). Moreover, murine offspring that were treated with antibiotics shortly after birth exhibited altered T cell frequencies, such as a diminished frequency of intestinal Tregs and Th17 cells (Atarashi et al., 2011; Ozkul et al., 2020; V. E. Ruiz et al., 2017; S. L. Russell et al., 2012). In this context, further studies of infants demonstrated that newborns having a microbial dysbiosis within the first 3 months of life also exhibited an altered T cell function and an increased risk for immune-mediated diseases, such as asthma or IBD later in life (Arrieta et al., 2014, 2015; Laforest-Lapointe & Arrieta, 2017; Miyoshi et al., 2017; Olin et al., 2018). Microbial dysbiosis, in turn, is associated with stressors like pre- and postnatal antibiotic use (Arrieta et al., 2014, 2015; Fouhy et al., 2012; Laforest-Lapointe & Arrieta, 2017; Miyoshi et al., 2017; Olin et al., 2018; Zwittink et al., 2018).

The aim of this study is to investigate the influence of early-life stressors on CD4$^+$ T cells in children. For this, flow cytometric data from blood-derived lymphocytes of children at 5 years of age who participated in the PRINCE study were investigated (Figure 6). The associations between CD4$^+$ T cell subsets and stressors were assessed using univariable and multivariable regression models. Starting in 2011, the PRINCE study is a prospective longitudinal pregnancy study, conducted at the Department of Obstetrics and Prenatal Medicine of the UKE. Women with a maternal age of 18 or higher and a viable pregnancy of 12-14 weeks of gestation were included in this study. Exclusion criteria for the study included women with infections, such as HIV or hepatitis B/C, a history of drug or alcohol abuse, and multiple pregnancies or

pregnancies conceived through ART. Data was collected at the end of each trimester and annually after birth, in the month of birth, on the anthropometry and health status of the mother and child. To date (April 2024), the PRINCE study involves 749 study participants. At the beginning of the analysis of this study in May 2021, 233 children participated in the 5-year examination. Out of these children, the flow cytometric data from blood-derived lymphocytes of the first 118 5-year-old children who participated in the PRINCE study were made available and included in this study.

4.1.1 Participant characteristics

The demographic characteristics of the mothers from the 118 children included in this study are presented in Table 2. Overall, the women were in relatively good health. The age range of the mothers was between 24 and 46 years, with a mean age of 32 years. 20 (17%) of the mothers reported having a chronic disease, including 12 with thyroid disease and 5 with asthma/emphysema. The BMI was calculated from the mothers' measured height and weight in the first trimester. Almost two-thirds of the mothers had a BMI in the normal range (n = 74, 63%; BMI > 18.4 to < 25). Only 3 mothers had a low weight with a BMI below 18.5 (3%). However, more than 30% had a high weight (n = 37, 31%; BMI > 24.9 to < 30), and 9 mothers were classified as obese (8%; BMI ≥ 30). At the end of the first trimester, 9 women reported smoking or were smoking within the last 6 months (data not shown). Maternal perceived stress was assessed using the PSS-14 at each trimester. The PSS-14 is a 14-item survey in which the participant appraise their stress perception (Cohen et al., 1983). The survey generates a total score ranging from 0 to 56, with higher values indicating a greater level of self-appraised stress. In the literature, there is no defined threshold characterizing a PSS-14 score as high stress. As Polinski *et al*., (2020), the total PSS-14 score in this study was categorized as low to moderate perceived stress (PSS-14 score 0–26) and high perceived stress (PSS-14 score >26). On average, the participants exhibited a low to moderate level of stress, with an average score of just under 20 for each trimester. However, the scores of the study population varied widely, ranging from a minimum of 2 to a maximum score of 39. About 13 to 18% of the women in this study had a PSS-14 score >26 during pregnancy (Table S1). Of all the women in this study, 48 (41%) used medication during their pregnancy, with levothyroxine (L-thyroxine) being the most commonly used drug (n = 19; 40%). Furthermore, 58% (n = 68) of the women reported experiencing at least one infection during their pregnancy. Of these, 59% (n = 40) had flu symptoms or a severe cold, 24% (n = 16) reported a gastrointestinal infection, 16% (n = 11) a urinary tract infection, and 15% (n = 10) a genital tract infection at least once.

Table 2: Characteristics of the mothers of the children included in the PRINCE study.

Demographic variables	*N (total N=118)*	*%*	*M*	*SD*	*Range*
Maternal age in first trimester, years			32.0	3.7	24-46
Chronic diseases					
Yes	20	17			
No	96	83			
Missing	2				
Most frequent (N>4)					
Thyroid disease	12	60			
Asthma / Emphysema	5	25			
BMI at 1st trimester (12.-14. GW), kg/m²					
BMI <18.5	3	3			
BMI ≥18.5 to <25	74	63			
BMI ≥25 to <30	37	31			
BMI ≥30 to <35	5	4			
BMI ≥35 to <40	4	3			
Perceived Stress Scale (PSS)-14 score					
1st trimester (12.-14. GW) missing (N): 14			19.5	6.7	3-39
2nd trimester (24. GW) missing (N): 5			19.9	7.0	4-35
3rd trimester (34.-36. GW) missing (N): 5			18.6	7.1	2-39
Medication during pregnancy					
Yes	48	41			
No	70	59			
Most frequently used (N>4)					
Levothyroxine	19	40			
Infections during pregnancy					
Yes	68	58			
No	50	42			
Most frequent (N>4)					
Flu/severe cold	40	59			
Gastrointestinal infection	16	24			
Urinary tract infection	11	16			
Genital tract infection	10	15			
Pregnancy complications					
Yes	22	19			
No	94	81			
Missing	2				
Mode of delivery					
C-section	24	20			
Vaginal	94	80			

Values are N (%) or mean ± SD including range of values (minimum and maximum). C-section = caesarean section.

Pregnancy complications can also impact the development of the child's immune system and can encompass a variety of health problems that may arise during pregnancy and affect the health of the mother and the fetus (Gomez-lopez et al., 2019; J. Henderson, Carson, & Redshaw, 2016; Hernández-Díaz, Toh, & Cnattingius, 2009; Jensen et al., 2003; McIntyre et al., 2019; D. Miller, Gershater, Slutsky, Romero, & Gomez-Lopez, 2020; Tanner et al., 2022; van Esch, van Heijst, de Haan, & van der Heijden, 2017). In this study, 22 women (19%) have reported experiencing a complication during their pregnancy. Among the reported complications were gestational diabetes (n =4), preterm labor (n = 4), fetal growth retardation (n = 3), and pre-eclampsia (n = 2). In addition, 6 women reported requiring hospitalization with 3 of them due to pregnancy complications and 3 for other reasons (data not shown). Out of all mothers in this present study, 20% (n = 24) gave birth by caesarean section (C-section).

Table 3: Characteristics of children included in the PRINCE study at 5 years of age.

Demographic variables	*N (total N=118)*	*%*	*M*	*SD*	*Range*
Sex					
Female	51	43			
Male	67	57			
Gestational age, weeks			39.3	1.15	36-41
Birthweight, g					
< 2500	3	3			
≥ 2500 to <4000	104	88			
≥ 4000	11	9			
APGAR score					
0-4	/	/			
5-7	1	1			
8-10	114	99			
Missing	3				
Antibiotic use in the first year of life					
Yes	31	31			
No	70	69			
Missing	17				
Abdominal pain, headaches, and nausea at 5 years of age					
Yes	24	24			
No	77	76			
Missing	17				

Values are N (%) or mean ± SD including range of values (minimum and maximum).

Table 3 shows the characteristics of the 118 children who participated in the PRINCE study and were included in this study. As their mothers, the children were generally in good health. Males accounted for 57% (n = 67) of children, slightly more than females. The gestational age (GA) of the children ranged from 36 to 41 weeks, with an average GA of 39.3 weeks. Only 3 children had a GA of 36, which categorizes them as moderate to late preterm newborns according to the World Health Organization (WHO; Howson, Kinney, & Lawn, 2012). The majority of children (n = 104; 88%) had a birthweight between 2500 and 4000 g. Only 3 children

(3%) had a birthweight below 2500, and 11 (9%) had a birthweight greater than 4000 g. The APGAR test is a standardized health assessment that was performed on newborns 5 minutes after birth. The test evaluates the baby's heart rate, breathing, muscle tone, reflexes, and skin color. The score ranges from 0 to 10, with a higher score indicating a better state of health. In the present study, almost all reported APGAR scores (99%) fell between 8 and 10. Within the first year of life, 31% (n = 31) of the children received antibiotics, indicative of severe infections. Only one child was reported to have received gentamicin and tobramycin 7 days after birth. For the other children, the type of antibiotic and time point of intake was not documented. In the annual examination of 5-year-old children, 24 children had reported abdominal pain, nausea or headaches in the last 6 months. Of the 101 participants who provided this information, this represents about a quarter (n = 24; 24%) of the children in this study. Allergies were only reported in 4 children. These included 2 children with food intolerance, 1 child with an insect allergy and 1 child with both allergic rhinitis/hay fever and a drug allergy (data not shown). Asthma was reported in 3 children, with one child's asthma being confirmed by a doctor (data not shown).

4.1.2 Selection of early-life stressors and their association with CD4$^+$ T cell subsets in children at the age of 5 years

Based on the literature, 7 types of early-life stressors were selected: maternal chronic diseases, maternal obesity (BMI ≥ 30) at first trimester (GW 12-14), maternal stress (PSS-14 score >26) at first trimester (GW 12-14), second trimester (GW 24), and third trimester (GW 34-36), maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and systemic antibiotic use in the infant's first year of life. As a first step, univariable linear regression models were performed to examine the association between stressors and the frequencies of CD4$^+$ T cell subsets in children at 5-years of age. Table 4A and 4B present the results of the univariable linear regression models. Overall, the analyses revealed that associations between individual stressors and T-cell types were rare. Only two types of stressors were associated with specific T-cell subsets. CXCR5^{hi} cells were negatively associated with maternal stress. Specifically, children born to mothers with a PSS-14 score >26 in the third trimester had lower frequencies of CXCR5^{hi} cells compared to children born to mothers with a PSS14 score <27 (coefficient -3.024, P = 0.049; Table 4A; Figure S1A). Furthermore, maternal medication use during pregnancy was negatively associated with CRTh2$^+$CCR4^{hi} cells and positively associated with CCR6$^+$CCR4$^+$CD161$^+$ cells. Children born to mothers receiving medication during pregnancy had reduced frequencies of CRTh2$^+$CCR4^{hi} cells (coefficient -0.586, P = 0.038; Table 4B; Figure S1B) and increased frequencies of CCR6$^+$CCR4$^+$CD161$^+$ cells (coefficient 0.430, P = 0.023; Table 4B; Figure S1C) compared to children who were not exposed to maternal medication during pregnancy.

Table 4A: Univariable linear regression analyses of early-life stressors with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Maternal chronic diseases		Maternal BMI ≥30 at 1st trimester (12.-14. GW)		PSS-14 score >26					
					1st trimester (12.-14. GW)		2nd trimester (24. GW)		3rd trimester (34.-36. GW)	
	N=20		*N=9*		*N=14*		*N=20*		*N=15*	
Total N:	*N=116*		*N=118*		*N=104*		*N=113*		*N=113*	
Baseline:	*No*		*BMI ≤ 30*		*PSS-14 score <27*					
	Coeff.	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*
• naive $CD4^+CD25^-$ cells	0.062	0.974	3.997	0.132	1.935	0.390	-1.877	0.326	-2.023	0.349
• $CD4^+CD25^-$ memory cells	-0.010	0.996	-3.967	0.135	-1.892	0.401	1.741	0.362	2.012	0.352
• $CXCR3^+CCR6^-$ cells	-0.781	0.677	4.547	0.083	1.107	0.616	1.275	0.491	1.630	0.445
• $CRTh2^+CCR4^{hi}$ cells	-0.290	0.439	0.207	0.696	-0.228	0.611	-0.543	0.151	0.801	0.059
• $CCR6^+CCR4^+CD161^+$ cells	-0.242	0.337	-0.025	0.945	-0.028	0.916	-0.342	0.181	0.253	0.376
• $CCR6^+CCR4^+CD161^-$ cells	-0.316	0.528	-0.711	0.318	-0.056	0.919	-0.601	0.227	0.580	0.309
• $CXCR5^{int}$ cells	0.968	0.316	1.900	0.160	0.527	0.642	-0.320	0.741	-0.077	0.943
• $CXCR5^{hi}$ cells	-1.409	0.307	1.536	0.433	0.140	0.931	0.959	0.498	*-3.024**	*0.049*
• $CD25^+CCR4^-$ $CD45RA^+$ cells	2.170	0.401	5.102	0.162	2.594	0.398	-0.863	0.733	-0.510	0.862
• $CD25^+CCR4^+CD45RA^-$ cells	0.507	0.803	-4.687	0.115	1.220	0.631	0.374	0.862	3.420	0.153

*Note. *P-value<0.05*

Table 4B: Univariable linear regression analyses of early-life stressors with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Maternal medication use		Maternal infections		Pregnancy complications		Antibiotic use in the first year of life	
	N=48		*N=68*		*N=22*		*N=31*	
Total N:	*N=118*		*N=118*		*N=116*		*N=101*	
Baseline:	*No*		*No*		*No*		*No*	
	Coeff.	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*
• naive $CD4^+CD25^-$ cells	-1.940	0.175	-0.224	0.876	-0.081	0.965	-0.745	0.665
• $CD4^+CD25^-$ memory cells	1.886	0.187	0.162	0.910	-0.066	0.972	0.580	0.736
• $CXCR3^+CCR6^-$ cells	-1.997	0.157	0.408	0.773	1.313	0.461	0.051	0.975
• $CRTh2^+CCR4^{hi}$ cells	*-0.586**	*0.038*	0.028	0.922	-0.010	0.978	-0.150	0.661
• $CCR6^+CCR4^+CD161^+$ cells	*0.430**	*0.023*	-0.122	0.520	-0.216	0.379	0.302	0.192
• $CCR6^+CCR4^+CD161^-$ cells	0.657	0.085	0.063	0.868	-0.469	0.340	0.308	0.504
• $CXCR5^{int}$ cells	0.033	0.964	0.561	0.440	0.054	0.954	-0.110	0.894
• $CXCR5^{hi}$ cells	0.644	0.541	0.258	0.806	-1.339	0.323	-0.731	0.565
• $CD25^+CCR4^-$ $CD45RA^+$ cells	-0.500	0.800	-0.443	0.821	0.162	0.949	-3.400	0.130
• $CD25^+CCR4^+CD45RA^-$ cells	-1.010	0.529	0.486	0.761	-1.323	0.517	3.101	0.094

*Note. *P-value<0.05*

To assess whether the significant associations of these 3 stressors and immune cell types remained significant after adjustment for multiple testing, a false positive *P*-value probability of 5% was considered. After applying Benjamini-Hochberg (BH) correction, no significant associations were identified between the stressors and the $CD4^+$ T cell subpopulations (Table S2). Specifically, the BH-adjusted *P*-value of $CXCR5^{hi}$ cells associated with a maternal PSS-14 score >26 in the third trimester was 0.441 (Table S2). Moreover, the BH-adjusted *P*-values of $CRTh2^+CCR4^{hi}$ cells and $CCR6^+CCR4^+CD161^+$ cells associated with maternal medication use were determined to be 0.266 and 0.207, respectively (Table S2).

4.1.3 Association of multiple stressors and $CD4^+$ T cell subsets in children at 5 years of age

A "*two-hit*" model has been proposed by a growing number of preclinical and clinical studies as a potential cause of developmental alterations in infants (Feigenson et al., 2014; Hsueh et al., 2018; Korzeniewski et al., 2014; Leviton et al., 2013; Shimizu et al., 2021; Verstraeten et al., 2019). This model, also referred to as "*two-hit*" hypothesis, "*dual-hit*" or "*multiple-hit*" model, is based on the hypothesis that exposure to an initial stressor primes an alteration and exposure to a second or more stressors subsequently triggers the manifestation of the alteration and the actual onset of the disease (Bayer et al., 1999; Gundacker et al., 2023; X. Li et al., 2017). For instance, a study by Leviton *et al.* in 2013 discovered an association of small for gestational age and postnatal systemic inflammation with the development of brain damage in preterm newborns. Other studies showed that double hit of inflammation prenatal and later in life, also in combination with postnatal hypoxia, can lead to the onset of neurological disorders like schizophrenia and autism-like behavior (Feigenson et al., 2014; van Tilborg et al., 2018). A potential prenatal mechanism and disease primer for these alterations might be MIA (Jain et al. 2021; Shimizu et al. 2021, 2023; Terasaki and Schwarz 2016). In human epidemiological studies, MIA as a response to stressors has been associated with altered immunity, including the emergence of type 1 diabetes mellitus and allergic diseases, and neurodevelopmental disorders in offspring (Atladóttir et al., 2010; Brown, 2012; McKeever et al., 2002; Yue et al., 2018). Animal studies further suggest that in addition to MIA, a secondary inflammatory stimulation can enhance the occurrence of alterations in the offspring's immune system (Hsueh et al., 2018; Shimizu et al., 2021).

In this study, a cumulative stressor variable was generated to examine the association between exposure to multiple stressors and $CD4^+$ T cell subsets in 5-year-olds, independently of the type of stressors children were exposed to. The following stressors were recorded cumulatively in this variable: maternal obesity, PSS-14 score >26 at third trimester, maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and systemic antibiotic use in the infant's first year of life. Only participants with a PSS-14 score >26

in the third trimester were selected, as univariable regression analyses did not show any *P*-values ≤ 0.150 for the first and second trimesters (Table 4A). In this study, 75% of the women with a chronic disease were also taking medication during pregnancy (data not shown). Due to the close similarity of the stressor types, the stressor "maternal chronic diseases" was excluded from the generation of the cumulative variable. In summary, 21 children were not exposed to any of the selected stressors, 33 were exposed to one stressor, 41 were exposed to two types of stressors, 16 were exposed to three types of stressors, and 5 were exposed to 4 types of stressors in this study (Table 5).

Table 5: Univariable linear regression analyses of cumulative stressors with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

Total N=118 *Baseline: No cumulative stressors (N=21)*	1 stressor *N=33*		2 stressors *N=41*		3 stressors *N=16*		4 stressors *N=5*	
	Coeff.	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*	*Coeff.*	*P-value*
• naive $CD4^+CD25^-$ cells	-1.255	0.556	-2.657	0.199	-3.513	0.167	-2.891	0.449
• $CD4^+CD25^-$ memory cells	1.228	0.564	2.592	0.211	3.472	0.172	2.241	0.557
• $CXCR3^+CCR6^-$ cells	3.275	0.124	2.463	0.232	3.231	0.201	-0.130	0.973
• $CRTh2^+CCR4^{hi}$ cells	0.020	0.963	0.367	0.376	-0.012	0.982	-0.492	0.520
• $CCR6^+CCR4^+CD161^+$ cells	-0.129	0.653	0.272	0.328	0.112	0.742	0.467	0.363
• $CCR6^+CCR4^+CD161^-$ cells	0.282	0.625	0.537	0.339	0.256	0.710	0.957	0.356
• $CXCR5^{int}$ cells	1.637	0.135	1.671	0.116	1.480	0.255	0.557	0.776
• $CXCR5^{hi}$ cells	-0.719	0.649	-1.027	0.502	-2.362	0.210	1.916	0.499
• $CD25^+CCR4^-$ $CD45RA^+$ cells	0.959	0.745	-2.553	0.373	-2.383	0.497	-0.052	0.992
• $CD25^+CCR4^+CD45RA^-$ cells	-0.961	0.692	0.662	0.778	0.629	0.827	1.768	0.684

Note. 5 stressors: N=2

Table 5 presents the results of the univariable linear regression model with $CD4^+$ T cell subpopulations of 5-year-olds and the cumulative stressor variable. The analyses revealed that there were no significant associations between the number of stressors to which children were exposed and the frequencies of their $CD4^+$ T cell subsets (Table 5). However, the results of the analyses indicated that there were trends ($P < 0.15$) towards an association between specific T-cell subsets and the exposure to either one or two stressors. Specifically, $CXCR3^+CCR6^-$ cells were positively associated with exposure to 1 stressor (coefficient 3.275, $P = 0.124$) and $CXCR5^{int}$ cells were positively associated with the exposure to 1 (coefficient

1.637, *P* = 0.135) and 2 stressors (coefficient 1.671, *P* = 0.116). Although no further associations between T cell subtypes and exposure to 2 or more stressors could be demonstrated, it is still possible that specific interactions between stressors may have occurred. A simple cumulative stressor variable as used above does not capture such specific interactions. Consequently, multivariable linear regression models with $CD4^+$ T cell subsets and interaction between specific stressors were performed to evaluate the interactions between multiple stressors and $CD4^+$ T cells in 5-year-old children.

The stressor variables included in these models were maternal obesity, PSS-14 score >26 at third trimester, maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications, and systemic antibiotic use in the infant's first year of life. The results of the multivariable linear regression models with interactions between two prenatal stressors and $CD4^+$ T cells of 5-year-olds are listed in Table 6A and 6B. In total, the analyses revealed that exposure to two prenatal stressors was rarely associated with $CD4^+$ T-cell types of 5-year-olds. Only exposure to high maternal stress in the third trimester together with the occurrence of pregnancy complications were associated with specific T-cell subsets. Specifically, a PSS-14 score >26 in the third trimester and pregnancy complications was positively associated with $CRTh2^+CCR4^{hi}$ cells and negatively associated with $CXCR5^{hi}$ cells. The 5 children who were exposed to these two stressors had higher frequencies of $CRTh2^+CCR4^{hi}$ cells (coefficient 1.555, *P* = 0.027, Table 6B) and lower frequencies of $CXCR5^{hi}$ cells (coefficient -5.369, *P* = 0.037) compared to children not exposed to both stressors (Table 6B; Figure S2). However, after adjustment for multiple testing, the associations between these stressors and the $CD4^+$ T cell subsets was not significant (BH-adjusted *P* of $CRTh2^+CCR4^{hi}$ cells = 0.162; BH-adjusted *P* of $CXCR5^{hi}$ cells = 0.222; Table 6).

Following these analyses, multivariable linear regression models with interactions between prenatal stressors and systemic antibiotic use in the first year of life were performed. Analyses of interactions between early antibiotic use and maternal obesity as well as early antibiotic use and maternal stress in the third trimester (PSS-14 score >26) were not performed since the number of participants exposed to these combinations was smaller than 5. Of the children who received antibiotics, 17 were also exposed to maternal medication use, 20 were exposed to infections during pregnancy, and 8 were exposed to pregnancy complications. The analysis showed that the use of medication by mothers during pregnancy and the intake of antibiotics early in life were negatively associated with $CRTh2^+CCR4^{hi}$ cells and positively associated with $CCR6^+CCR4^+CD161^+$ cells in children at 5-years of age (Table 7). These children had reduced frequencies of $CRTh2^+CCR4^{hi}$ cells (coefficient -0.931, *P* = 0.035) and increased frequencies of $CCR6^+CCR4^+CD161^+$ cells (coefficient 0.661, *P* = 0.030) as compared to children who were not exposed to maternal medication and antibiotics within the first year of life (Table 7).

Table 6A: Multivariable linear regression analyses of $CD4^+$ T cell subsets with the interaction of two individual prenatal stressors in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Maternal medication use								
	+ Maternal infections			+ PSS-14 sc. >26 (T3)			+ Pregnancy complications		
	N=32			*N=5*			*N=12*		
Baseline: No stressors	*N=34*			*N=56*			*N=58*		
	Coeff.	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	-1.858	0.325	0.923	-0.461	0.897	0.923	-1.391	0.572	0.923
• $CD4^+CD25^-$ memory cells	1.756	0.352	0.944	0.447	0.901	0.944	1.155	0.639	0.944
• $CXCR3^+CCR6^-$ cells	-1.392	0.454	0.683	1.543	0.667	0.800	-1.764	0.455	0.683
• $CRTh2^+CCR4^{hi}$ cells	-0.483	0.193	0.386	-0.317	0.649	0.649	-0.791	0.100	0.300
• $CCR6^+CCR4^+CD161^+$ cells	0.268	0.278	0.626	0.632	0.184	0.626	0.085	0.791	0.791
• $CCR6^+CCR4^+CD161^-$ cells	0.623	0.216	0.648	1.193	0.212	0.648	0.012	0.985	0.985
• $CXCR5^{int}$ cells	0.475	0.616	0.859	-0.668	0.716	0.859	-1.408	0.251	0.790
• $CXCR5^{hi}$ cells	0.799	0.567	0.758	-2.023	0.434	0.758	0.857	0.632	0.758
• $CD25^+CCR4^-$ $CD45RA^+$ cells	-0.810	0.756	0.972	5.350	0.277	0.972	-0.121	0.972	0.972
• $CD25^+CCR4^+CD45RA^-$ cells	-0.423	0.842	0.842	-1.739	0.663	0.842	-2.299	0.403	0.842

*Note. *P-value<0.05; BH-adjusted P-value = Benjamini-Hochberg-adjusted P-value; PSS-14 sc. >26 (T3) = High maternal stress level (PSS14>26) at 3rd trimester. Maternal obesity + maternal medication: N=4; Maternal obesity + maternal infections: N=3; Maternal obesity + PSS-14 sc. >26 (T3): N=0; Maternal obesity +* pregnancy complications: *N=4.*

Table 6B: Multivariable linear regression analyses of $CD4^+$ T cell subsets with the interaction of two individual prenatal stressors in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Maternal infections						PSS-14 sc. >26 (T3)		
	+ PSS-14 sc. >26 (T3)			+ Pregnancy complications			+ Pregnancy complications		
	N=9			*N=14*			*N=5*		
Baseline: No stressors	*N=42*			*N=40*			*N=80*		
	Coeff.	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	-0.551	0.848	0.923	-0.608	0.803	0.923	-0.347	0.923	0.923
• $CD4^+CD25^-$ memory cells	0.536	0.852	0.944	0.375	0.877	0.944	0.252	0.944	0.944
• $CXCR3^+CCR6^-$ cells	3.538	0.215	0.683	-0.070	0.976	0.976	3.790	0.281	0.683
• $CRTh2^+CCR4^{hi}$ cells	0.441	0.429	0.515	-0.425	0.365	0.515	*1.555**	*0.027*	0.162
• $CCR6^+CCR4^+CD161^+$ cells	-0.373	0.313	0.626	-0.163	0.611	0.733	-0.363	0.447	0.671
• $CCR6^+CCR4^+CD161^-$ cells	-0.436	0.551	0.893	-0.076	0.906	0.985	-0.503	0.595	0.893
• $CXCR5^{int}$ cells	1.511	0.292	0.790	0.030	0.981	0.981	1.539	0.395	0.790
• $CXCR5^{hi}$ cells	-2.542	0.216	0.648	-0.306	0.863	0.863	*-5.369**	*0.037*	0.222
• $CD25^+CCR4^-$ $CD45RA^+$ cells	0.544	0.890	0.972	-0.336	0.920	0.972	-0.175	0.972	0.972
• $CD25^+CCR4^+CD45RA^-$ cells	1.421	0.653	0.842	-0.727	0.788	0.842	2.962	0.456	0.842

*Note. *P-value<0.05; BH-adjusted P-value = Benjamini-Hochberg-adjusted P-value; PSS-14 sc. >26 (T3) = High maternal stress level (PSS14>26) at 3rd trimester. Maternal obesity + maternal medication: N=4; Maternal obesity + maternal infections: N=3; Maternal obesity + PSS-14 sc. >26 (T3): N=0; Maternal obesity +* pregnancy complications: *N=4.*

Table 7: Multivariable linear regression analyses of $CD4^+$ T cell subsets with the interaction between a prenatal stressor and antibiotic use in the first year of life in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Infant antibiotic use (1st year)								
	+ Maternal medication use			+ Maternal infections			+ Pregnancy complications		
	N=17			*N=20*			*N=8*		
Baseline: No stressors	*N=45*			*N=30*			*N=57*		
	Coeff.	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	-2.698	0.232	0.696	-0.181	0.937	0.937	1.513	0.615	0.923
• $CD4^+CD25^-$ memory cells	2.493	0.270	0.777	-0.043	0.985	0.985	-1.943	0.518	0.777
• $CXCR3^+CCR6^-$ cells	-2.790	0.198	0.594	-0.665	0.759	0.759	0.992	0.733	0.759
• $CRTh2^+CCR4^{hi}$ cells	*-0.931**	*0.035*	0.105	-0.161	0.722	0.722	-0.227	0.708	0.722
• $CCR6^+CCR4^+CD161^+$ cells	*0.661**	*0.030*	0.090	0.219	0.475	0.713	0.027	0.947	0.947
• $CCR6^+CCR4^+CD161^-$ cells	0.839	0.168	0.504	0.490	0.425	0.638	-0.258	0.749	0.749
• $CXCR5^{int}$ cells	-0.287	0.795	0.907	-0.128	0.907	0.907	0.336	0.818	0.907
• $CXCR5^{hi}$ cells	-0.033	0.984	0.984	-0.058	0.973	0.984	-0.925	0.678	0.984
• $CD25^+CCR4^-$ $CD45RA^+$ cells	-4.285	0.149	0.447	-2.747	0.357	0.536	0.468	0.905	0.905
• $CD25^+CCR4^+CD45RA^-$ cells	1.899	0.432	0.648	3.047	0.212	0.636	0.866	0.787	0.787

*Note. *P-value<0.05; BH-adjusted P-value = Benjamini-Hochberg-adjusted P-value; Inf. antibiotic use. (1st year)= infant antibiotic intake during 1st year of life; Inf. antibiotic use. (1st year) + Maternal obesity: N=3; Inf. antibiotic use. (1st year) + PSS-14 sc. >26 at 3rd trimester: N=3.*

In analyses adjusted for multiple testing, associations between both stressors and $CD4^+$ T cell subsets were not statistically significant (BH-adjusted *P* of $CRTh2^+CCR4^{hi}$ cells = 0.105; BH-adjusted *P* of $CCR6^+CCR4^+CD161^+$ cells = 0.090; Table 7). However, there was still a strong trend (BH-adjusted *P* = 0.090) towards increased frequencies of $CCR6^+CCR4^+CD161^+$ cells in children exposed to maternal medication during pregnancy and systemic antibiotic intake within the first year of life. Specifically, the median frequency of $CCR6^+CCR4^+CD161^+$ cells was higher in children exposed to both stressors (1.83%) as compared to those exposed to none (0.66%), to only maternal medication use (0.79%), or only infant antibiotic use (0.59%; Figure 8).

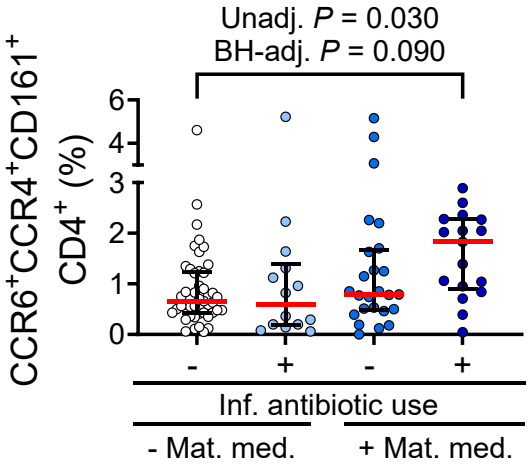


Figure 8: Association of maternal medication use (Mat. med.) during pregnancy and systemic antibiotic use in the first year of life (Inf. antibiotic use) with frequencies of $CCR6^+CCR4^+CD161^+$ cells of 5-year-old children enrolled in the PRINCE study (FLTR: n = 45; 14; 25; 17). Data presented as median percentage (± IQR).

4.1.4 Maternal medication use during pregnancy and antibiotic use within the first year of life are significantly associated with $CCR6^+CCR4^+CD161^+$ $CD4^+$ T cells in 5-year-old children reporting abdominal pain, nausea or headaches

Abdominal pain is one of the most frequent reasons for children to consult a pediatrician (Gieteling et al., 2011; Krause et al., 2019). In the annual PRINCE survey, it was reported that 24 out of the surveyed 5-year-olds experienced abdominal pain, nausea or headaches occasionally to regularly in the last 6 months. Nausea and headache are both symptoms that often occur in association with functional abdominal pain disorders (FADPs; Carson et al. 2011; Kovacic et al. 2013; Léa et al. 2021; Russell, Stone, and Walker 2017; Santucci 2020; Yeom et al. 2013) (de Bruijn, Geijtenbeek, Browne, Benninga, & Vlieger, 2023). As previously described, T cells in the gut play an important role in maintaining intestinal homeostasis (Inagaki-Ohara et al., 2006; Schreurs et al., 2019). Additionally, it has been demonstrated that

altered peripheral blood and intestinal intraepithelial T cells are associated with IBD (Eastaff-Leung et al., 2010; Jaeger et al., 2021).

To examine the association between the reported symptoms experienced by children who participated in the PRINCE study, stressors, and the frequencies of $CD4^+$ T cell subsets, linear regression models were generated. In the following, the children's complaints of abdominal pain, nausea or headaches are referred to as abdominal symptoms or headaches (abdominal symptoms/headaches).

Table 8: Univariable linear regression analyses of abdominal symptoms/headaches with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

Total N=101	Abdominal symptoms/ headaches *Yes*	
Baseline: No abdominal symptoms (N=74)	*N=24*	
	Coeff.	*P-value*
• naive $CD4^+CD25^-$ cells	-0.223	0.903
• $CD4^+CD25^-$ memory cells	0.212	0.908
• $CXCR3^+CCR6^-$ cells	-0.690	0.717
• $CRTh2^+CCR4^{hi}$ cells	-0.537	0.169
• $CCR6^+CCR4^+CD161^+$ cells	0.396	0.091
• $CCR6^+CCR4^+CD161^-$ cells	0.332	0.509
• $CXCR5^{int}$ cells	-1.117	0.212
• $CXCR5^{hi}$ cells	-0.576	0.665
• $CD25^+CCR4^-$ $CD45RA^+$ cells	-4.742	0.059
• $CD25^+CCR4^+CD45RA^-$ cells	3.242	0.117

First, univariable linear regression models were used to assess whether there was a direct association between abdominal symptoms/headaches and $CD4^+$ T cell subsets in 5-year-old children (Table 8). Overall, no significant associations were observed. However, there was a trend ($P < 0.15$) towards a positive association between $CCR6^+CCR4^+CD161^+$ cells and abdominal symptoms/headaches (coefficient 0.396, $P = 0.091$), a trend towards a negative association between $CD25^+CCR4^-CD45RA^+$ cells and abdominal symptoms/headaches (coefficient -4.742, $P = 0.059$) and a positive association between $CD25^+CCR4^+CD45RA^-$ cells and abdominal symptoms/headaches (coefficient 3.242, $P = 0.117$). Specifically, children with

abdominal symptoms/headaches tended to have increased frequencies of $CCR6^+CCR4^+CD161^+$ cells, reduced frequencies of $CD25^+CCR4^-CD45RA^+$ cells and increased frequencies of $CD25^+CCR4^+CD45RA^-$ cells as compared to children without abdominal symptoms/headaches.

Second, binary logistic regression models were conducted to examine an association between individual stressors and abdominal symptoms/headaches in 5-year-olds. Analyses revealed a positive association between maternal chronic diseases and abdominal symptoms/headaches (coefficient 1.3, $P = 0.018$), suggesting that women with chronic diseases are 3.79 times more likely to have a child with abdominal symptoms/headaches at the age of 5 (Table 9). Although subsequent multiple testing correction showed that the *P*-value was no longer statistically significant, there was a trend ($P < 0.15$) towards this association (BH-adjusted $P = 0.072$). Furthermore, there was a trend towards a negative association between maternal infections and abdominal symptoms/headaches (coefficient -0.833, $P = 0.082$, BH-adjusted $P = 0.109$) and a trend towards a positive association between infant systemic antibiotic use and abdominal symptoms/headaches (coefficient 0.937, $P = 0.069$, BH-adjusted $P = 0.109$; Table 9). Analyses of the interaction between abdominal symptoms/headaches and maternal obesity, high maternal stress at the first, second and third trimesters (PSS 14 score >26) and pregnancy complications, respectively, were not performed since the group sizes were smaller than 5.

Table 9: Logistic regression analyses of abdominal symptoms/headaches and exposure to maternal chronic diseases, maternal medication use, maternal infections and infant antibiotic use (1st year of life), respectively, in children included in the PRINCE study at 5 years of age.

	Coeff.	*Odds ratio (OR)*	*95% CI (OR)*		*P-value*	*BH-adjusted P-value*
			Lower endpoint	*Upper endpoint*		
Chronic diseases	1.333	3.793	1.255	11.462	*0.018*	0.072
Maternal medication use	0.102	1.108	0.435	2.827	0.829	0.829
Maternal infections	-0.833	0.435	0.170	1.111	0.082	0.109
Infant antibiotic use (1st year)	0.937	2.552	0.928	7.016	0.069	0.109

Note. P-value<0.05; BH-adjusted P-value = Benjamini-Hochberg-adjusted P-value.

The next step was to investigate whether $CD4^+$ T-cell populations were associated with stressors and abdominal symptoms/headaches in children. This was done by using multivariable linear regression models with $CD4^+$ T-cell subsets and interaction between individual stressors and abdominal symptoms/headaches. Overall, the analyses revealed that two types of stressors were associated with specific T-cell subsets in 5-year-olds with abdominal symptoms/headaches. Particularly, analyses showed a significant positive

association between $CCR6^+CCR4^+CD161^+$ cells in children with abdominal symptoms/headaches born to mothers taking medication during pregnancy (Table 10). Children with these characteristics had higher frequencies of $CCR6^+CCR4^+CD161^+$ cells compared to children without abdominal symptoms/headaches and without exposure to maternal medication during pregnancy (coefficient 0.872, P = 0.014, BH-adjusted P = 0.028; Table 10). Specifically, 5-year-olds with abdominal symptoms/headaches and exposure to maternal medication had a higher median frequency (2.1%) compared to children with no symptoms and no exposure to maternal medication (0.7%), children with only exposure to maternal medication (0.8%), and children with only abdominal symptoms/headaches (0.7%; Figure 9A). In this study, L-thyroxine was the most commonly used drug by women during pregnancy (n = 19; Table 2). Multivariable linear regression models with T cell subsets and interaction between maternal L-thyroxine use and abdominal symptoms/headaches in 5 year-olds also revealed a significant positive association between $CCR6^+CCR4^+CD161^+$ cells, L-thyroxine use and abdominal symptoms/headaches (coefficient 1.259, P = 0.005, BH-adjusted P = 0.010; Table S3). Figure 10 illustrates the significant higher median frequency of $CCR6^+CCR4^+CD161^+$ cells (2.2%) in children with abdominal symptoms/headaches and maternal L-thyroxine exposure compared to children with no abdominal symptoms/headaches and no L-thyroxine exposure (0.8%). In addition, $CRTh2^+CCR4^{hi}$ cells were negatively associated with maternal medication and abdominal symptoms/headaches, showing decreased frequencies of $CRTh2^+CCR4^{hi}$ cells in children exposed to maternal medication and reporting abdominal symptoms/headaches (coefficient -1.138, P = 0.043; Table 10). However, the association was not statistically significant after adjustment for multiple testing (BH-adjusted P = 0.172; Table 10). Furthermore, multivariable linear regression analyses revealed that $CCR6^+CCR4^+CD161^+$ cells were positively associated with systemic antibiotic use in the first year of life and abdominal symptoms/headaches at 5 years of age. Accordingly, children with abdominal symptoms/headaches showed significant higher frequencies of $CCR6^+CCR4^+CD161^+$ cells when they received antibiotics for a severe infection early in life than children without abdominal symptoms/headaches who did not receive antibiotics (coefficient 0.999, P = 0.004, BH-adjusted P = 0.016; Table 10). Specifically, compared to 5-year-olds without abdominal symptoms/headaches and no early antibiotic use (median frequency = 0.8%), children with both, abdominal symptoms/headaches and early antibiotic use, had significant higher frequencies of $CCR6^+CCR4^+CD161^+$ cells (median frequency = 1.9%; Figure 10B). No significant association was found between $CD4^+$ T cell subsets in children with abdominal symptoms/headaches and maternal chronic diseases or infections during pregnancy (Table 10).

Table 10: Multivariable linear regression analyses of abdominal symptoms/headaches and early-life stressors with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Abdominal symptoms/headaches											
	+ Chronic diseases			+ Maternal medication use			+ Maternal infections			+ Inf. antibiotic use (1st year)		
	N=8			*N=10*			*N=10*			*N=10*		
Baseline: No stressors and no abdominal symptoms	*N=64*			*N=45*			*N=28*			*N=49*		
	Coeff.	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*	*Coeff.*	*P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	1.196	0.690	0.690	-3.430	0.201	0.690	2.218	0.439	0.690	-1.111	0.681	0.690
• $CD4^+CD25^-$ memory cells	-1.232	0.682	0.713	3.441	0.201	0.713	-2.302	0.424	0.713	0.996	0.713	0.713
• $CXCR3^+CCR6^-$ cells	-1.775	0.570	0.900	-3.262	0.242	0.900	-0.375	0.900	0.900	-0.364	0.896	0.900
• $CRTh2^+CCR4^{hi}$ cells	-0.411	0.515	0.515	*-1.138**	*0.043*	0.172	-0.489	0.425	0.515	-0.731	0.209	0.418
• $CCR6^+CCR4^+CD161^+$ cells	0.056	0.881	0.881	*0.849**	*0.014*	*0.028*	0.091	0.803	0.881	*0.999***	*0.004*	*0.016*
• $CCR6^+CCR4^+CD161^-$ cells	-0.437	0.589	0.785	1.143	0.122	0.244	0.098	0.901	0.901	1.351	0.069	0.244
• $CXCR5^{int}$ cells	-0.776	0.589	0.985	-0.024	0.985	0.985	-0.227	0.869	0.985	-1.283	0.295	0.985
• $CXCR5^{hi}$ cells	-1.942	0.357	0.986	-1.110	0.572	0.986	-0.038	0.986	0.986	-0.632	0.749	0.986
• $CD25^+CCR4^-$ $CD45RA^+$ cells	0.534	0.893	0.893	-5.569	0.131	0.262	-1.916	0.618	0.824	-6.548	0.067	0.268
• $CD25^+CCR4^+CD45RA^-$ cells	-0.685	0.827	0.827	1.569	0.600	0.800	2.103	0.512	0.800	4.872	0.100	0.400

*Note. *P-value<0.05; **P-value<0.01; BH-adjusted P-value = Benjamini-Hochberg-adjusted P-value; Inf. antibiotic use. (1st year) = infant antibiotic intake during 1st year of life; abdominal symptoms/headaches + Maternal obesity: N=3; abdominal symptoms/headaches* + PSS-14 sc. >26 *at 1st trimester: N=1; abdominal symptoms/headaches* + PSS-14 sc. >26 *at 2nd trimester: N=4; abdominal symptoms/headaches* PSS-14 sc. >26 *at 3rd trimester: N=1; abdominal symptoms/headaches + pregnancy complications: N=3.*

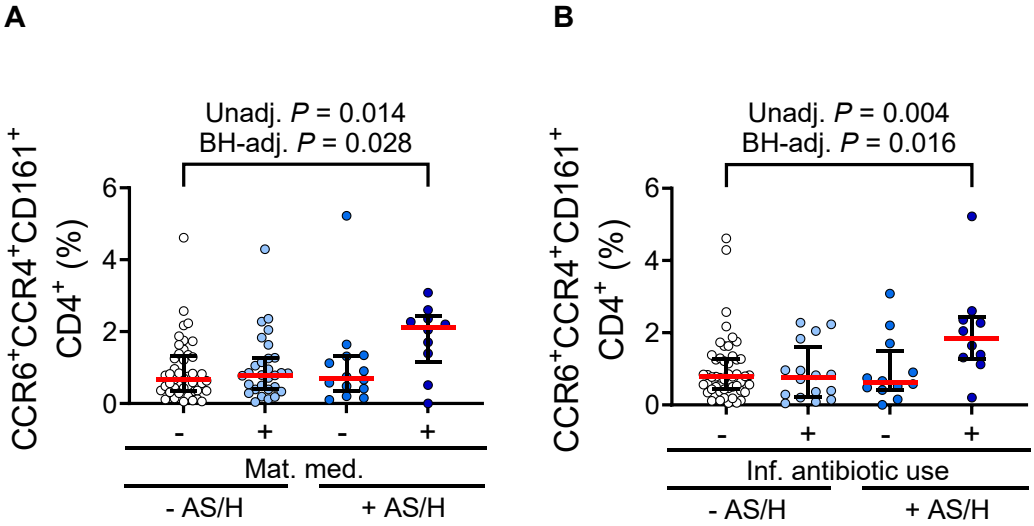


Figure 9: Altered frequencies of CCR6⁺CCR4⁺CD161⁺ CD4⁺ cells in 5-year-old children with abdominal symptoms/headaches (AS/H) are associated with (A) maternal medication use during pregnancy (Mat. med.; FLTR: n = 45; 29; 14; 10), and (B) antibiotic use in infants within the first year of life (Inf. antibiotic use; FLTR: n = 49; 16; 12; 10). Data presented as median percentage (± IQR).

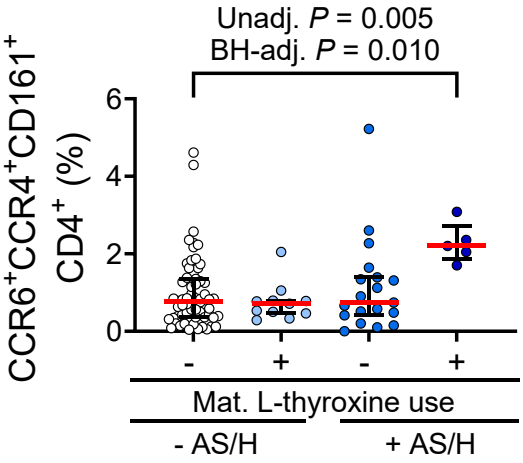


Figure 10: Altered frequencies of CCR6⁺CCR4⁺CD161⁺ CD4⁺ cells in 5-year-old children with abdominal symptoms/headaches (AS/H) are associated with maternal levothyroxine (L-thyroxine) intake (FLTR: n = 63; 11; 19; 5). Data presented as median percentage (± IQR).

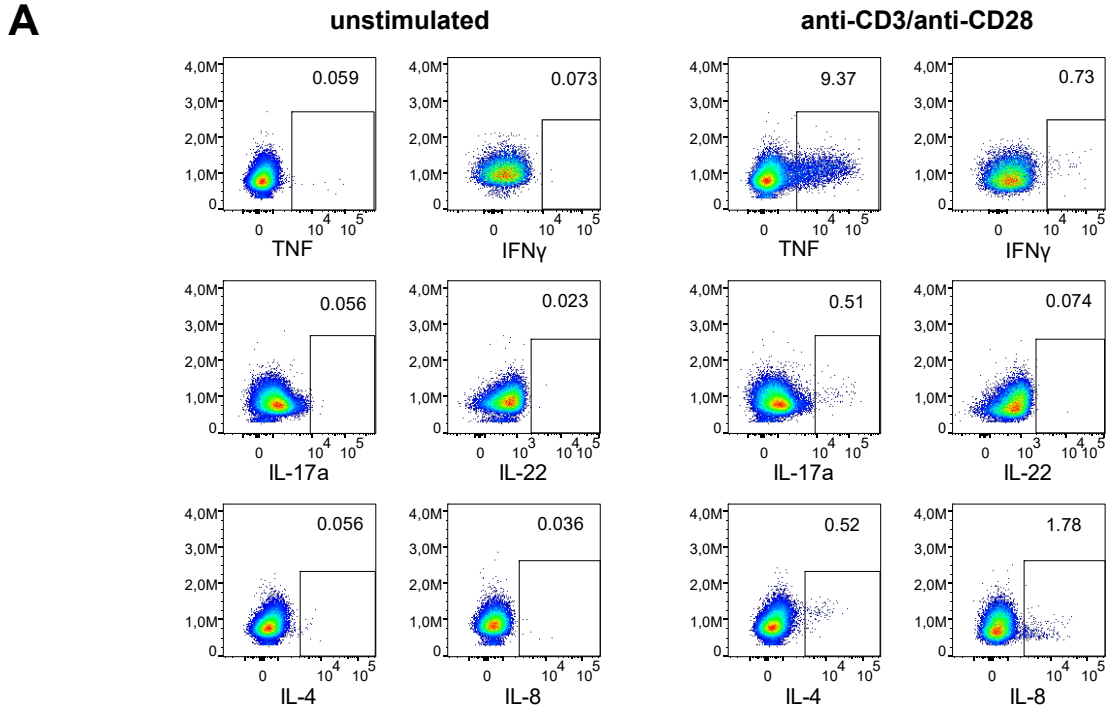
4.2 Functional analyses of CD4$^+$ T cells of 5-year-old children with abdominal symptoms/headaches who had been exposed to maternal medication during pregnancy or infant antibiotic use

The objective of the first part of this study was to examine the association between early-life stressors and the frequencies of CD4$^+$ T cell subpopulations of children at 5 years of age. For this, univariable and multivariable linear regression analyses were conducted using the flow cytometric and demographic data of 118 5-year-old children participating in the PRINCE study. The results indicated that the two stressors, maternal medication use during pregnancy and systemic antibiotic use within the first year of life, were associated with altered frequencies of CD4$^+$ T cell subsets in 5-year-old children. Specifically, children who experienced abdominal symptoms/headaches, exhibited significantly elevated frequencies of CCR6$^+$CCR4$^+$CD161$^+$ cells when they were exposed to maternal medication use during pregnancy or antibiotic use in the first year of life (Table 10; Figure 9). This observation prompted the question whether T cells of children with these exposures and phenotypic changes related to functional properties upon stimulation. To assess the functionality of CD4$^+$ T cells, cytokine production was determined upon TCR-specific stimulation. Based on their receptor expression, CCR6$^+$CCR4$^+$CD161$^+$ cells can be described as Th17-like cells (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Cosmi et al., 2008; Kleinschek et al., 2009). It was therefore of particular relevance to investigate the expression of Th17 cell-related cytokines, such as IL-17A, in comparison to other pro-inflammatory cytokines. For this analysis, isolated PBMCs were assessed from 5-year-old children with abdominal symptoms/headaches and exposure to early-life stressors, namely maternal medication use during pregnancy or infant systemic antibiotic use (symptomatic children; n = 7) and compared to 5-year-old children without abdominal symptoms/headaches and without exposure to both stressors (non-symptomatic children; n = 12). In this analyses, healthy adult PBMC samples were examined as a control to assess the cytokine production upon stimulation (Adults, n = 5). To assess the cytokine production, PBMCs from all groups were stimulated for 18 hours with 2 µg/ml anti-CD3 and anti-CD28 antibodies (anti-CD3 and anti-CD28) to induce TCR-specific activation. Subsequently, intracellular cytokine production of TNF, IFNγ, IL-17A, IL-22, IL-8 and IL-4 was determined by CD4$^+$FOXP3$^-$ memory T cells and CD4$^+$FOXP3$^+$ memory T cells using flow cytometry.

4.2.1 Cytokine production by CD4$^+$FOXP3$^-$ memory T cells of 5-year-old children with abdominal symptoms/headaches and exposure to early-life stressors

Figure 11 presents the response of CD4$^+$FOXP3$^-$ memory T cells following stimulation with anti-CD3 and anti-CD28. Analyses of TNF revealed comparable median frequencies of TNF-producing CD4$^+$FOXP3$^-$ memory T cells in symptomatic children compared to non-

symptomatic children upon TCR-specific stimulation (non-symptomatic children: 3.89 %, IQR 3.18-4.80%; symptomatic children: 3.68%, IQR 1.28-5.61%; *P* = 0.920; Figure 11B). Additionally, the frequencies observed in both groups of 5-year old children were significantly lower than the median frequency observed in adults (8.80%, IQR 6.72-10.70%, non-symptomatic children: *P* = 0.002; symptomatic children: *P* = 0.048). The analysis of IFNγ production yielded similar findings to those observed for TNF production. In particular, there were no significant differences in the median frequencies of IFNγ-producing $CD4^+FOXP3^-$ memory T cells between non- and symptomatic children upon TCR activation (non-symptomatic children: 0.27%, IQR 0.16-0.32%; symptomatic children: 0.26%, IQR 0.07-0.57%, *P* = 0.696; Figure 11B). Compared to adults (1.20%, IQR 0.66-2.79%), the frequencies of IFNγ-producing $CD4^+FOXP3^-$ memory T cells were significantly lower in 5-year old children (non-symptomatic children: *P* = 0.0003; symptomatic children: *P* = 0.0025). Although not significant, the analyses revealed a trend (*P* < 0.15) towards increased frequencies of IL-17A-producing $CD4^+FOXP3^-$ memory T cells (symptomatic group: 0.30%, IQR 0.09-0.55%, *P* = 0.096) and IL-22-producing $CD4^+FOXP3^-$ memory T cells (symptomatic children: 0.05%, IQR 0.03-0.10%, *P* = 0.114) in the symptomatic group as compared to the non-symptomatic group (non-symptomatic children: IL-17A 0.16%, IQR 0.10-0.21%; non-symptomatic children: IL-22 0.03%, IQR 0.01-0.06%; Figure 11B). In addition, IL-17A production as well as IL-22 production observed in the symptomatic group was similar to the production observed in adults (IL-17A: 0.32%, IQR 0.10-0.39%; IL-22: 0.05%, IQR 0.02-0.09%; Figure 11B).



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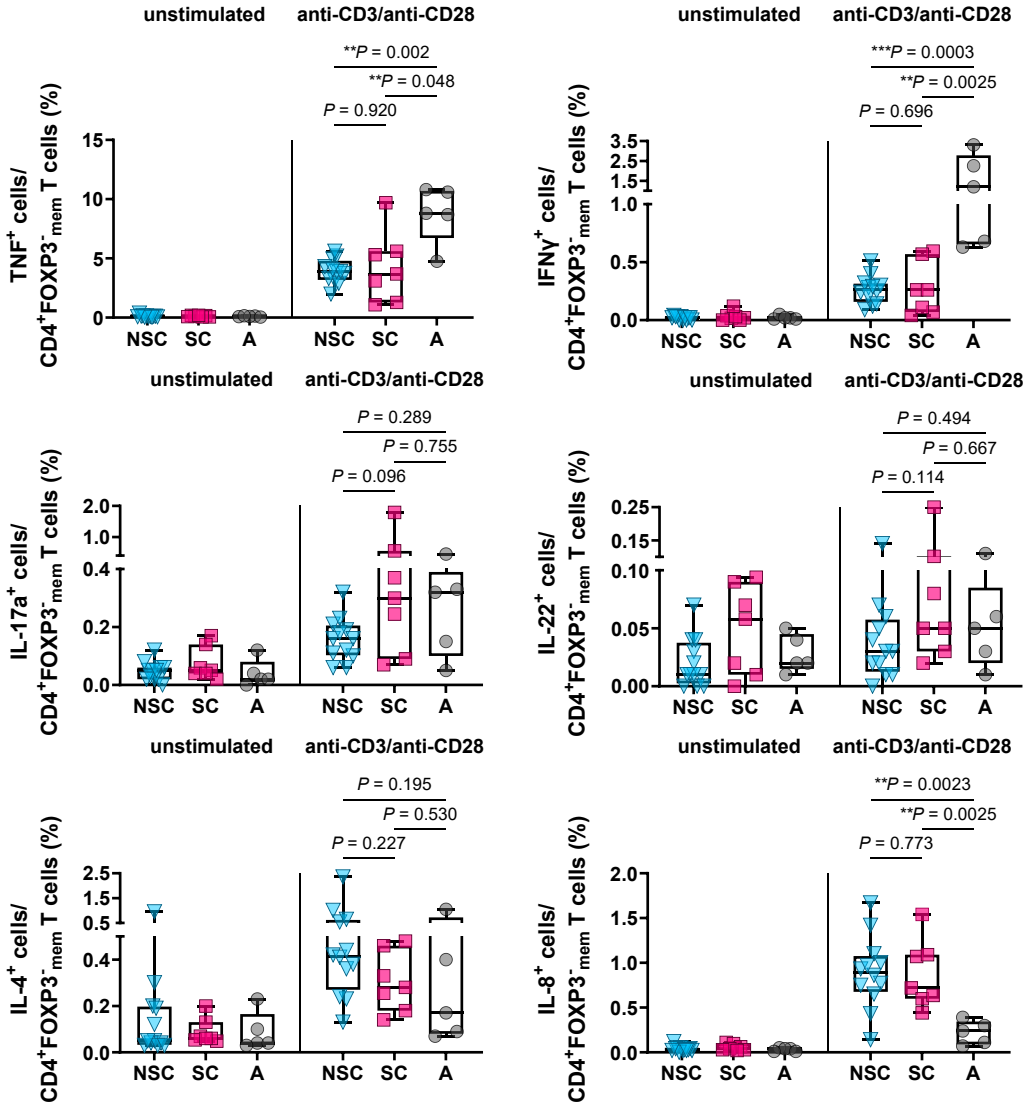


Figure 11: Cytokine production of CD4⁺FOXP3⁻ memory T cells from 5-year-old children with abdominal symptoms/headaches and exposed to early-life stressors, including prenatal maternal medication use or antibiotic use in the 1st year of life (symptomatic children (SC)). Cytokine production of CD4⁺FOXP3⁻ memory T cells from 5-year-olds who were not exposed to stressors and did not have abdominal symptoms/headaches (non-symptomatic children (NSC)), and from adults (A) were used as comparisons. The cytokine response was quantified following 18-hour stimulation with 2 µg/ml anti-CD3 and anti-CD28. **A** Representative flow cytometric plots of TNF-, IFNγ-, IL-17A-, IL-22-, IL-4-, and IL-8-production by CD4⁺FOXP3⁻memory T cells from 5-year-olds upon stimulation with anti-CD3 and anti-CD28 or unstimulated. **B** Frequencies (%) of TNF-, IFNγ-, IL-17a-, IL-22-, IL-4, and IL-8-producing CD4⁺FOXP3⁻ memory T cells. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum/maximum ranges. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

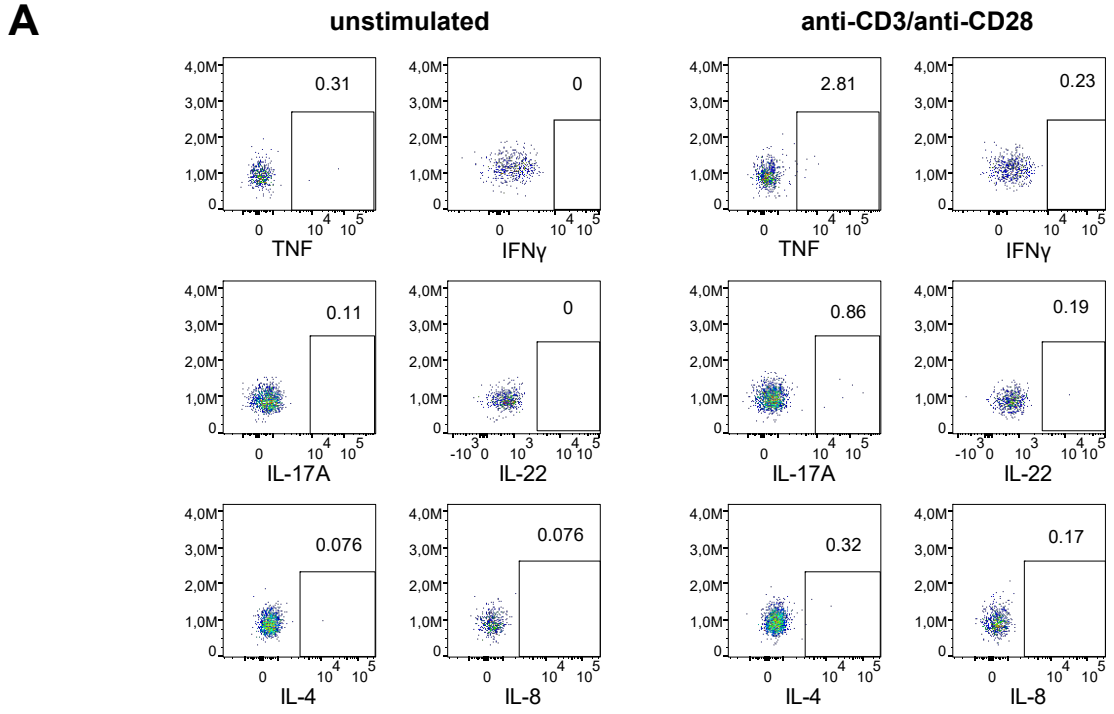
Nevertheless, it is important to note that upon anti-CD3 and anti-CD28 stimulation, IL-22 production was low across all groups, as also previously described by Affandi *et al.* (Affandi et al., 2018). Although not statistically significant, IL-4 production by CD4⁺FOXP3⁻ memory T cells from symptomatic children was slightly lower upon stimulation compared to the non-symptomatic children (non-symptomatic children: 0.42%, IQR 0.27-

0.62%; symptomatic children: 0.28%, IQR 0.18-0.46%, P = 0.227; Figure 11B). Furthermore, the median frequency of IL-4 producing $CD4^+FOXP3^-$ memory T cells in adults was lower compared to 5-year old children (adults: 0.17%, IQR 0.08-0.73%). However, these differences were not statistically significant (non-symptomatic children: *P* = 0.195; symptomatic children: *P* = 0.530). IL-8 production by $CD4^+FOXP3^-$ memory T cells of children was significantly increased upon TCR activation in comparison to adults (adults: 0.24%, IQR 0.09-0.35%; non-symptomatic children: *P* = 0.0023; symptomatic children: *P* = 0.0025). This observation has also been demonstrated in previous studies by Gibbons *et al.* (2014) and Thome *et al*. (2016), which have shown an enhanced production of IL-8 by neonatal and pediatric T cells compared to adult T cells upon stimulation (Gibbons et al., 2014; Thome et al., 2016). However, induction of IL-8 did not significantly differ between the symptomatic and non-symptomatic group upon TCR-specific stimulation in this study (non-symptomatic children: 0.90%, IQR 0.68-1.08%; symptomatic children: 0.72%, IQR 0.60-1.09%; *P* = 0.773; Figure 11B). In sum, TCR stimulation of $CD4^+$ T cells resulted in upregulation of pro-inflammatory cytokines, with a trend towards increased IL-17A and IL-22 production in symptomatic children compared to non-symptomatic children.

4.2.2 Cytokine production by $CD4^+FOXP3^+$ memory T cells of 5-year-old children with abdominal symptoms/headaches and exposure to early-life stressors

Multiple studies have demonstrated that the differentiation towards $Foxp3^+$ Tregs is not a fixed process and that plasticity allows Tregs to differentiate into Th17 cells and vice versa (Beriou et al., 2009; Koenen et al., 2008; Omenetti & Pizarro, 2015). The plasticity of Tregs and Th17 cells is mainly regulated by the cytokine milieu, but also by other environmental factors such as the gut microbiota (Ivanov et al., 2009, 2008; LiLi Xu et al., 2007; L. Zhou et al., 2008). Recent studies reported the presence of IL-17-producing Tregs in human peripheral blood, lymphoid tissue and organs (Hanna et al., 2023; Kluger et al., 2016; Plaza-Sirvent et al., 2021; Voo et al., 2009). These cells have been widely considered as intermediate cells during Treg/Th17 transformation (Cui et al., 2024; Gagliani et al., 2015; Omenetti & Pizarro, 2015). Under physiological conditions, IL-17-producing Tregs can also exert immunosuppressive functions (Beriou et al., 2009; Voo et al., 2009). However, IL-17-producing Tregs are also described to be involved in the pathogenesis of various inflammatory diseases, such as IBD and colitis-associated colorectal cancer (Blatner et al., 2012; Cui et al., 2024; Mitsialis et al., 2020; Quandt et al., 2021; Rizzo et al., 2018). Therefore, following the observed significant increased frequencies of Th17-like cells in 5-year-olds with abdominal symptoms/headaches and exposure to maternal medication or infant antibiotic treatment in the first part of this study, the cytokine response of $CD4^+FOXP3^+$ memory T cells was next assessed upon TCR-specific stimulation (Figure 12).

Similar to CD4⁺FOXP3⁻ memory T cells, there were no significant differences in cytokine production between CD4⁺FOXP3⁺ memory T cells in non- and symptomatic children (Figure 12B). Upon TCR- specific stimulation with anti-CD3 and anti-CD28, TNF and IFNγ production by CD4⁺FOXP3⁺ memory T cells did not differ between both pediatric groups (non-symptomatic children: TNF 1.41%, IQR 1.08-2.23%, and symptomatic children: TNF1.79%, IQR 1.27-2.98%, *P* = 0.385; non-symptomatic children: IFNγ 0.21%, IQR 0.12-0.30%, and symptomatic children: IFNγ 0.20%, IQR 0.14-0.48%, *P* = 0.853; Figure 12B). In adults, median frequencies of TNF and IFNγ-producing CD4⁺FOXP3⁺ memory T cells following TCR-activation were increased in comparison to 5-year-olds (adults: TNF 2.97%, IQR 1.02-6.67%; adult: IFNγ 0.48%, IQR 0.25-1.50%; Figure 12B). However, the statistical analysis did not reveal a significant difference between the group of adults and the two groups of 5-year old children (non-symptomatic children (TNF): *P* = 0.234; symptomatic children (TNF): *P* = 0.530; non-symptomatic children (IFNγ): *P* = 0.109; symptomatic children (IFNγ): *P* = 0.202). Similar to CD4⁺FOXP3⁻ memory T cells, the flow cytometry analyses revealed a trend towards increased frequencies of IL-17A-producing CD4⁺FOXP3⁺ memory T cells in symptomatic children following stimulation with anti-CD3 and anti-CD28, as compared to non-symptomatic children (non-symptomatic children: 0.12%, IQR 0.03-0.21%; symptomatic children: 0.29%, IQR 0.12-0.56%, *P* = 0.058; Figure 12B). Furthermore, similar to the production of IL-17A by CD4⁺FOXP3⁻ memory T cells, the IL-17A production by CD4⁺FOXP3⁺ memory T cells of symptomatic children was comparable to that observed in adults (adults: 0.30%, IQR 0.06-0.50%; Figure 12B).



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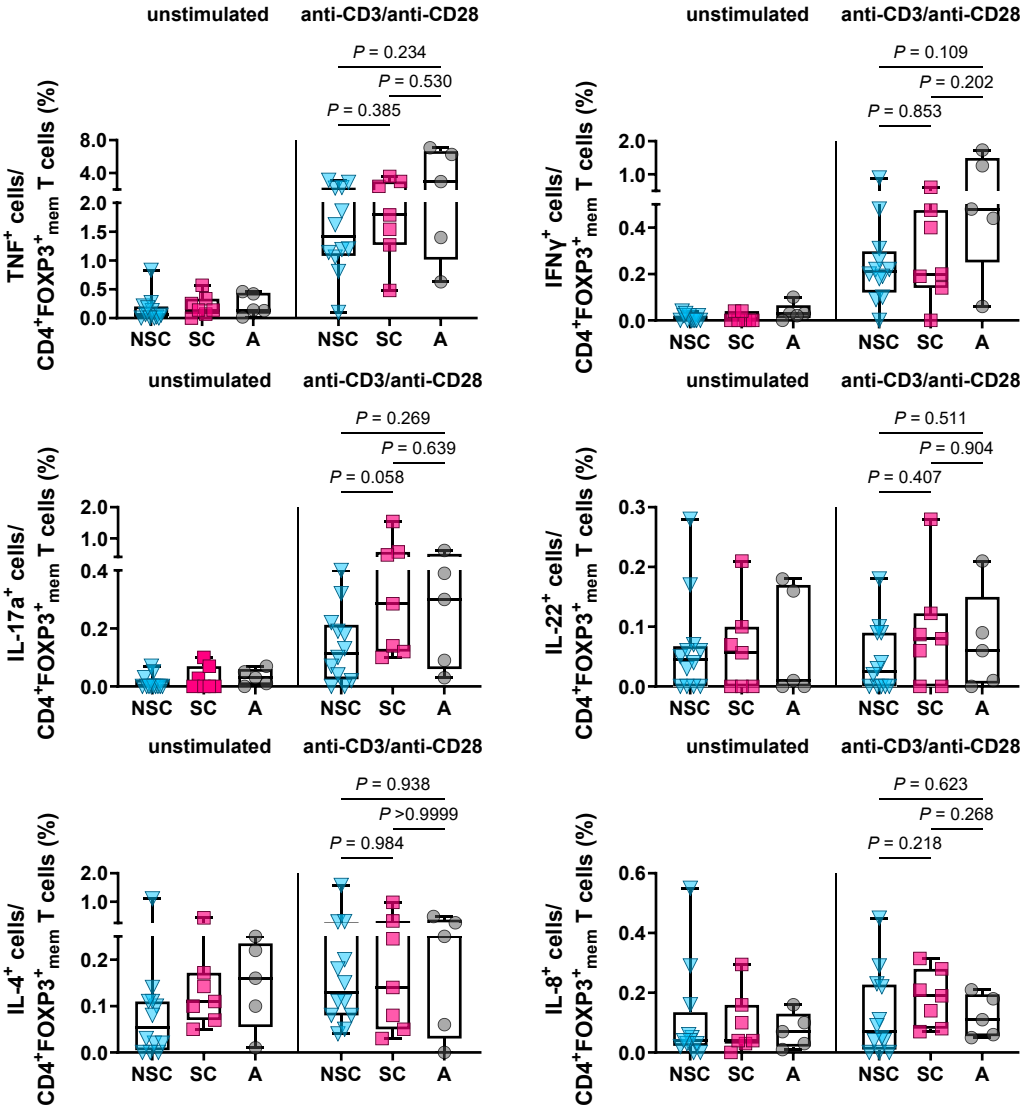

Figure 12: Cytokine production of CD4⁺FOXP3⁺ memory T cells from 5-year-old children with abdominal symptoms/headaches and exposed to early-life stressors, including prenatal maternal medication use or antibiotic use in the 1st year of life (symptomatic children (SC)). Cytokine production of CD4⁺FOXP3⁺ memory T cells from 5-year-olds who were not exposed to stressors and did not have abdominal symptoms/headaches (non-symptomatic children (NSC)), and from adults (A) were used as comparisons. The cytokine response was quantified following 18-hour stimulation with 2 µg/ml anti-CD3 and anti-CD28. **A** Representative flow cytometric plots of TNF-, IFNγ-, IL-17A-, IL-22-, IL-4-, and IL-8-production by CD4⁺FOXP3⁺ memory T cells from 5-year-olds upon stimulation with anti-CD3 and anti-CD28 or unstimulated. **B** Frequencies (%) of TNF-, IFNγ-, IL-17a- IL-22-, IL-4, and IL-8-producing CD4⁺FOXP3⁺ memory T cells. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile ranges and whiskers indicate minimum/maximum ranges. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

Induction of IL-22, IL-4 and IL-8-production upon TCR-specific stimulation did not differ between the two pediatric groups (non-symptomatic children (IL-22): 0.03%, IQR 0.00-0.09%, and symptomatic children (IL-22): 0.08%, IQR 0.00-0.12%, *P* = 0.407; non-symptomatic children (IL-4): 0.13%, IQR 0.08-0.27%, and symptomatic children (IL-4): 0.14%, IQR 0.05-0.32%, *P* = 0.984; non-symptomatic children (IL-8): 0.07%, IQR 0.01-0.23%, and symptomatic

children (IL-8): 0.19%, IQR 0.08-0.28%, *P* = 0.218; Figure 12B). In general production of IL-22, IL-4 and IL-8 by $CD4^+FOXP3^+$ memory T cells was low suggesting little production of these cytokines by $CD4^+FOXP3^+$ memory T cells (Figure 12A).

4.2.3 IL-6 in $CD14^+$ monocytes and $CD19^+$ B cells, and IL-6 receptor expression on $CD4^+FOXP3^-$ memory T cells and $CD4^+FOXP3^+$ memory T cells in children and adults

One of the earliest recognized cytokines associated with maternal immune activation is IL-6 (Buka et al., 2001; Ye Li et al., 2023; Spann et al., 2018). In response to tissue injury or infections, IL-6 is secreted by various cell types upon pattern recognition receptor activation (Greenhill et al., 2011; D. Li & Wu, 2021). Besides stromal cells, these cell types also include immune cells, such as neutrophils, monocytes and B cells (Chalaris et al., 2007; Hughes, Onore, Careaga, Rogers, & Ashwood, 2022; Linge et al., 2022; Ospelt et al., 2009; Villar-Fincheira et al., 2021; West, 2019). IL-6 plays a pivotal role in the differentiation of Th17 cells and the secretion of IL-17A in the presence of IL-21, IL-23, IL-1β and transforming growth factor-β (TGF-β; Yang et al., 2009; Zhou et al., 2007). Furthermore, elevated levels of IL-6 and enhanced frequencies of Th17 cells are associated with inflammatory disorders such as IBD (Fujino et al., 2003; Gross, Andus, Caesar, Roth, & Schölmerich, 1992; Hyams, Fitzgerald, Treem, Wyzga, & Kreutzer, 1993; Mudter & Neurath, 2007). In the present study, multivariable linear regression analyses revealed a significant association between increased frequencies of $CCR6^+CCR4^+CD161^+$ cells in 5-year-old children with abdominal symptoms/headaches and exposure to maternal medication use during pregnancy or systemic antibiotic use within the first year of life. As described above, $CCR6^+CCR4^+CD161^+$ cells can be described as Th17-like cells based on their receptor expression (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Cosmi et al., 2008; Kleinschek et al., 2009). To assess whether exposure to an altered production of IL-6 might be associated with the elevated presence of $CCR6^+CCR4^+CD161^+$ cells in these children, the intracellular IL-6 production in monocytes and B cells of these children was examined by flow cytometry. To assess IL-6 production upon stimulation, PBMCs of non- and symptomatic children were stimulated for 18 hours with PMA and ionomycin (PMA/ionomycin). In this analysis, monocytes were discriminated as $CD14^+$ cells and B cells as $CD19^+$ cells. Given that the $CD14^+$ monocyte population undergoes a decline due to differentiation to macrophages following 18 hours of PMA/ionomycin treatment (data not shown), the presence of IL-6 in $CD14^+$ monocytes was solely evaluated under unstimulated conditions (R. J. Phillips, Lutz, & Premack, 2005). B cell populations did not change after PMA/ionomycin stimulation, therefore the expression of IL-6 by $CD19^+$ B cells was assessed under unstimulated conditions and following stimulation with PMA/ionomycin stimulation (Figure S3B, S3C). To determine intracellular IL-6 expression, adult $CD4^+FOXP3^-$ cells and

adult $CD14^+CD19^-$ monocytes were used as negative and positive gating controls, respectively (Figure S3A).

Overall, the analyses revealed no significant differences in IL-6 presence in $CD14^+$ monocytes and $CD19^+$ B cells between non- and symptomatic children (Figure S3). Specifically, the median frequency of IL-6-expressing $CD14^+$ monocytes of symptomatic children were comparable to the median frequency observed in non-symptomatic children (non-symptomatic children: 1.92%, IQR 0.38-4.27%, and symptomatic children ($CD14^+CD19^-$ monocytes): 2.28%, IQR 0.30-11.30%, $P = 0.773$; Figure S3C). However, whether this would be similar observed in stimulated monocytes and macrophages remains to be determined. Similarly, comparable median frequencies of IL-6-expressing $CD19^+$ B cells were observed between non- and symptomatic children, both unstimulated and upon PMA/Ionomycin stimulation (non-symptomatic children (unstimulated): 1.06%, IQR 0.72-1.41%, and symptomatic children (unstimulated): 0.92%, IQR 0.51-1.62%, $P = 0.0482$; non-symptomatic children (PMA/Ionomycin): 1.40%, IQR 0.78-3.43%, and symptomatic children (PMA/Ionomycin): 1.15%, IQR 0.48-4.10%, $P = 0.711$; Figure S3C). The latter results generally indicate a low IL-6-stimulatory effect after 18 hours of PMA/ionomycin. In this regard, it is important to note that, due to the small cell numbers, multiple stimulations tailored to monocytes such as TLR stimulation and B cells with CD40L and IL-4 could not be performed. Therefore, these findings must be interpreted in this light and further studies are needed.

An enhanced sensitivity of $CD4^+$ T cells for IL-6 due to altered expression of the IL-6Rα may underlie elevated frequencies of Th17-like cells. The binding of IL-6 to the membrane-bound IL-6Rα and the membrane-bound signal transducing subunit glycoprotein 130 (gp130) is described as classical IL-6 signaling, which plays a crucial role in differentiation and maintenance of Th17 cells (Harbour, Ditoro, et al., 2020; Stefan Rose-John, Jenkins, Garbers, Moll, & Scheller, 2023; Taga et al., 1989). To determine IL-6Rα expression on $CD4^+FOXP3^-$ memory T cells and $CD4^+FOXP3^+$ memory T cells, $CD8^+CD4^-$ cells and $CD14^+$monocytes were used as negative and positive gating controls, respectively (Figure 13A).

Analyses of IL-6Rα expression on $CD4^+FOXP3^-$ memory T cells and $CD4^+FOXP3^+$ memory T cells revealed no significant difference between non-symptomatic and symptomatic children under unstimulated and TCR stimulated conditions (Figure 13). In particular, in unstimulated samples, the median frequency of $IL\text{-}6R\alpha^+CD4^+FOXP3^-$ memory T cells observed in samples from symptomatic children was similar to the frequency observed in samples from non-symptomatic children (non-symptomatic children: 0.57%, IQR 0.13-1.44%, symptomatic children: 0.49%, IQR 0.13-0.68%, $P > 0.999$; Figure 13B). Upon anti-CD3 and anti-CD28 stimulation, $CD4^+FOXP3^-$ memory T cells from symptomatic 5-year old children showed a slightly higher expression of IL6-Rα than $CD4^+FOXP3^-$ memory T cells from non-symptomatic

5-year olds (non-symptomatic children: 0.46%, IQR 0.33-1.51%; symptomatic children: 0.87%, IQR 0.22-1.66%; Figure 13B). However these findings were not considered significant (*P* = 0.855).

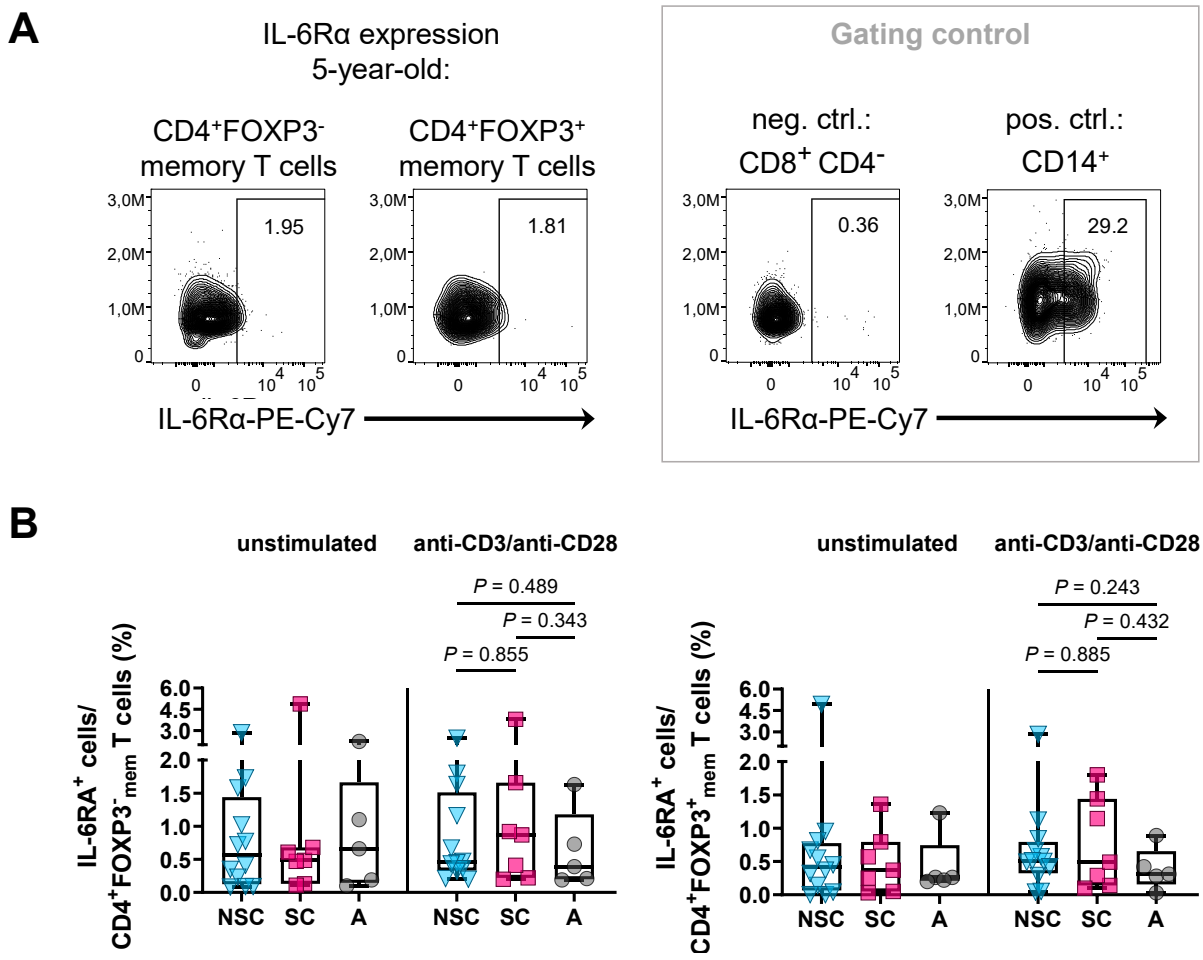

Figure 13: IL-6Rα-expression of CD4⁺FOXP3⁻ memory T cells and CD4⁺FOXP3⁺ memory T cells from 5-year-old children with abdominal symptoms/headaches and exposed to early-life stressors, including prenatal maternal medication use or antibiotic use in the 1st year of life (symptomatic children (SC)). IL-6Rα expression of CD4⁺FOXP3⁻ memory T cells and CD4⁺FOXP3⁺ memory T cells from 5-year-olds who were not exposed to stressors and did not show abdominal symptoms/headaches (non-symptomatic children (NSC)), and from adults (A) were used as comparisons. IL-6Rα-expression was quantified following 18-hour stimulation with 2 µg/ml anti-CD3 and anti-CD28 or unstimulated. **A** Representative flow cytometric plots of the gating strategies to identify IL-6Rα-positive CD4⁺FOXP3⁻ memory T cells and CD4⁺FOXP3⁺ memory T cells. **B** Frequencies (%) of IL-6Rα-expressing CD4⁺FOXP3⁻ memory T cells and CD4⁺FOXP3⁺ memory T cells. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile range and whiskers indicate minimum/maximum range. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

Similarly, the IL-6Rα expression on unstimulated CD4⁺FOXP3⁺ memory T cells of symptomatic children was comparable to the expression determined on unstimulated CD4⁺FOXP3⁺ memory T cells of non-symptomatic children (non-symptomatic children: 0.41%, IQR 0.07-0.78%, and symptomatic children: 0.37%, IQR 0.05-0.80%, *P* = 0.984; Figure 13B). Furthermore, the median frequency of IL-6Rα⁺CD4⁺FOXP3⁻ memory T cells observed in samples from symptomatic children did also not differ from the median frequency of IL-

6Rα^{+}CD4^{+}FOXP3^{-} memory T cells observed in samples from non-symptomatic children upon TCR-specific stimulation (non-symptomatic children: 0.51%, IQR 0.32-0.80%, symptomatic children: 0.49%, IQR 0.14-1.44%, *P* = 0.718; Figure 13B).

4.2.4 Thyroid receptor expression in CD4^{+} T cells of 5-year-old children with abdominal symptoms/headaches and exposure to early-life stressors

Out of the 118 women included the PRINCE study who were examined in the course of this study, 48 participants have been taking medication during pregnancy (Table 2). Among these, 19 participants were taking the thyroid medication L-thyroxine (Table 2). Multivariable linear regression models subsequently indicated a significant association between the exposure of maternal L-thyroxine intake during pregnancy and increased frequencies of CCR6^{+}CCR4^{+}CD161^{+} cells in 5-year old children with the reported abdominal symptoms/headaches (Table S3; Figure 10). L-thyroxine mimics the thyroid hormone, thyroxine (T4), and is used to treat hypothyroidism and other conditions associated with thyroid dysfunction (Chalmers, Dickson, Elks, & Hems, 1949; Harington & Barger, 1927; Kahaly & Gottwald-Hostalek, 2022). In the organism, T4 or L-thyroxine is converted into the physiologically active thyroid hormone triiodothyronine (T3, Boye & Laurberg, 1984)). T3 binds in the nucleus to thyroid receptor (TR) proteins, including the TR isoforms TRα and TRβ (Sap et al., 1986; Weinberger et al., 1986). The complex binds to other cofactors and induces the transcription of specific target genes, thereby playing an important role in the maintenance of vital processes, including growth, brain function, food metabolism, and body temperature (Kahaly & Gottwald-Hostalek, 2022). Furthermore, *in vitro* and *in vivo* models indicated a direct effect of thyroid hormones on T cell immunity by inducing proliferation, activation and apoptosis of T cells (Aoki, Wakisaka, & Nagata, 1976; Barreiro Arcos et al., 2011; Mihara et al., 1999; Wenzek et al., 2022). In this study, a flow cytometry antibody targeting the TR isoforms TRα1 and TRβ1 (TRα1/β1) was used to assess the TR expression in CD4^{+}FOXP3^{-} cells and CD4^{+}FOXP3^{+} cells of symptomatic and non-symptomatic 5-year-old children and adults. Receptor expression was examined in unstimulated samples as well as upon TCR-specific stimulation. To determine thyroid receptor expression, CD19^{+} B cells were used as a negative gating control and CD8^{-}CD4^{-} cells, which likely are NK cells, were used as a positive gating control (Figure 14A).

Assessment of TRα1/β1-expression in CD4^{+}FOXP3^{-} cells and CD4^{+}FOXP3^{+} cells revealed no significant differences between non- and symptomatic children (Figure 14B). Specifically, in unstimulated samples, frequencies of TRα1/β1^{+}CD4^{+}FOXP3^{-} cells from symptomatic children was comparable to frequencies observed in samples from non-symptomatic children (non-symptomatic children: 0.77%, IQR 0.19-3.43%, and symptomatic children: 0.64%, IQR 0.19-4.56%, *P* = 0.902). A similar observation was made for the TRα1/β1-expression in unstimulated CD4^{+}FOXP3^{+} cells between 5-year old symptomatic children and 5-year-old non-

symptomatic children (non-symptomatic children: 1.13%, IQR 0.65-2.60%, and symptomatic children: 0.85%, IQR 0.44-3.12%, *P* = 0.522). However, upon TCR-specific stimulation, the median frequencies of TRα1/β1$^+$CD4$^+$FOXP3$^-$ cells and TRα1/β1$^+$CD4$^+$FOXP3$^+$ cells were slightly higher in samples of symptomatic children compared to samples of non-symptomatic children (non-symptomatic children (CD4$^+$FOXP3$^-$ cells): 0.56%, IQR 0.30-0.92%, and symptomatic children (CD4$^+$FOXP3$^-$ cells): 1.25%, IQR 0.08-2.28%, *P* = 0.837; non-symptomatic children (CD4$^+$FOXP3$^+$ cells): 0.70%, IQR 0.38-4.06%, and symptomatic children (CD4$^+$FOXP3$^+$ cells): 1.28%, IQR 0.22-2.64%, *P* = 0.712).

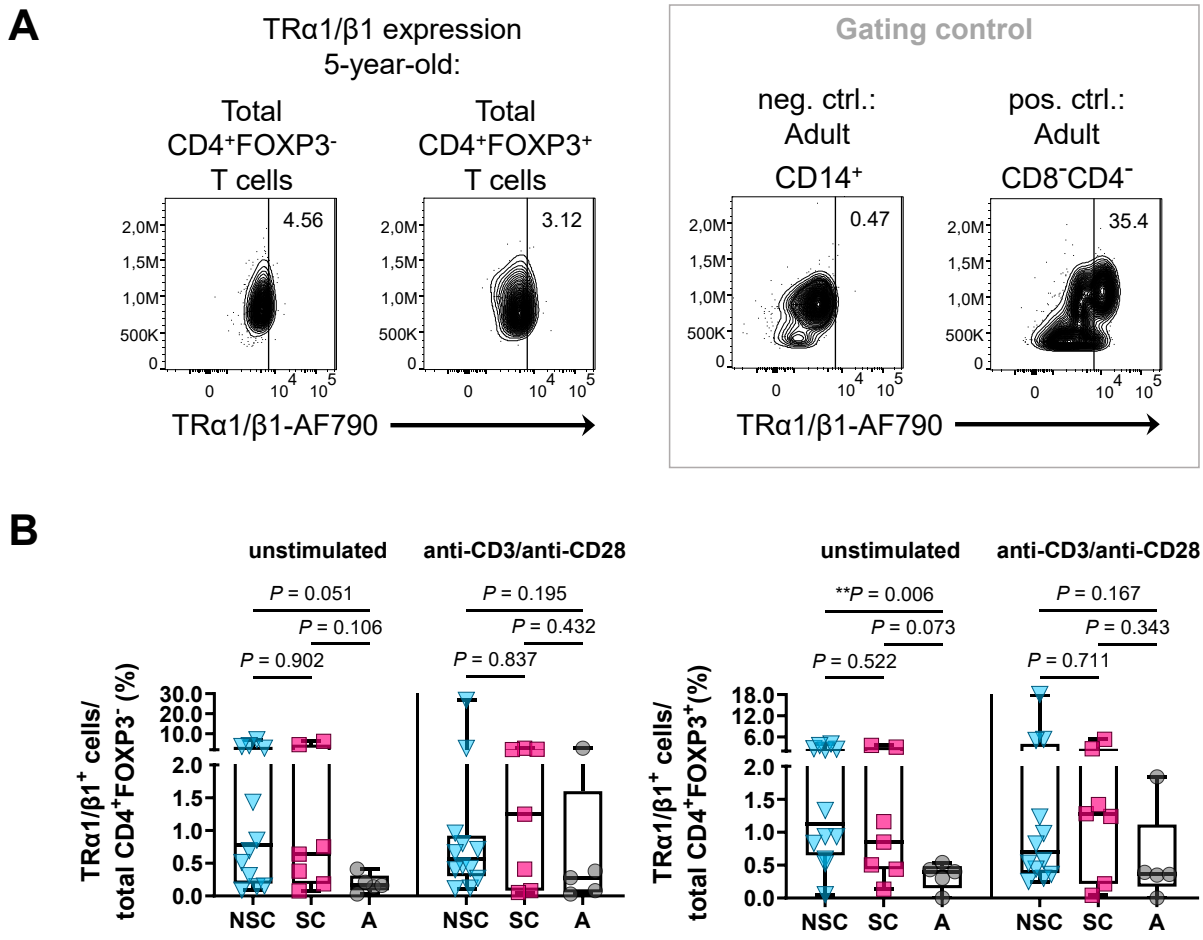

Figure 14: Thyroid receptor α1 and β1 expression (TRα1/β1) expression of total CD4$^+$FOXP3$^-$ T cells and total CD4$^+$FOXP3$^+$ T cells from 5-year-old children with abdominal symptoms/headaches and exposed to early-life stressors, including prenatal maternal medication use or antibiotic use in the 1st year of life (symptomatic children (SC)). TRα1/β1 expression of total CD4$^+$FOXP3$^-$ T cells and total CD4$^+$FOXP3$^+$ T cells from 5-year-old children who were not exposed to stressors and did not show abdominal symptoms/headaches (non-symptomatic children (NSC)), and from adults (A) were used as comparisons. TRα1/β1-expression was quantified following 18-hour stimulation with 2 µg/ml anti-CD3 and anti-CD28 or unstimulated. **A** Representative flow cytometric plots of the gating strategies to identify TRα1/β1-positive CD4$^+$FOXP3$^-$ T cells and CD4$^+$FOXP3$^+$ T cells. **B** Frequencies (%) of TRα1/β1-expressing CD4$^+$FOXP3$^-$ T cells and CD4$^+$FOXP3$^+$ T cells. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile range and whiskers indicate minimum/maximum range. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

Compared to 5-year-olds, TRα1/β1 expression in adult CD4$^+$FOXP3$^-$ cells and in adult CD4$^+$FOXP3$^+$ cells was lower, both unstimulated and TCR-specific stimulated (adult

$CD4^+FOXP3^-$ cells (unstimulated): 0.16%, IQR 0.09-0.31%, and stimulated: 0.28%, IQR 0.06-1.60%; adult $CD4^+FOXP3^+$ cells (unstimulated): 0.40%, IQR 0.15-0.49%, and stimulated: 0.36%, IQR 0.18-1.12%; Figure 14B).

4.3 Altered cytokine levels in third-trimester serum samples of mothers of children exposed to early-life stressors

A number of epidemiological studies have led to the formulation of the developmental origins of health and disease (DOHaD; Barker, Osmond, Winter, Margetts, & Simmonds, 1989; Barker et al., 1993; Barker, 2007; Barker & Osmond, 1986; Shimizu, Sakata-Haga, Saikawa, & Hatta, 2023). This theory hypothesizes that at critical time points during gestation, the fetus is more susceptible to developmental alterations following exposure to adverse intrauterine factors (Barker, 2007). One potential mechanism underlying the DOHaD hypothesis is MIA (Jain et al., 2021; Shimizu et al., 2023, 2021; Terasaki & Schwarz, 2016). During pregnancy, the maternal immune system can be activated due to various stressors, such as maternal infections, high psychological stress, or autoimmune diseases (Andersson, Li, Mills, Ly, Nomura, Hospital, et al., 2016; Antonson et al., 2020; H. J. Chen et al., 2020; Jain et al., 2021; Lim et al., 2021; Shimizu et al., 2023, 2021). This results in an increased production of cytokines that can be transmitted to the fetus via amniotic fluid, placenta, or maternal serum (Brynge et al., 2022; Paraschivescu et al., 2020; S. E. P. Smith et al., 2007; Urakubo et al., 2001; Zaretsky et al., 2004). MIA is linked to aberrant immunity in offspring, including exaggerated immune responses or immune deficiency, and the emergence of immunological disorders (Atladóttir et al., 2010; Brown, 2012; Hodyl et al., 2007; McKeever et al., 2002; Onore et al., 2014; Shimizu et al., 2023; Yue et al., 2018). In this study, children exposed to early-life stressors, including maternal medication during pregnancy and infant systemic antibiotic use in the first year of life, exhibited altered frequencies of $CCR6^+CCR4^+CD161^+$ cells at 5 years of age. Furthermore, these 5-year-olds reported more frequently abdominal pain, headache and nausea (abdominal symptoms/headaches; Table 10; Figure 9). In order to assess whether these children had been exposed to MIA during pregnancy, concentrations of specific cytokines and effector molecules associated with inflammation were determined in the serum samples of their mothers taken at the third trimester. The analysis included the serum samples of all mothers from the symptomatic and non-symptomatic children that had been examined in the second part of this study (see section 4.2), with serum samples from mothers of symptomatic children (MSC; n = 7) and serum samples from mothers of non-symptomatic children (MNSC; n = 12). In addition, serum samples from 5 non-pregnant women were used as non-pregnant controls (NP; n = 5). To quantify the concentration of CRP, IL-6, soluble IL-6Rα, soluble gp130, CXCL5, CXCL10, IL-1β, IL-2, IL-4, IL-6, IL-17A, IL-18 and TNF, multiplex-bead assays were performed. However, the values of IL-6, IL-1β, IL-2, TNF, IL-17A, were below the reliable detection range and could therefore not be subjected to interpretation.

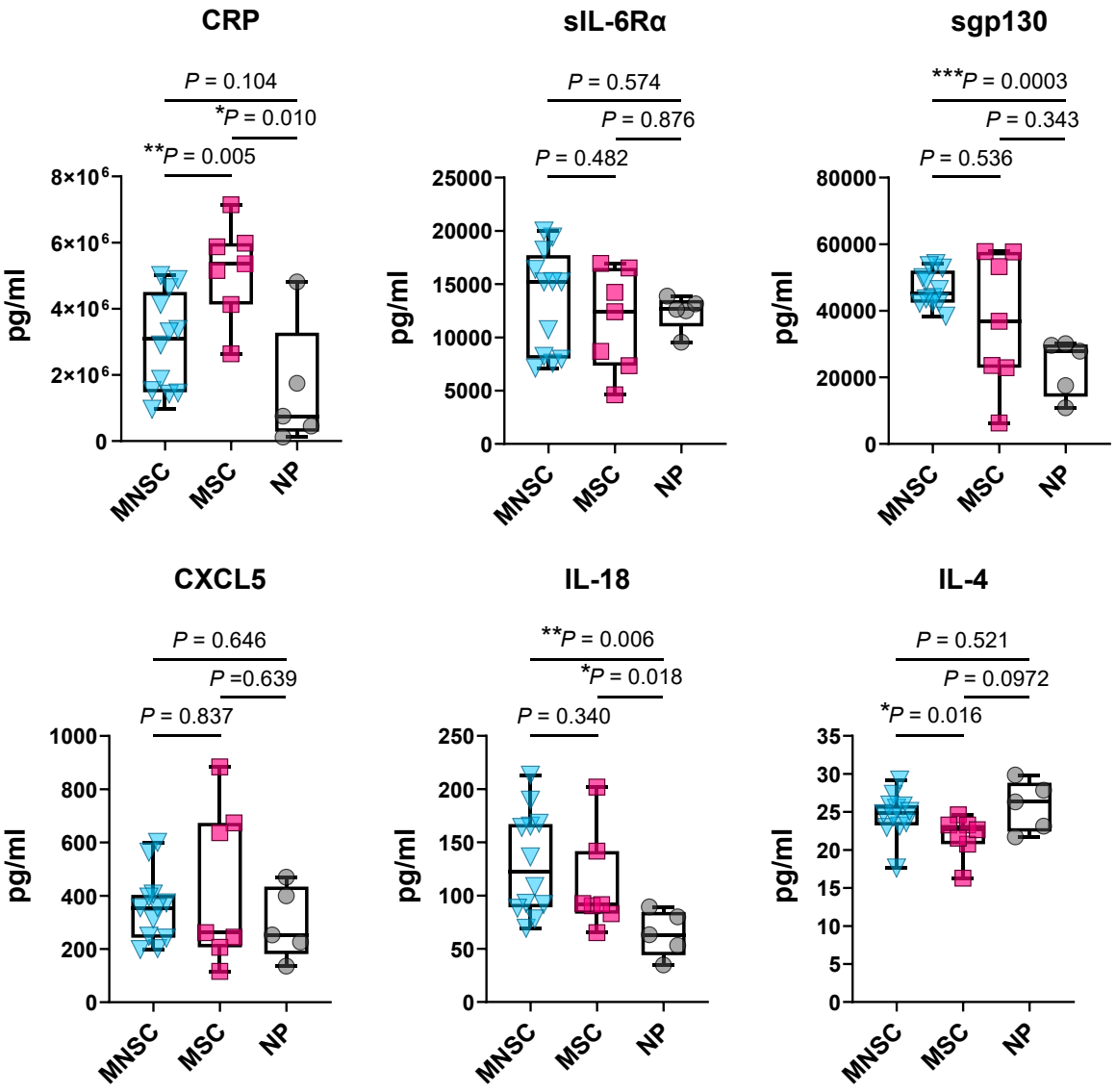

Figure 15: Altered cytokine concentrations in third trimester serum samples from women whose children experienced abdominal symptoms/headaches at the age of 5 and exposed to early-life stressors, including prenatal maternal medication use or antibiotic use in the 1st year of life (mothers of symptomatic children (MSC)). Cytokine concentrations in third trimester serum samples from women whose children were not exposed to stressors and did not show abdominal symptoms/headaches (mothers of non-symptomatic children (MNSC)), and from non-pregnant women (NP) were used as comparisons. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile range and whiskers indicate minimum/maximum range. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

The results of the cytokine measurements are presented in Figure 15. Mothers of symptomatic children had significantly increased levels of CRP compared to mothers of non-symptomatic children (MNSC: 3,103,150 pg/ml, IQR 1,473,775 – 4,509,100 pg/ml; MSC: 5,358,600 pg/ml, IQR 4,130,600 – 5,979,000 pg/ml, *P* = 0.005). As also described by previous studies, the CRP levels in non-pregnant women were lower than those observed in pregnant women of both groups in this study (NP: 751,613 pg/ml, IQR 287,537 – 3,279,350 pg/ml; MNSC: *P* = 0.104; MSC: *P* = 0.010; Belo et al., 2005; Jarmund et al., 2021). In general, CRP is primarily

synthesized by liver hepatocytes in response to IL-6 following the onset of inflammation, infection, or tissue damage (Boras et al., 2014; Geyer et al., 2021; Rhodes, Fürnrohr, & Vyse, 2011). IL-6 signaling can occur in multiple forms. Trans-signaling is initiated by the binding of IL-6 to a soluble form of the IL-6 receptor (sIL-6Rα) and the membrane-bound signal transducing subunit gp130 (Mülberg et al., 1993; S. Rose-John & Heinrich, 1994). A soluble form of gp130 (sgp130), in turn, had been shown to block trans-signaling via IL-6/ sIL-6Rα and is associated with a reduction in IL-6-induced pro-inflammatory effects (Hong et al., 2016; Jostock et al., 2001; Narazaki et al., 1993). To assess these IL-6-signaling associated mechanisms, the concentrations of sIL-6Rα and sgp130 were quantified in the maternal serum samples. The results showed that the levels of both, sIL-6Rα and sgp130, did not significantly differ between mothers of symptomatic and non-symptomatic children (sIL-6Rα: $P = 0.482$; sgp130: $P = 0.536$). Specifically, the median concentration of sIL-6Rα in the MSC group was 12,408 pg/ml (IQR 7,344 – 16,529 pg/ml,) and 15,200 pg/ml (IQR 8,016 – 17,726 pg/ml) in the MNSC group samples. For sgp130, a median concentration of 36,825 pg/ml (IQR 22,902-57,672 pg/ml,) was measured in serum samples of mothers of symptomatic children and a median concentration of 45,195 pg/ml (IQR 42,421 – 52,100 pg/ml) was quantified in samples of mothers of non-symptomatic children. The levels of sIL-6Rα quantified in the serum of non-pregnant women were comparable to those of the pregnant women (NP: 12,646 pg/ml, IQR 11,029 – 13,513 pg/ml), whereas sgp130 levels were significantly lower in non-pregnant women as compared to pregnant women in the MNSC group (NP: 27,873 pg/ml, IQR 14,185 – 29,886 pg/ml; MNSC: $P = 0.0003$). The differences in sgp130 serum-concentrations between pregnant women in the MSC group and non-pregnant women did not reach statistical significance ($P = 0.343$). Moreover, CXCL5 levels were not significantly different between mothers of symptomatic and non-symptomatic children (MNSC: 355.0 pg/ml, IQR 242.5 – 403.2 pg/ml; MSC: 261.1 pg/ml, IQR 206.2 – 673.9 pg/ml; $P = 0.837$) and also did not significantly differ from those observed in the serum of non-pregnant women (252.7 pg/ml, IQR 181.0 – 434.7 pg/ml; MNSC: $P = 0.646$; MSC: $P = 0.639$). Also, IL-18 levels were similar between the two groups of pregnant women (MNSC: 122.40 pg/ml, IQR 88.98 – 167.20 pg/ml; MSC: 91.45 pg/ml, IQR 83.08 – 141.70 pg/ml; $P = 0.340$). In addition, the levels of IL-18 quantified in the serum of pregnant women in both groups, mothers of symptomatic and non-symptomatic children, were significantly higher than those observed in the serum of non-pregnant women (NP: 63.19 pg/ml, IQR 44.07 – 84.69 pg/ml; MNSC: $P = 0.006$; MSC: $P = 0.018$). This observation is also in line with a study by Ida *et al.* (2000) who previously demonstrated the upregulation of IL-18 concentrations in the serum of pregnant women when compared to non-pregnant women (Ida et al., 2000). Furthermore, a significant difference was observed in serum IL-4 levels between the MSC and MNSC groups of pregnant women in this study ($P = 0.016$). In particular, the median concentration of IL-4 was notably lower in the

serum of mothers of symptomatic children in comparison to mothers of non-symptomatic children (MNSC: 24.91 pg/ml, IQR 23.24 – 25.88 pg/ml; MSC: 22.66 pg/ml, IQR 20.75 – 23.27 pg/ml). Both serum IL-4 levels did not significantly differ from serum IL-4 levels determined in the non-pregnant control group (NP: 26.33 pg/ml, IQR 22.42 – 28.85 pg/ml; MNSC: $P = 0.521$; MSC: $P = 0.097$). In conclusions, serum levels of pregnant women giving birth to children with early-life stressors and abdominal symptoms/headaches had significant increased levels of CRP and decreased levels of IL-4 compared to pregnant women giving birth to children without exposure to early-life stressors and abdominal symptoms/headaches.

5 Discussion

Emerging data suggest that the early phase of life, particularly the gestational period and the initial postnatal year, represents a critical window of vulnerability to stressors for the developing child (Barker, 2007; Fouhy et al., 2019; Olin et al., 2022). In particular, recent studies in humans and rodents have reported that the occurrence of early-life stressors are associated with an altered immunity in offspring (Atègbo et al., 2006; Ing Lim et al., 2021; Leibowitz et al., 2012; Ye Li et al., 2023; Ozkul et al., 2020; V. E. Ruiz et al., 2017; Shimizu et al., 2023, 2021). Findings build on data from murine models, where the offspring exposed to prenatal stressors, such as maternal stress, maternal infections and maternal obesity, displayed alterations in lymphocyte frequencies, accompanied by altered inflammatory responses (Garcia-Flores et al., 2020; Hsiao et al., 2012; Mandal et al., 2010; Myles et al., 2013; S. L. Russell et al., 2012; Shimizu et al., 2021). Especially, $CD4^+$ T cells have been suggested to be affected by stressors, as for instance shown by a diminished frequency of $CD4^+Foxp3^+CD25^+$ Tregs and a preferential development of Th17 cells, including an increased production of IL-17 (Hsiao et al., 2012; Mandal et al., 2010). $CD4^+$ T cells play a fundamental role in adaptive immunity, including the activation of B cells and regulation of $CD8^+$ T cells, the mediation of immune regulation, as well as the maintenance of tissue homeostasis and repair (D'Alessio et al., 2019; Garside et al., 1998; Kawabe et al., 2011; Okada et al., 2005; Phares et al., 2012; Zaiss et al., 2013; Zeng et al., 2012). The various functions are carried out by different subsets of $CD4^+$ T cells, each of which performs a number of specialized effector functions (Acosta-Rodriguez et al., 2007; Chevalier et al., 2011; Rivino et al., 2004; Sallusto, 2016). However, $CD4^+$ T cells also play a decisive role in the development of allergies and autoimmune diseases when their functionality is dysregulated (Ballesteros-Tato et al., 2016; Eastaff-Leung et al., 2010; Knight et al., 2007; Künzli & Masopust, 2023; Ulrich et al., 2022). Epidemiological studies of pregnant women and their children have indicated an association between stressors, such as prenatal infections, and the onset of immune-related disorders in offspring, including asthma, type 1 diabetes and allergic diseases (Collier et al., 2013; Fuchs & von Mutius, 2013; McKeever et al., 2002; Yue et al., 2018). These observations support the hypothesis that there is a link between early-life stressors and the development of altered $CD4^+$ T-cell responses that may contribute to the onset of autoimmune diseases or other immune-related disorders. In addition to the fetal development, the initial postnatal period is also suggested to have a heightened susceptibility to stressors (Fouhy et al., 2019; Olin et al., 2022, 2018; Shimizu et al., 2023). In this context, murine offspring that were treated with antibiotics shortly after birth exhibited altered T cell frequencies, such as a diminished frequency of intestinal Tregs and Th17 cells (Atarashi et al., 2011; Ozkul et al., 2020; V. E. Ruiz et al., 2017; S. L. Russell et al., 2012). Studies in human and in mice further demonstrated that pre- and postnatal antibiotic use is associated with microbial dysbiosis in offspring, which in turn is associated with an

altered T cell function and an increased risk for immune-mediated diseases later in life (Arrieta et al., 2014, 2015; Fouhy et al., 2012; Laforest-Lapointe & Arrieta, 2017; Miyoshi et al., 2017; Olin et al., 2018; Zwittink et al., 2018). Nevertheless, there is still a paucity of longitudinal human studies examining the potential long-term consequences of early-life stressors on $CD4^+$ T cell immunity in children beyond infancy. The majority of the investigations were conducted using cross-sectional studies, which have inherent limitations in terms of their ability to establish causal relationships. For these reasons, the objective of the present study was to examine the influence of early-life stressors on the $CD4^+$ T cell immunity in children at the age of 5. The data for this study was obtained from 118 children included in the PRINCE study, a prospective, longitudinal cohort of mothers and their children. The data encompassed the flow cytometric data from blood-derived lymphocytes of these children at the age of 5, as well as their and their mother's demographic and anthropometric data.

5.1 Classification of the study population in the national context

In general, the demographic and anthropometric data indicate that the women and children of this study are in relatively good health. Most of the characteristics of the 118 children and their mothers were comparable to the national average or other similar study populations. For instance, the average age of the mothers in this study was 32 years at the first trimester, which is comparable to the German average maternal age at birth of 31.7 years (Statistisches Bundesamt, 2022). Regarding the BMI of women at the outset of pregnancy, 63% of women in this study had a BMI in the normal range (BMI ≥ 18.5 to < 25), which was even notably higher than the national average in Germany of 53% (IQTIG – Institut für Qualitätssicherung und Transparenz im Gesundheitswesen, 2022). Additionally, only 7% of women in this study were classified as obese (BMI ≥ 30), which is considerably below the national average of 17.89% (IQTIG – Institut für Qualitätssicherung und Transparenz im Gesundheitswesen, 2022). In the present study, chronic diseases in women occurred in 17% of cases. Similarly, a study by Kersten et al., published in 2014, including 5320 women from north-east Germany, examined the prevalence of maternal chronic diseases with regard to birth outcomes and identified that 21.4% of pregnant women reported having a chronic disease (Kersten et al., 2014a). Furthermore, the proportion of women who had a caesarean section (C-section) in this study (20%) was notably lower compared to the latest published C-section rate in Germany in 2021 (30.9%; Statistisches Bundesamt (Destatis), 2023).

The data pertaining to the children's demographic and anthropometric characteristics also indicated an overall good health status from birth. The average GA at birth of children in this study was 39.3 ± 1.15 weeks, which is considered full-term (Quinn et al., 2016; Spong, 2013). According to the International Classification of Diseases, term birth is defined as delivery between 37 weeks 0 days and 41 weeks 6 days and is generally considered to be the optimal

time for delivery to ensure a healthy outcome for both, mother and newborn (Blencowe, Hug, Moller, You, & Moran, 2024; World Health Organization, 2022). Furthermore, 99% of the children in this study had an APGAR score of over 8, indicating that the majority of children were in a healthy initial condition at birth (Apgar, 2015). In this study, 31% of children received systemic antibiotics due to an infection in their first year of life, which is comparable to a study by Stam *et al*., published in 2012. The study examined the antibiotic use in infants in the first year of life in five European countries and showed that 33% of children in Germany received antibiotics at least once in the first year of life (Stam et al., 2012). Similarly, a study by Dawson-Hahn and Rhee (2019) involving 586 children from California, USA, demonstrated that 39.42% of children received antibiotics during the first year, with 33.79% receiving 1–2 antibiotic courses and 5.63% receiving 3 or more antibiotic courses (Dawson-Hahn & Rhee, 2019). Although the number observed in the present study is not above average, it remains a relatively high number of children receiving systemic antibiotics in the first year of life and emphasizes the clinical relevance of this stressor considering the effects of systemic antibiotics on the gut microbiota.

5.2 Associations between early-life stressors and the frequencies of $CD4^+$ T cell subsets in 5-year-old children

The PRINCE database, which includes demographic and anthropometric data from mothers and their children, along with flow cytometric data of blood-derived lymphocytes from the children at age 5, allowed to investigate whether exposure to single or multiple stressors is associated with altered $CD4^+$ T cell immunity in children. For the present study, the exposure to the following early-life stressors were assessed: maternal chronic diseases, maternal obesity (BMI ≥ 30) at first trimester (GW 12-14), maternal stress (PSS-14 score >26) at first trimester (GW 12-14), second trimester (GW 24), and third trimester (GW 34-36), maternal medication use during pregnancy, maternal infections during pregnancy, pregnancy complications and infant systemic antibiotic use in the first year of life.

In a first step, univariable linear regression analyses were performed to examine potential associations between exposure to individual stressors and $CD4^+$ T-cell subsets in 5-year-old children. Overall, no significant associations were identified in the adjusted model, indicating that exposure to one of the aforementioned stressors might not be sufficient to induce alterations in T cells. Emerging data indicate a "*two-hit*" or "*multiple-hit*" model, which suggests that exposure to an initial stressor primes for alterations, but then exposure to a second or more stressors ultimately triggers the actual onset of a phenotypic alteration or disease (Feigenson et al., 2014; Hsueh et al., 2018; Korzeniewski et al., 2014; Leviton et al., 2013; Shimizu et al., 2021; Verstraeten et al., 2019). Therefore, regression models were fitted to study whether exposure to multiple hits of the aforementioned stressors are associated with

altered frequencies of T cells from 5-year-olds. In this study, the model of exposure to multiple stressors was analyzed using two approaches. One consideration was that it is not necessarily the type of stressor that is decisive, but the actual number of stressors to which a child has been exposed. Following the theory of the "*multiple-hit"* model, it could be assumed that the more stressors a child has been exposed to, the more likely it is that alterations will occur. However, analyses including a variable that cumulatively summarized the exposure to the different types of stressors examined in this study did not confirm this hypothesis. The second approach was based on the hypothesis that it is not the number of stressors that leads to alterations, but rather the occurrence of two specific stressors. Indeed, multivariable linear regression models suggested that the two stressors, maternal medication use during pregnancy and systemic antibiotic use within the first year of life, were negatively associated with CRTh2^{+}CCR4^{hi} cells and positively associated with CCR6^{+}CCR4^{+}CD161^{+} cells in 5-year-old children. These associations were significant in the unadjusted model and continued to demonstrate a strong trend in the FDR-adjusted model. Specifically, these results indicated a strong trend towards increased frequencies of CCR6^{+}CCR4^{+}CD161^{+} cells in children at the age of 5 who were exposed to both stressors. Following the "*two-hit–hypothesis*", the use of medications during pregnancy would be considered the first hit, priming incipient alterations, while the use of antibiotics, including the underlying bacterial infection, in the first year of life and the consequences for the microbiota and derived metabolites would be the second hit, ultimately inducing the alterations in the aforementioned CD4^{+} T cell subsets.

Based on the utilized linear regression models, this study showed that apart from the findings regarding maternal medication use during pregnancy and infant antibiotic use in the first year of life, the majority of stressors examined were not associated with CD4^{+} T cells of children at 5 years of age. First and foremost, these results positively indicate that children generally exert a considerable resilience towards stressors. In addition, alterations resulting from stressors during pregnancy or in the initial postnatal period may also normalize as the child develops. In this regard, recent findings indicate that children undergo a developmental convergence process, whereby initial developmental differences converge over time, suggesting an inherent robustness (Olin et al., 2018; Wernroth et al., 2022). In particular, a study by Olin et al., published in 2018, examined the development of the immune system of term and preterm newborns within the first 100 days of life and discovered that although the immune system between preterm and term infants differed quiet strongly after birth, the development converged thereafter within the first 3 months (Olin et al., 2018). Another potential explanation for the absence of associations between stressors and CD4^{+} T cell subsets in 5-year-old children is that different stressor types may possess varying levels of impact. With regard to prenatal stressors, Shimizu et al. (2023) reviewed the impact of MIA following stressors in animal and human studies and suggested that clinical features in offspring may depend on

various factors, such as the time point of MIA during gestation, the magnitude of the inflammatory response, and the type of inflammation involved (Shimizu et al., 2023). The present study also revealed that the degree of resilience and susceptibility to stressors varies from individual to individual. For instance, although a distinct group of children who had been exposed to maternal medication use during pregnancy and antibiotics, including the underlying bacterial infection, in the first year of life exhibited notably elevated frequencies of Th17 cells, some children with the same exposures did not have increased frequencies of Th17 cells at the age of 5. Consequently, the impact of stressors cannot be generalized to all children. Even in animal models of MIA with the same genetic background and controlled environmental factors, offspring exhibited disparate outcomes (Estes et al., 2021; V. X. Han, Patel, Jones, & Dale, 2021; Mueller et al., 2021). A number of studies suggest that epigenetic alterations through complex gene-exposome interactions prenatal or postnatal may be involved in inter-individual variability and the development of alterations (Estes et al., 2021; V. X. Han, Patel, Jones, & Dale, 2021; Meyer, 2019; Wadhwa, Buss, Entringer, & Swanson, 2009). In this regard, recent findings from studies conducted on human populations have indicated that genome-wide demethylations of critical immune genes occur during the early stages of life (Acevedo et al., 2015, 2021; Olin et al., 2022). This age-associated DNA methylation may render the immune system particularly susceptible to stressors and environmental factors at specific time points. Furthermore, data from Perera *et al.* (2009) and Morales *et al.* (2012) suggest that in early-life altered methylation levels of inflammatory genes may persist over the course of years, potentially contributing to the development of diseases such as asthma and wheezing in childhood (Acevedo et al., 2015; Morales et al., 2012; Perera et al., 2009). Applying these findings to the present study, children who e.g. have been exposed to antibiotics in the first month of life may have a different susceptibility for alterations in the methylation status of certain immune genes than children exposed to antibiotics at 10 months of age. Thus, the specific timing of stressor exposure in conjunction with susceptibility to DNA methylation alterations may be critical factors for the onset of developmental alterations in children later in life, and serve as a potential explanation for the observed variation in individuals' susceptibility to stressors and the inter-individual occurrence of alterations. In general, a subject that merits further exploration in future research. Other possibilities for the lack of associations between the stressors analyzed in this study and $CD4^+$ T cells may be attributed to the relatively limited size of the study population and the composition of the stressor variables, as discussed in more depth in the Strengths and Limitations section below (section 5.5).

An indication of the potential consequences of altered $CD4^+$ T-cell immunity due to exposure to stressors might be the occurrence of frequent health complaints in children at 5 years of age. Abdominal pain is one of the most frequent reasons for children to consult a pediatrician

(Gieteling et al., 2011; Krause et al., 2019). In this context, $CD4^+$ T cells play a crucial role in mediating intestinal homeostasis and inflammation (Inagaki-Ohara et al., 2006; Schreurs et al., 2019). In gastrointestinal disorders, such as IBS and IBD, altered intestinal intraepithelial $CD4^+$ T cells promote chronic intestinal inflammation resulting in symptoms such as abdominal pain, fatigue or diarrhea (Choghakhori, Abbasnezhad, Hasanvand, & Amani, 2017; Eastaff-Leung et al., 2010; Jaeger et al., 2021; Perler et al., 2019; M. Singh, Singh, Schurman, Colombo, & Friesen, 2020). Besides abdominal pain, the occurrence of nausea or headache, either separately or in conjunction with abdominal pain, is increasingly reported in children (Carson et al., 2011; de Bruijn et al., 2023; Krause et al., 2019; Saps et al., 2016). In the present study, about a quarter of the children complained about abdominal pain, nausea or headaches (abdominal symptoms/headaches) at the age of 5. As the PRINCE study did not record the occurrence of each of the three symptoms separately, it is not possible to distinguish exactly which of the three symptoms occurred most frequently and in what percentage. Based on data from the nationwide German Health Interview and Examination Survey for Children and Adolescents (KiGGS Wave 2, 2014–2017), recurrent abdominal pain is most prevalent among the 3 to 6-year-old children, affecting about one third of girls (32.0%) and about one quarter of boys (23%; Krause et al., 2019). In comparison to abdominal pain, the prevalence of recurrent headaches among the 3 to 6-year-olds was lower, with 10.7% of girls and 9.8% of boys according to the KIGGS Wave 2 study (Krause et al., 2019). A study by Saps *et al.* (2016) assessed the prevalence of nausea among 8 to 15 year-old children in Latin America and discovered that nausea was present in 15.9% of 1,137 children (Saps et al., 2016). Besides, the study revealed that nausea was significantly more common in children with functional gastrointestinal disorders (Saps et al., 2016). In light of the prevalence of abdominal pain, headache and nausea observed in the aforementioned studies, it can be assumed that abdominal pain was the primary presenting symptom among children in the present study.

Interestingly, this study demonstrated that exposure to maternal medication use during pregnancy and infant antibiotic use in the first year of life, were each significantly associated with increased frequencies of Th17-like cells in children at the age of 5 who experienced abdominal symptoms/headaches. Subsequent functional analyses of $CD4^+$ T cells from children with these exposures showed a strong trend towards an increased frequency of IL-17A-producing $CD4^+$ T cells upon TCR-specific stimulation, which was similar to the frequency observed in adults. Moreover, the mothers of the children exhibited significantly elevated levels of the non-specific inflammatory marker CRP and significantly reduced levels of the cytokine IL-4 in the serum during the third trimester. These findings indicate that early-life stressors, including maternal medication use during pregnancy and infant antibiotic use, including the underlying infection, in the first year of life, may contribute to the development of Th17 cells in

children, which in turn may lead to the emergence of abdominal symptoms/headaches at the age of 5.

5.3 Mechanisms enhancing Th17 cell polarization

Th17 cells are a functionally diverse subgroup of $CD4^+$ T cells that can perform both homeostatic and pathogenic functions (Brockmann et al., 2023; Nizzoli et al., 2018; Patterson et al., 2021; Pickert et al., 2009; Schnell et al., 2023). Several cytokines are involved in the differentiation of Th17 cells, whereby IL-6 in combination with TGF-β are essential to drive Th17 cell differentiation (Schnell et al., 2023; L. Yang et al., 2009; L. Zhou et al., 2007). Upon binding to the IL-6R, IL-6 induces the downstream activation of STAT3, leading to the expression of RORγt, the hallmark transcription factor of Th17 cells (Harbour, DiToro, et al., 2020). In mucosal tissues, such as the intestinal mucosa, Th17 cells provide immunity towards extracellular pathogenic bacteria and fungi by releasing cytokines, including IL-17A, IL-17F and IL-22 (Amezcua Vesely et al., 2019; Bacher et al., 2019; Conti et al., 2009; Ishigame et al., 2009; S. C. Liang et al., 2006). Specifically, these cytokines can induce the recruitment of neutrophils to the site of inflammation and the expression of antimicrobial peptides, such as β-defensin, by epithelial cells (Huber et al., 2012; S. C. Liang et al., 2006; Pelletier et al., 2010). Furthermore, studies have demonstrated that moderate quantities of IL-17A, IL-17F and IL-22 can directly promote epithelial cell proliferation, tissue repair and increase the expression of tight junction components, thereby promoting the intestinal epithelial barrier integrity (J. S. Lee et al., 2015; Patterson et al., 2021; Pickert et al., 2009; Schnell et al., 2023; Sugimoto et al., 2008). In fulfilling all of the aforementioned functions, Th17 cells contribute to the maintenance of mucosal tissue homeostasis. Besides Tregs, Th17 cells are the most abundant $CD4^+$ T cell subsets in the intestinal lamina propria (Ivanov et al., 2006; Maynard et al., 2007; Schnell et al., 2023). In a non-pathological state, there is a balance between Th17 cells and Tregs, whereby Tregs express co-inhibitory surface molecules and produce anti-inflammatory cytokines, such as IL-10, to regulate the activity of Th17 cells (Diefenhardt et al., 2018; Eisenstein & Williams, 2009; P. Gu et al., 2012; D. Kim et al., 2024; Kurtulus et al., 2015; Qureshi et al., 2011; L. Sun et al., 2023; Tekguc et al., 2021). However, a perturbed Th17/Treg balance with an increased prevalence of Th17 cells and elevated IL-17 levels is associated with dysregulated homeostasis. As discussed in greater detail in section 5.4, consequences resulting from increased Th17 cells frequencies involve the exacerbation of mucosal inflammation and the induction of immune-mediated inflammatory diseases (Eastaff-Leung et al., 2010; Schnell et al., 2023). In addition, a growing number of studies suggest that Th17 cells can take on a pathogenic Th17 cell state, which is responsible for auto-inflammatory processes (Harbour et al., 2015; Nizzoli et al., 2018; Schnell et al., 2023; Wen et al., 2024). IL-23 has been proposed as the main cytokine driving pathogenicity of Th17 cells (Annunziato et al., 2007; Ghoreschi et al., 2010; Y. Lee et al., 2012; Nizzoli et al., 2018). The presence of IL-

23, in combination with either IL-6 and IL-1β or IL-6 and TGFβ, results in the differentiation of CXCR6$^+$ pathogenic Th17 cells (Ghoreschi et al., 2010; Y. Lee et al., 2012; Nizzoli et al., 2018). These cells secrete, in addition to IL-17A, the pro-inflammatory cytokines IFNγ and GM-CSF, which enhance intestinal tissue inflammation (Hirota et al., 2011; Langrish et al., 2005; Y. Lee et al., 2012; Schnell et al., 2021). Specifically, a study by Nizzoli et al., published in 2018, showed that IFNγ secreted by IL-17$^+$ T cells increase epithelial barrier permeability by affecting the expression of the tight junction ZO-1 and reducing the expression of anti-inflammatory cytokine IL-10 by intestinal CD4$^+$ T cells (Nizzoli et al., 2018). GM-CSF can activate phagocytic myeloid cells, such as neutrophils, who in turn can promote tissue damage by the release of pro-inflammatory cytokines or ROS (Becher, Tugues, & Greter, 2016; Subramaniam et al., 2014; Zhan et al., 2012).

5.3.1 Functionality of CD4$^+$ T cells from 5-year old children with abdominal symptoms/headaches exposed to early-life stressors

Following the findings in this study that children with abdominal symptoms/headaches had significantly increased frequencies of Th17-like cells at 5 years of age when exposed maternal medication use during pregnancy or infant systemic antibiotic use in the first year of life, the question prompted whether CD4$^+$ T cells of children with these exposures also exhibit functional changes. Interestingly, functional analyses revealed that children with the aforementioned exposures (symptomatic children) showed a strong trend towards an increased frequency of IL-17A-producing and IL-22-producing CD4$^+$FOXP3$^-$ memory T cells in response to TCR-specific stimulation, when compared to children without these exposures (non-symptomatic children). These findings may indicate a trend towards heightened pro-inflammatory immune responses. Although IL-22 plays an important protective role in tissue inflammation, IL-22 has been also shown to elicit pro-inflammatory effects in inflamed tissue by inducing the CXCL1, a neutrophil-recruiting chemokine, and serum amyloid A (SAA)-1/2, an acute-phase response protein, which in turn enhance the activation of pro-inflammatory Th17 cells (Bernshtein et al., 2019; Dudakov et al., 2012; Hanash et al., 2012; J. Y. Lee et al., 2020; S. C. Liang et al., 2010; Sano et al., 2015; Saxton et al., 2021). Therefore, the observed trend towards increased frequencies of IL-22$^+$CD4$^+$FOXP3$^-$ memory T cells may contribute to intestinal dysregulation. As mentioned above, a number of studies have shown that pathogenic Th17 cells secrete the pro-inflammatory cytokine IFNγ in addition to IL-17A, thereby promoting intestinal tissue inflammation (Hirota et al., 2011; Langrish et al., 2005; Y. Lee et al., 2012; Nizzoli et al., 2018; Schnell et al., 2021). In this study, no significant differences in IFNγ production by CD4$^+$FOXP3$^-$ memory T cells were observed between symptomatic and non-symptomatic children. These findings may indicate that pathogenic Th17 cells are not necessarily present within the CD4$^+$FOXP3$^-$ memory T cell subset. Nevertheless, future studies may benefit from the inclusion of additional pathogenic Th17 cell markers, such as

GM-CSF, to gain a more profound understanding of the pathogenic state of Th17 cells in symptomatic children. Analyses of further cytokines, including TNF, IL-8 and IL-4, also revealed no differences between non- and symptomatic children. These findings suggest that, with the exception of IL-17A, $CD4^+FOXP3^-$ memory T cells of symptomatic children exhibit no altered functionality.

Furthermore, functional analyses showed a trend towards augmented frequencies of IL-17A-producing $CD4^+FOXP3^+$ memory T cells among symptomatic children upon TCR-specific stimulation, in comparison to non-symptomatic children. IL-17-producing Tregs have been widely considered as intermediate cells during the transformation of $Foxp3^+$ Tregs to Th17 cells (Cui et al., 2024; Gagliani et al., 2015; Omenetti & Pizarro, 2015). Although these cells have been described to exert immunosuppressive functions under physiological conditions, they are also been reported to be associated in the pathogenesis of various inflammatory diseases, such as IBD and colitis-associated colorectal cancer (Beriou et al., 2009; Blatner et al., 2012; Cui et al., 2024; Mitsialis et al., 2020; Quandt et al., 2021; Rizzo et al., 2018; Voo et al., 2009). The elevated presence of IL-17A-producing $CD4^+FOXP3^+$ memory T cells among symptomatic children may therefore indicate an increased transformation of Tregs to Th17 cells, which may have contributed to the increased presence of Th17-like cells and the onset of abdominal symptoms/headaches in these children. Besides IL-17A, there were no remarkably differences in the production of TNF, IFNγ, IL-22, IL-4, and IL-8-production by $CD4^+FOXP3^+$ memory T cells between non-and symptomatic children. As with $CD4^+FOXP3^-$ memory T cells, these findings collectively suggest that $CD4^+FOXP3^+$ memory T cells of symptomatic children exhibit no aberrant functionality.

Another possibility for elevated frequencies of Th17-like cells might be an altered expression of the IL-6Rα on $CD4^+$ T cells. The binding of IL-6 to the membrane-bound IL-6Rα and the membrane-bound signal transducing subunit gp130 is referred to as classical IL-6 signaling, which plays a pivotal role in the differentiation and maintenance of Th17 cells (Harbour, Ditoro, et al., 2020; Stefan Rose-John et al., 2023; Taga et al., 1989). However, analyses of IL-6Rα expression on $CD4^+FOXP3^-$ memory T cells and $CD4^+FOXP3^+$ memory T cells revealed no significant differences between non-symptomatic and symptomatic children under unstimulated and under TCR-specific stimulation. Nevertheless, the differentiation of Th17 cells may have been promoted by trans-signaling of IL-6. Trans-signaling is initiated by the binding of IL-6 to the sIL-6Rα and the membrane-bound signal transducing subunit gp130 (Mülberg et al., 1993; S. Rose-John & Heinrich, 1994). Specifically, a study by Jones *et al.* (2010) showed that T cells from IL-6R-deficient mice maintain and promote the presence of IL-17A-secreting $CD4^+$ T cells in response to IL-6 via trans-signaling (G. W. Jones et al., 2010). Therefore, an increased Th17 cell differentiation via IL-6 trans-signaling in symptomatic children cannot be excluded. One way to investigate this in future studies could be an

examination of the concentration of sIL-6Rα in the serum of 5-year-old children with the aforementioned exposures and symptoms. Furthermore, it would be both interesting and beneficial to carry out the studies with a larger number of participants in the future.

5.3.2 Serum immune profile during pregnancy of mothers from children exposed to early-life stressors and with abdominal symptoms/headaches at 5 years of age

The maternal immune system undergoes a series of systemic immunological adaptations during pregnancy, enabling the maintenance of tolerance to the semi-allogeneic fetus while preserving immunity to protect against pathogens (Abu-Raya et al., 2020; Aghaeepour et al., 2017; Berry et al., 2021; Mor et al., 2017). In this context, the 3 trimesters are also referred to as the 3 immunological stages of pregnancy (Mor et al., 2017; Mor, Cardenas, Abrahams, & Guller, 2011). The first trimester contains local inflammation stages that are necessary for the implantation of the blastocyst and placentation (Jarmund et al., 2021; Mor et al., 2017; Pongcharoen et al., 2007). The second trimester is considered as anti-inflammatory or Th2 type environment to provide fetal growth (Jarmund et al., 2021; Mor et al., 2017; Somerset, Zheng, Kilby, Sansom, & Drayson, 2004). During the third trimester, the anti-inflammatory status shifts towards a pro-inflammatory or Th1 status, which is regarded as essential for the onset of labor and the subsequent birth (Jarmund et al., 2021; Mor et al., 2017; Somerset et al., 2004). Cytokines are important mediators to enable these dynamic changes (Jarmund et al., 2021). Maternal serum cytokines consist of cytokines released by immune cells and tissues, including placental tissue, and serve as a reflection of collective immunological processes during pregnancy (Jarmund et al., 2021). Abnormal cytokine levels can provide an indication of the maternal systemic inflammatory status or immunological disturbances (Jarmund et al., 2021; Lobo et al., 2018; Mullins, Prior, Roberts, & Kumar, 2012). MIA during pregnancy is characterized by increased maternal pro-inflammatory cytokine levels that can be transmitted to the fetus and are associated with developmental alterations in the offspring (Atladóttir et al., 2010; Brown, 2012; Brynge et al., 2022; Hodyl et al., 2007; McKeever et al., 2002; Onore et al., 2014; Paraschivescu et al., 2020; Shimizu et al., 2023; S. E. P. Smith et al., 2007; Urakubo et al., 2001; Yue et al., 2018; Zaretsky et al., 2004). In this study, concentrations of specific cytokines and effector molecules associated with inflammation were determined in third trimester serum samples from the mothers of the aforementioned symptomatic children to assess the maternal immune profile during their pregnancies. Interestingly, the results of the analyses indicated an altered serum cytokine status in mothers of symptomatic children compared to mothers of non-symptomatic children, which may have contributed to the increased prevalence of Th17 cells and symptoms observed in the 5-year-old subjects.

Specifically, mothers of symptomatic children had significantly increased concentrations of CRP compared to mothers of non-symptomatic children. CRP is an acute-phase protein and

the most commonly used marker for nonspecific systemic inflammation in the clinical context (Baumeister, Akhtar, Ciufolini, Pariante, & Mondelli, 2016; Pradhan, Manson, Rifai, Buring, & Ridker, 2001; Tillett & Francis, 1930; World Health Organization, 2014). Following the onset of inflammatory processes, infections, or tissue damage, CRP is primarily synthesized by liver hepatocytes in response to IL-6, and secreted into the bloodstream (Boras et al., 2014; Geyer et al., 2021; Rhodes et al., 2011). In general, an increase in the concentration of CRP in plasma occurs relatively quickly after the increase in the concentration of IL-6 during acute inflammation (Del Giudice & Gangestad, 2018; Schmit & Vincent, 2008). The increase in CRP levels is typically observed within the first 4-6 hours post-infection, reaching its maximum level approximately 1 to 2 days later (Del Giudice & Gangestad, 2018; Schmit & Vincent, 2008). In severe systemic infections, for instance, CRP levels can escalate from less than 1 µg/ml to up to 1000 µg/ml (Del Giudice & Gangestad, 2018; Schmit & Vincent, 2008). CRP plays an important role in the induction of the complement system and the promotion of phagocytosis by neutrophils and macrophages (Bíró et al., 2007; Bodman-Smith et al., 2002; Crowell, Du Clos, Montoya, Heaphy, & Mold, 1991; Kilpatrick & Volanakis, 1985; Siegel, Rent, & Gewurz, 1974; S. K. Singh, Ngwa, & Agrawal, 2020). During pregnancy, CRP levels are elevated with peak levels during the second trimester (Belo et al., 2005; Ferguson, McElrath, Chen, Mukherjee, & Meeker, 2014; Jarmund et al., 2021). Jarmund *et al.* (2021) suggested that elevated CRP levels in conjunction with minimal pro-inflammatory cytokine concentrations, including IL-1β and TNF, during the second trimester promote tissue repair and anti-inflammatory effects, potentially through the induction of M2 macrophages (Del Giudice & Gangestad, 2018; Jarmund et al., 2021). This is thought to contribute to the anti-inflammatory state of the second trimester (Del Giudice & Gangestad, 2018; Jarmund et al., 2021). Nevertheless, CRP is also a marker for low-grade inflammation during pregnancy (Subhabrata Basu, Haghiac, et al., 2011; Subhabrata Basu, Leahy, et al., 2011; Fink et al., 2019; Hauta-alus et al., 2024). Factors such as maternal obesity are associated with higher IL-6 and CRP levels along with tissue inflammation (Subhabrata Basu, Leahy, et al., 2011; Ingvorsen et al., 2015; Jarmund et al., 2021). Although CRP is considered to not cross the placental barrier due to the heavy molecular mass and the lack of Fc receptors, studies indicated an association between high maternal CRP levels during pregnancy and high CRP levels in children 6 months and 6-8 years after birth (Fink et al., 2019; Ghezzi et al., 2002; Hauta-alus et al., 2024; Malek et al., 2006; Raio et al., 2019). This may be indicative of a potential indirect impact on the immune status of the developing fetus, which could result in low-grade systemic inflammation in the child, also later in life (Fink et al., 2019).

To date, there is no evidence of a direct effect of CRP on Th17 cell differentiation. However, previous studies indicated a positive correlation between elevated CRP levels and augmented Th17 cell concentrations in a number of diseases, including rheumatoid arthritis and aortic

dissection (Al-Saadany, Hussein, Gaber, & Zaytoun, 2016; M. Song et al., 2022). Furthermore, it has been shown that CRP can in turn induce monocytes to secrete the pro-inflammatory cytokines TNF, IL-1β, IL-6 and IL-23 (Geyer et al., 2021). As described above, in particular IL-23 in combination with IL-6 and IL-1β promote the differentiation and proliferation of pathogenic Th17 cells (Ghoreschi et al., 2010; Y. Lee et al., 2012; Nizzoli et al., 2018). As several studies have indicated that maternal monocytes can passage through the placenta, elevated CRP levels may have the potential to prime maternal monocytes to produce pro-inflammatory cytokines at the maternal-fetal interface in the placenta, which in turn may induce alterations in fetal T cell development, including a potential enhanced development of fetal Th17 precursor cells (Dahlgren et al., 2001; Faas, Spaans, & De Vos, 2014; Ingvorsen et al., 2015; Mellembakken et al., 2002; Mold et al., 2008; Wienecke et al., 2012). Besides, given that CRP is largely synthesized in response to IL-6, elevated CRP levels may also be an indication of preceding heightened IL-6 levels. IL-6 is one of the earliest recognized and most frequently cytokine associated with MIA (Buka et al., 2001; Ye Li et al., 2023; Spann et al., 2018; Zaretsky et al., 2004). In addition, IL-6 has the potential to cross the placenta and enter the fetal circulation, thereby potentially causing developmental alterations in the fetus (Brynge et al., 2022; Hsiao & Patterson, 2011; Paraschivescu et al., 2020; S. E. P. Smith et al., 2007; Urakubo et al., 2001; Zaretsky et al., 2004; Zawadzka et al., 2021). In this study, the concentrations of IL-6, as well as of IL-1β, IL-2, TNF and IL-17A in the maternal serum samples were below the reliable detection range of the multiplex-bead assay. For these cytokines, highly sensitive ELISAs would be beneficial for future analyses to accurately determine differences in the low cytokine levels.

With regards to IL-6, the concentration of sIL-6Rα and sgp130 in the maternal serum may provide conclusions on IL-6 signaling. As described above, trans-signaling is initiated by the binding of IL-6 to a sIL-6Rα and the membrane-bound signal transducing subunit gp130 (Mülberg et al., 1993; S. Rose-John & Heinrich, 1994). In turn, sgp130 had been shown to block trans-signaling via IL-6/sIL-6Rα and is associated with a reduction in IL-6-induced pro-inflammatory effects (Hong et al., 2016; Jostock et al., 2001; Narazaki et al., 1993). Interestingly, the sgp130 levels in serum samples of mother from non-symptomatic children were significantly higher compared to the sgp130 levels determined in non-pregnant women. These elevated levels in pregnant women may suggest a protective function of sgp130 in preventing augmented pro-inflammatory IL-6 trans-signaling (Hong et al., 2016; Jostock et al., 2001; Narazaki et al., 1993; Stefan Rose-John et al., 2023). In serum samples of mothers from symptomatic children, the sgp130 levels exhibited considerable inter-donor variability. Thereby, the markedly diminished sgp130 levels observed in samples of mothers of symptomatic children may suggest the possibility of an impaired sgp130 function.

Furthermore, the results of the serum analyses revealed significant reduced concentrations of IL-4 in maternal serum samples from mothers of symptomatic children compared to mothers of non-symptomatic children. IL-4 is a pleiotropic cytokine that plays, among others, an important role in the promotion of B cell proliferation and antibody class switching, as well as the differentiation of naive $CD4^+$ T cells into a Th2 cell phenotype (Fallon et al., 2002; Hsieh, Heimberger, Gold, O'Garra, & Murphy, 1992; Keegan, Warren, & Zhu, 2021; Le Gros et al., 1990; Morawetz et al., 1996). Reduced levels of IL-4 are associated with severe pregnancy outcomes including preeclampsia and spontaneous abortion (Cottrell et al., 2019; Piccinni et al., 1998). During gestation, IL-4 is considered to play an important role in regulating maternal-fetal tolerance by inhibiting pro-inflammatory cytokines like IL-6 and TNF (P. Chatterjee, Chiasson, Bounds, & Mitchell, 2014; Cottrell et al., 2019). Furthermore, several studies have been demonstrated that IL-4 promotes and maintains the differentiation of Tregs by inducing FoxP3 mRNA expression via STAT6 signaling (Sanchez-Guajardo et al., 2007). Moreover, a study by Guenova *et al.* (2015) showed that IL-4 inhibits IL-23 production in both, mouse and human DCs, thereby indirectly preventing their capacity to induce and maintain IL-17–producing Th17 cells (Guenova et al., 2015). The diminished concentrations of IL-4 in the serum of mothers of symptomatic children may, in addition to CRP, contribute to a cytokine milieu that is conducive to the differentiation of fetal Th17 precursor cells.

5.3.3 Influence of maternal medication during pregnancy and antibiotic use for severe infections in the first year of life on increased prevalence of Th17 cells in 5-year-old children with abdominal symptoms/headaches

The medications taken by pregnant women in this study encompassed a broad spectrum of medicinal products. Interestingly, half of the women who reported taking medication and whose children showed abdominal symptoms/headaches at the age of 5 years, were taking L-thyroxine. Subsequent multivariable linear regression models confirmed that exposure to maternal L-thyroxine use was significantly positive associated with Th17-like cells in 5-year-olds with reported abdominal symptoms/headaches. Although the number of mother-child pairs with these characteristics in this study is relatively small, the findings strongly suggest that the administration of L-thyroxine or the underlying maternal thyroid hormone dysregulation may exert a notable influence on the offspring immune system.

L-thyroxine mimics the thyroid hormone T4, and is used to treat thyroid hormone deficiency (Chalmers et al., 1949; Harington & Barger, 1927; Kahaly & Gottwald-Hostalek, 2022). At the beginning of pregnancy, maternal T4 concentrations normally increase and remain at this level throughout the pregnancy (R. H. Lee et al., 2009). However, some women are unable to produce the required amount of thyroid hormones, which can result in a relative hypothyroid state (Dash et al., 2022). In such cases, it is necessary to substitute L-thyroxine to maintain optimal thyroid hormone levels (Dash et al., 2022; Runkle et al., 2021). In other cases,

hypothyroidism is attributable to a pre-existing thyroid disease, so that L-thyroxine was already been taken before pregnancy. Thyroid hormones are crucial for the maternal health and for the development of the child, in particular for the fetal growth and fetal brain development (Alvarez-Dolado et al., 1999; Bernal, 2000; Haddow, 1999; Korevaar et al., 2016; Richard et al., 2020; C. Zhang et al., 2019). The fetus depends on the transplacental transfer of the maternal thyroid hormones, especially in the first 16 weeks of gestation (Calvo et al., 2002; Costa et al., 1991; Obregon, Calvo, Escobar Del Rey, & Morreale De Escobar, 2007; J. Patel, Landers, Li, Mortimer, & Richard, 2011). Only then does the fetus begins to produce its own thyroid hormones (Jatin Patel, Landers, Li, Mortimer, & Richard, 2011). The transfer of biological substances, such as T4 or L-thyroxine, from the maternal circulation to the fetal circulation is mainly regulated by trophoblast cells in the placenta and their cell membrane transporters (Loubière et al., 2010; McKinnon, Li, Richard, & Mortimer, 2005; A. M. Mitchell, Manley, & Mortimer, 1992). Within the trophoblast cell, intracellular thyroid hormone levels are mediated by deiodinases, such as DIO1, which are able to convert T4 into the physiologically active thyroid hormone T3 (Boye & Laurberg, 1984). In the nucleus, T3 forms a complex with a TR and a retinoic acid receptor (Kakizawa et al., 1997; Walfish, Yang, Ypganathan, Chang, & Butt, 1996; Yen, 2001). This complex then binds to T3-response elements, thereby inducing the transcription of specific target genes (Mangelsdorf et al., 1991). In addition to the canonical TR signaling, T3 has the capacity to bind to TRs in the cytoplasm, thereby inducing the activation of signaling pathways, including the PI3K/AKT and MAPK/ERK pathways (non-canonical signaling; Cao, Kambe, Moeller, Refetoff, & Seo, 2005; Cao, Kambe, Yamauchi, & Seo, 2009; G. Sebastian Hönes et al., 2017; Georg Sebastian Hönes et al., 2024; Kalyanaraman et al., 2014). In these ways, thyroid hormones can exert their effect on fetal development in different tissues. Besides tissues, thyroid hormones have been also described to play a regulatory role of innate and adaptive immune cells (Wenzek et al., 2022). *In vitro* and *in vivo* models indicated a direct effect of thyroid hormones, including L-thyroxine, on T cell immunity by inducing proliferation, activation and apoptosis of T cells (Aoki et al., 1976; Barreiro Arcos et al., 2011; Mihara et al., 1999; Wenzek et al., 2022). Furthermore, recently published studies in mice by Hönes *et al.* (2024) and Wenzek *et al.* (2024) indicated that TR signaling plays an important role in T cell differentiation (Georg Sebastian Hönes et al., 2024; Wenzek et al., 2024). Specifically, the studies demonstrated that the absence of TRα signaling specifically increased the frequency of Tregs and diminished Th17 cell differentiation (Wenzek et al., 2024). In addition, the study showed that TRα-mediated thyroid hormone signaling inhibits NFκB activation induced by TCR-specific stimulation, which is a crucial pathway in the differentiation of Tregs (Levine, Arvey, Jin, & Rudensky, 2014; Long, Park, Strickland, Hayden, & Ghosh, 2009; Wenzek et al., 2024). Given that the polarization of Th17 cells and Tregs occurs in opposite directions, the authors proposed that TRα-mediated thyroid hormone

signaling might, in turn, promote pro-inflammatory Th17 cell responses, thereby constraining Treg differentiation (Eisenstein & Williams, 2009; Ichiyama et al., 2008; Takaki et al., 2008; Wenzek et al., 2024; L. Zhou et al., 2008). Interestingly, the present study showed that the median frequency of TRα1/β1^{+}CD4^{+} T cells of symptomatic children tended to increase upon TCR-specific stimulation, whereas the median frequency of TRα1/β1^{+}CD4^{+} T cells of non-symptomatic children tended to decline upon stimulation. This finding was observed in both, CD4^{+}FOXP3^{-} T cells and CD4^{+}FOXP3^{+} T cells. Considering the findings of the aforementioned studies by Hönes *et al.* and Wenzek *et al.*, the increase in TRα1/β1 expression may enhance the responsiveness of CD4^{+} T cells from symptomatic children to thyroid hormones upon activation, which may in turn favors Th17 cell development and responses. Whether and how exposure to early-life stressors, including prenatal exposure to L-thyroxine or maternal thyroid hormone dysregulation, may have induced an altered TRα1/β1 expression in the children's T cells that promoted Th17 cell development later in life remains open and requires further investigation. Moreover, studies by Alamino et al. (2019, 2015) also showed an indirect effect of thyroid hormones on T cell function mediated by DCs. Specifically, *in vitro* T3 treated murine bone marrow-derived DCs displayed a pro-inflammatory DC phenotype that promoted IL-17 mediated immune responses, such as an increased secretion of Th17-polarizing cytokines, including IL-23, IL-1β, IL-6 and TGF-β (Alamino et al., 2019). In addition, co-cultures with T3-stimulated DCs and splenocytes resulted in an elevated frequency of CD4^{+}IL-17^{+} splenocytes with increased intracellular IL-17 levels and a reduced frequency of CD25^{+}FoxP3^{+} Tregs (Alamino et al., 2019). According to these studies, it might be possible that elevated thyroid hormone levels during pregnancy promoted alterations that led to a preferential development of Th17 cells and the onset of abdominal symptoms/headaches in children at the age of 5. Thereby, the precise mechanism by which thyroid hormones, including L-thyroxine might have induced the observed alterations in children remains unclear. One possibility could be that thyroid hormones directly affect the fetal immune system by entering the fetal circulation via transplacental delivery. According to the aforementioned studies, fetal T cells could be directly affected by thyroid hormones or the actually tolerogenic fetal DCs could be induced to a pro-inflammatory state by thyroid hormones, thus priming the fetal T cells towards a Th17 T cell direction. Another possibility for introducing alterations in fetal T cells might be due to an altered maternal-fetal immune cross-talk (Garcia-flores et al., 2024). Hence, thyroid hormones may alter maternal DCs resulting in the secretion of pro-inflammatory cytokines that could be transmitted to the fetus and thereby prime the fetal immune system to IL-17 mediated immune responses later in life. In the fetal immune system, a Th17-like cell phenotype as found in adults based on specific hallmark chemokine receptors, such as CD4, CXCR3, CCR6, and CD161, have not been identified by previous studies. However, analyses of cord blood samples identified several T-cell types that could be considered as precursors of Th17 cells.

Specifically, previous studies of term infant cord blood determined the presence of $RORC^{+}CCR6^{+}$ effector memory cells and an enriched presence of $CD161^{+}$ CD4 T cells, which *in vitro* preferentially differentiate to Th17 cells (Cosmi et al., 2008; Elze Rackaityte & Halkias, 2020; Xiaoming Zhang et al., 2014). Furthermore, fetal $CD4^{+}$ T cells expressing the transcription factor PLZF showed the capacity to produce IL-17 in the mesenteric lymph node and in the small intestine (Halkias et al., 2019; Elze Rackaityte & Halkias, 2020). Accordingly, dysregulated enhanced thyroid hormone levels may have promoted the development of fetal Th17 precursor cells during pregnancy. In this context, analyses of cord blood and maternal blood samples to determine the T cell and DC status of children included in this study and their mothers during pregnancy as well as measurements of maternal thyroid hormone levels during pregnancy may provide a more comprehensive picture and may be an interesting consideration for future studies.

A potential link between the early-life stressors, elevated Th17-like cell frequencies and the emergence of abdominal symptoms/headaches may also be attributed to alterations in the intestinal microbiota. The gut microbiota plays an important role in regulating Th17 cell differentiation. Specific microbiota in the intestine, such as, *cytophaga-flavobacter-bacteroidetes (CFB) bacteria*, epithelial-adhering *SFB* or *bifidobacterium adolescentis*, have been reported to induce the generation of intestinal Th17 cells (Guan et al., 2016; Ivanov et al., 2009, 2008). Thereby, the induction of Th17 cells can occur via different pathways. Bacteria antigens have the capacity to bind to TLRs, such as TLR2, on Th17 cells or to be presented to naive $CD4^{+}$ T cells via MHC-II-dependent manner by intestinal APCs, such as DCs (Goto et al., 2014; Marks et al., 2021; C. Y. Sun et al., 2023; Y. Yang et al., 2014). For instance, the gut resident *SFBs* have been shown to promote the generation of Th17 cells by the *SFB*-specific antigens, such as *SFB* protein P3340, or *SFB* flagellins (Y. Wang et al., 2019; Y. Yang et al., 2014). Furthermore, a study by Ladinsky *et al*. (2019) showed that *SFBs* transfer T cell antigens into intestinal epithelial cells via a microbial adhesion-triggered endocytosis to induce *SFB*- specific Th17 cells (Ladinsky et al., 2019). Under non-aberrant conditions, resident gut microbiota, such as *SFBs*, promote in these ways a homeostatic Th17 cell type (Ladinsky et al., 2019; M. Xu et al., 2018). In contrast, microbial dysbiosis is associated with an altered induction of Th17 cells and an exacerbation of autoimmune diseases (Yuan Li et al., 2021; López et al., 2016; Paik et al., 2022; C. Y. Sun et al., 2023). Longitudinal studies have demonstrated that infants who have been exposed to antibiotics within the first year of life exhibited an altered microbiome composition and an elevated risk of developing asthma, allergies and IBDs in comparison to children who have not been exposed to antibiotics (Arrieta et al., 2015; Fouhy et al., 2012; Korpela et al., 2016; Penders et al., 2011; Tanaka et al., 2014). In studies of murine models, early postnatal antibiotic exposure increased the permeability of the intestinal epithelial barrier and the expression of pro-inflammatory cytokines (Chaaban et

al., 2022; H. Huang, Jiang, Wang, Jiang, & Cao, 2024). An elevated intestinal permeability enables microbes and further foreign antigens to increasingly cross the intestinal epithelial barrier, which in turn results in an expansion of intestinal Th17 cells and an enhanced intestinal inflammation (Łoniewska et al., 2020; Suárez-Martínez, Santaella-Pascual, Yagüe-Guirao, & Martínez-Graciá, 2023; M. Yu et al., 2021). Moreover, the use of antibiotics has been linked to a notable decline in the levels of SCFAs, including butyrate and propionate (Dupraz et al., 2021). SCFAs are microbial metabolites that have been demonstrated to promote the differentiation of homeostatic Th17 cells and IL-10-secreting Tregs in the intestine (C. H. Kim, 2023; Ney et al., 2023; J. Park et al., 2015). In addition, an *in vitro* study using human intestinal concentrations of L-thyroxine, revealed that L-thyroxine also alters bacterial growth and SCFAs production of certain bacteria (Hammouda, Wasfi, & Abdeltawab, 2023). Therefore, a reduction in SCFAs levels may in turn promote an increased presence of pro-inflammatory Th17 cells.

It is likely that the child's microbiota is more susceptible to stressors during the prenatal period and the first year of life, especially as the child's microbiome is not yet fully developed (Arrieta et al., 2015; Wernroth et al., 2022). A number of studies indicate that microbial colonization of the fetal gut begins in utero and originates from the maternal microbiome, which is transferred to the fetus via the amniotic fluid, umbilical cord blood and fetal membranes (DiGiulio, 2012; Jimenez et al., 2005; H. E. Jones et al., 2009; Suárez-Martínez et al., 2023; X. Wang et al., 2013). Alterations in the maternal gut microbiota have been associated with prenatal stressors and altered fetal immune development (Boutin & Finlay, 2016; Gosalbes, Llop, & Vall, 2012; J. L. Kaplan, Walker, & Shi, 2011; M. Li, Wang, & Donovan, 2014; E Rackaityte et al., 2020; Suárez-Martínez et al., 2023; Tapiainen et al., 2018). With regard to L-thyroxine use during pregnancy, there is a possibility that dysregulated thyroid hormone levels during gestation may exert an influence on the maternal microbiome, which in turn may result in an altered fetal microbiome establishment (Hammouda et al., 2023). Following birth, microbial colonization proceeds to evolve, resulting in augmented microbial diversity and species abundance (Roswall et al., 2021; Stewart et al., 2018; Wernroth et al., 2022). Studies suggest that by the end of the first 3-5 years of life, the microbiota reaches an adult-like state (Roswall et al., 2021). However, until then, and especially during the first year of life, when microbial diversity is still very low (Wernroth et al., 2022), antibiotic use may have a profound effect on the gut microbiota, leading to an imbalance in microbial composition that may alter crosstalk with intestinal Th17 cells and persist into later life. On that point, a study by Fouhy *et al.* from (2019) observed that an altered gut composition early in life can retain up to the age of 4 (Fouhy et al., 2019). In this context, an analysis of the gut microbiome of the 5-year-old children included in this study using stool samples would be a promising method to gain more detailed information about possible early-life stressor-related microbial dysbiosis and its association with Th17 cells.

Beyond the effects of antibiotics, the rationale for their utilization, namely the severe bacterial infection, may also have exerted an influence on the elevated Th17 cell frequencies in 5-year-old children with abdominal symptoms/headaches. Bacterial infections can elicit strong immune responses against pathogens in children, especially within the first year of life when the functional capabilities of their immune system is still limited (Hill et al., 2020; Kollmann et al., 2009; Raymond, Rincon, et al., 2017; Raymond, Stortz, et al., 2017). For instance, *Staphylococcus aureus* (*S. aureus*) is the most prevalent pathogen of skin and soft tissue infections and it is a common bacterium found in children with healthcare-associated infections (Filleron et al., 2018; S. L. Kaplan, 2016; Ondusko & Nolt, 2018; Vogel et al., 2023). Infections with *S. aureus* have been demonstrated to induce excessive $CD4^+$ T-cell activation and aberrant inflammation, culminating in the secretion of elevated levels of cytokines into the circulation (Bae et al., 2021; Roetzer et al., 2020; Vogel et al., 2023). Dysregulated inflammation induced by *S. aureus* toxins can trigger a cytokine storm, which, in turn, can cause systemic and deleterious effects, including shock and multi-organ failure (Bae et al., 2021; Roetzer et al., 2020; Vogel et al., 2023). In addition, an *in vitro* study by Vogel *et al.* (2023) showed that neonatal $CD4^+$ T cells specifically increase the expression of IL17A and RORγt in response to *S. aureus* and *Staphylococcus epidermidis* (Vogel et al., 2023). Furthermore, the expression levels of IL17A and RORγt in neonatal T cells were found to be considerably higher compared to adult T cells, which may be attributed to a reduced activation threshold in neonatal T cells (Schmiedeberg et al., 2016; Vogel et al., 2023). Growing evidence indicate that exposure to certain bacterial pathogens in the first months of life may also increase the risk for developing immune-mediated disorders later in life. For instance, gastrointestinal infections with *Salmonella enteritidis, E. coli* and *Campylobacter species* during childhood have been associated with the onset of the functional gastrointestinal disorder IBS later in life (Cremon et al., 2014; Thabane et al., 2010; Thapar et al., 2020). Considering the aforementioned enhanced pro-inflammatory cytokine effects of the neonatal and infant immune system, it is possible that bacterial infections during the first year of life may have influenced the $CD4^+$ T cell development, potentially resulting in a predisposition of Th17 cells and the manifestation of abdominal symptoms/headaches in 5-year-old children in the present study. In this regard, further information on the severity of the infection, including the specific type of pathogen, would be beneficial and interesting to investigate the long-term consequences of certain infections in more detail in future studies.

5.4 Clinical relevance of increased Th17 cells and manifestation of abdominal symptoms/headaches in children

The results of this study suggest that exposure to maternal thyroid hormone dysregulation during pregnancy and exposure to antibiotics during the first year of life, including the underlying bacterial infection, may alter the $CD4^+$ T cell development during the early phase of

life, leading to an augmented differentiation of Th17 cells in 5-year-old children. This, in turn, may have, contributed to the onset of abdominal symptoms/headaches in these children at the age of 5.

As previously described in detail, Th17 cells are particularly abundant in mucosal tissues such as the intestinal lamina propria, where they provide host defense against extracellular bacterial and fungal pathogens by recruiting neutrophils, inducing antimicrobial peptides, stimulating mucus secretion, and supporting epithelial barrier integrity (Amezcua Vesely et al., 2019; Bacher et al., 2019; Conti et al., 2009; X. Fan et al., 2023; L. Han et al., 2015; Ishigame et al., 2009; S. C. Liang et al., 2006; Patterson et al., 2021; Schnell et al., 2023). However, an increased prevalence of Th17 cells including dysregulated Th17 cell functions can have detrimental consequences for the human organism (Eastaff-Leung et al., 2010; Schnell et al., 2023). Specifically, Th17 cell overactivation may lead to abnormal mucosal immune activation and chronic mucosal low-grade inflammation, which in turn may induce epithelial tissue damage (Di Nardo et al., 2023; Eastaff-Leung et al., 2010; Schnell et al., 2023). Consequently, these effects can trigger the onset of symptoms and the manifestation of disorders, such as IBS (Di Nardo et al., 2023). IBS is one of the most common functional gastrointestinal disorders, affecting about 2% to 23% of children worldwide (Aslan Doğan, Gokdemir, & Özçakar, 2024; Bouzios, Chouliaras, Chrousos, Roma, & Gemou-Engesaeth, 2017; Devanarayana et al., 2015; Di Nardo et al., 2023; Karabulut et al., 2013; Lewis, Palsson, Whitehead, & van Tilburg, 2016; P. L. Lu, Saps, Chanis, & Velasco-Benítez, 2016; Robin et al., 2018; Scarpato et al., 2018; Udoh, Devanarayana, Rajindrajith, Meremikwu, & Benninga, 2016). Based on Rome criteria IV, IBS is characterized by the presence of recurrent abdominal pain or abdominal discomfort associated with abnormal bowel habits and altered defecation, such as diarrhea, constipation or both (Hyams et al., 2016; M. Singh et al., 2020). Although the etiology, including the underlying pathophysiologic mechanisms, is not yet fully understood, emerging data suggest that IBS is a multifactorial disorder of the gut-brain axis (Di Nardo et al., 2023; M. Singh et al., 2020). Specifically, several gut-brain-system abnormalities have been reported in IBS, such as intestinal dysbiosis, increased intestinal permeability, mucosal immune activation, alterations in the central and the enteric nervous system, genetic predisposition, as well as psychologic dysfunction (Di Nardo et al., 2023; Eijsbouts et al., 2021; Mayer, Ryu, & Bhatt, 2023; M. Singh et al., 2020). In this context, a considerable number of 25% to 50% of individuals diagnosed with IBS also report the occurrence of headaches (Kawashima et al., 2020). Recent findings indicate the involvement of Th17 cells in the pathogenesis of IBS (M. Singh et al., 2020). A study by Singh et al. (2020) reported an increased mucosal density of Th17 cells in the colon of 8- to 17-year-olds with IBS compared to 8- to 17-year-olds without IBS (M. Singh et al., 2020). Furthermore, research conducted by Choghakhori and her colleagues showed that patients diagnosed with diarrhea-predominant

IBS exhibited elevated serum concentrations of IL-17A and TNF, concomitant with diminished IL-10 concentrations, suggesting a Th17/Treg imbalance (Burns, Talley, & Keely, 2022; Choghakhori et al., 2017). In light of these findings, the enhanced Th17 frequencies observed in children of the present study may have affected the gut-brain axis via mucosal tissue inflammation resulting in IBS-specific symptoms, such as indicated in this study by the report of abdominal symptoms/headaches. Although the Th17 cell frequencies measured in this study originated from peripheral blood and not directly from mucosal tissue samples, the frequencies can be indicative of conditions in the tissue. For instance, studies of patients with IBD demonstrated an increased frequencies of Th17 cells and decreased frequencies of Tregs, both in the intestinal mucosa and in the peripheral blood (Eastaff-Leung et al., 2010).

Moreover, the immune status during pregnancy may also directly exert permanent and tissue-specific impacts on the child's immunity (Lim et al., 2021). A recent study in mice reported that adult offspring exposed to high maternal IL-6 serum levels had elevated frequencies of Th17 cells in the small and large intestinal lamina propria (Lim et al., 2021). Furthermore, this study indicated that increased IL-6 levels can directly induce epigenetic changes on fetal intestinal epithelial stem cells, resulting in an enhanced protective immunity and resistance to gut infections (Lim et al., 2021). However, the authors also suggested that the augmented immune responses may also lead to an increased susceptibility to colitis-related inflammation in the adult offspring (Lim et al., 2021). If these findings are transferred to the present study, the high CRP levels in maternal serum samples and the in all probability preceding high IL-6 levels may also have potentially initiated epigenetic changes in the fetal epithelium and Th17 cell differentiation, which promoted the increased Th17 cell prevalence and the presence of abdominal symptoms/headaches in 5-year-olds.

5.5 Strengths and limitations

This study has several strengths and limitations. A particular strength is that the data used for the analyses in this study were derived from a prospective longitudinal cohort, with data collected on mother-child pairs from early pregnancy to the age of 5 years. Due to the broad data collection of the PRINCE study, this study afforded the possibility to examine a diverse range of stressors. There is still a paucity of human studies investigating the potential long-term effects of specific early-life stressors on the immune system in children. This study therefore provides a unique dataset to assess whether exposure to stressors is associated with $CD4^+$ T cells in 5-year-old children. A further strength is that the study cohort is relatively representative of the German national average, making it particularly relevant to investigate the potential impact of common early-life stressors.

Based on the utilized linear regression models, this study showed that apart from the findings regarding maternal medication use during pregnancy and infant systemic antibiotic use in the

first year of life, none of the other stressors examined were significantly associated with $CD4^+$ T cells of children at 5 years of age after adjustment for multiple testing. The absence of associations may be attributable to the size of the cohort and the composition of the stressor variables. In particular, it became apparent that the relatively small size of this study population entails limitations. Some stressors, such as maternal obesity, only occurred to a small number of women in this cohort, which makes it more difficult to identify possible associations with $CD4^+$ T cells from 5-year-olds. Moreover, the limited number of cases precluded the possibility of performing linear regression models with specific types of stressors, such as particular chronic diseases or specific types of pregnancy complications. This is also a reason why the majority of stressors, such as chronic diseases, maternal infections, maternal medications or pregnancy complications, were each primarily examined as collective stressor variables. The use of collective stressor variables also entails that a potential association between a specific type within a collective stressor variable and $CD4^+$ T cells may also interfere with a potential association between another specific type within a collective stressor variable and $CD4^+$ T cells. For example, it might be possible that preeclampsia and gestational diabetes within the collective pregnancy complication variable might have different associations with $CD4^+$ T cells. Consequently, these potential specific associations could cancel each other out within the collective stressor variable and thus be missed in the results of the linear regression analyses used. Therefore, a larger study population would be beneficial to investigate specific types of stressors in more detail.

In addition to the relatively small size of the cohort, the lack of information on some stressors also limited a more detailed investigation of exposure to certain stressors. The data collected on maternal infections during pregnancy in the PRINCE study lacked sufficient detail to ascertain the severity of the reported infections and the types of infections were not clearly verified. However, this information is crucial to investigate the potential impact of infections. In particular, a number of studies have indicated that children exposed to severe types of infection are at an increased risk of developing alterations, including the occurrence of neurological disorders, in comparison to those exposed to non-severe infections (Al-Haddad et al., 2019; Atladóttir et al., 2010; Hall, Willis, Rodriguez, & Schwarz, 2023). Due to the lack of information on the severity of the reported infections in this study, exposure to prenatal infections was assessed using a collective maternal infection variable, encompassing all reported infections. The aggregation of all infections is also likely to be the reason why no notable correlations between maternal infections and $CD4^+$ T cells were observed. The use of methods, such as polymerase chain reaction (PCR) testing to ascertain the specific type of pathogen and infection, and the establishment of a defined severity score for each infection may prove beneficial in future studies, as it would provide a more detailed basis for investigating the precise impact of prenatal infections on $CD4^+$ T cells in children.

The PRINCE study acquired data on the stress perception of women during pregnancy once per trimester using the instrument PSS-14. In this study, high prenatal maternal stress was defined as a PSS-14 score >26. Linear regression models in this study did not indicate an association between prenatal exposure to a PSS-14 score >26 at any trimester and $CD4^+$ T cells of 5-year-olds in the adjusted model. Although the PSS-14 questionnaire has been used in a large number of scientific studies, an important limitation of this instrument is that it only assesses the participant's subjective perception of stress and not the actual level of stress experienced. Furthermore, as the perception of stress was only recorded in total per trimester, it is not possible to provide a detailed account of the specific experiences of stress within each trimester. Thus, it is unclear whether the reported stress was experienced on single days or over an extended period of several weeks. Consequently, a more closely scheduled recording of the PSS-14 score may be a beneficial option. In addition, the use of further methods may enhance the validity. For instance, a number of studies indicated a significant association between stress perception and hair cortisol concentrations (HCC), suggesting HCC as an objective biomarker of chronic psychological stress (Ling, Xu, Robbins, & Meyer, 2020; Lynch et al., 2022). PSS-14 measurements in combination with HCC may therefore provide a potential avenue for more precise stress measurement.

In this study, the "*multiple-hit model*" was examined by assessing the exposure to multiple types of stressors using a cumulative stressor variable and multivariable linear regression models. However, multiple hits of a specific type of stressors was not included in the analysis of this study, as for example, the multiple occurrence of various infections during a pregnancy. However, it is likely that multiple exposures to a particular stressor may also result in the onset of alterations in offspring, which offers an intriguing avenue for future research (Zerbo et al., 2015).

In the present study, the investigation of exposure to stressors focused on the gestational period and the first year of life. In line with a number of studies, these periods are suggested as particular windows of susceptibility to stressors (Barker, 2007; Fouhy et al., 2019; Olin et al., 2022). Although the immune system matures with advancing age and thus builds up robustness against intrinsic and extrinsic factors, it is nevertheless possible that other factors may occur at a later stage in childhood and cause alterations in the immune system of children at the age of 5 years. To illustrate, a severe infection at the age of 3 years may act as a stressor or as a second stressor to a previous stressor during pregnancy, thereby priming or inducing developmental alterations. The lifestyle of the family may also play an important role in the development of the child. For instance, a study conducted by Olin *et al.* (2022) demonstrated discrepancies in the functional maturation of immune cells, particularly T cells, between children growing up in an anthroposophic lifestyle, where none of the children had developed IgE sensitization to allergens by the age of 5, and children growing up in a non-anthroposophic

lifestyle, where all of the children had developed IgE sensitization to allergens (Olin et al., 2022). The anthroposophic lifestyle is a holistic approach to infant care that includes e.g. home birth, less frequent body washing, prolonged breastfeeding and limited use of vaccinations (Alm, Swartz, Lilja, Scheynius, & Pershagen, 1999; Olin et al., 2022; Stenius et al., 2011). Olin *et al.* (2022) observed that children without IgE sensitization to allergens and raised in families with anthroposophic life style had a reduced production of pro-inflammatory cytokines in various cell types from birth until the age of 5 compared to children growing up in non-anthroposophic lifestyle with IgE sensitization (Olin et al., 2022). In addition to lifestyle, genetic and epigenetic factors, socio-economic aspects and the child's diet, other, as yet unidentified influencing factors may also play a decisive role (Acevedo et al., 2015; Olin et al., 2022; Stam et al., 2012; Verduci & Köglmeier, 2021). This gives rise to a wide range of research questions that can be explored in future studies.

6 Conclusion

The objective of the present study was to investigate the influence and potential long-term consequences of early-life stressors on $CD4^+$ T cell immunity in children beyond infancy.

Using univariable and multivariable linear regression models, this study demonstrated that exposure to medications, particularly L-thyroxine, during pregnancy and systemic antibiotics in the first year of life, were each significantly associated with increased frequencies of Th17-like cells in 5-year-old children who experienced abdominal symptoms/headaches. Subsequent functional analyses of $CD4^+$ T cells from children with these exposures showed a strong trend towards increased frequencies of IL-17A-producing $CD4^+$ T cells upon TCR-specific stimulation. Moreover, the mothers of these children exhibited significantly elevated levels of the non-specific inflammatory marker CRP and significantly reduced levels of the cytokine IL-4 in the serum during the third trimester, indicating altered maternal immune homeostasis during pregnancy.

The findings of this study suggest that exposure to maternal thyroid hormone dysregulation, as indicated by the need for the use of L-thyroxine, during pregnancy and exposure to systemic antibiotics during the first year of life, including their effects on the gut microbiota and the underlying bacterial infections, alter the $CD4^+$ T cell development during the early phase of life, resulting in an increased differentiation towards Th17 cells in 5-year-old children. Furthermore, an aberrant maternal cytokine profile may have played a pivotal role in the long-term enhanced Th17 cells and the onset of abdominal symptoms/headaches in these children at the age of 5.

Taken together, early-life stressors are associated with long-term changes in $CD4^+$ T cells in children and a clinical phenotype potentially indicating pediatric IBS. Further studies are needed to investigate the direct link with maternal thyroid hormone dysregulation or severe infection requiring systemic antibiotic treatment during infancy, immune development and the consequences for the child's health.

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Supplement

Table S1: Mothers of the children included in the PRINCE study with a PSS-14 score >26 during pregnancy.

	1st trimester (12.-14. GW)		2nd trimester (24. GW)		3rd trimester (34.-36. GW)	
Total N:	*N=104*		*N=113*		*N=113*	
	N	*%*	*N*	*%*	*N*	*%*
PSS-14 score >26	14	14	20	18	15	13

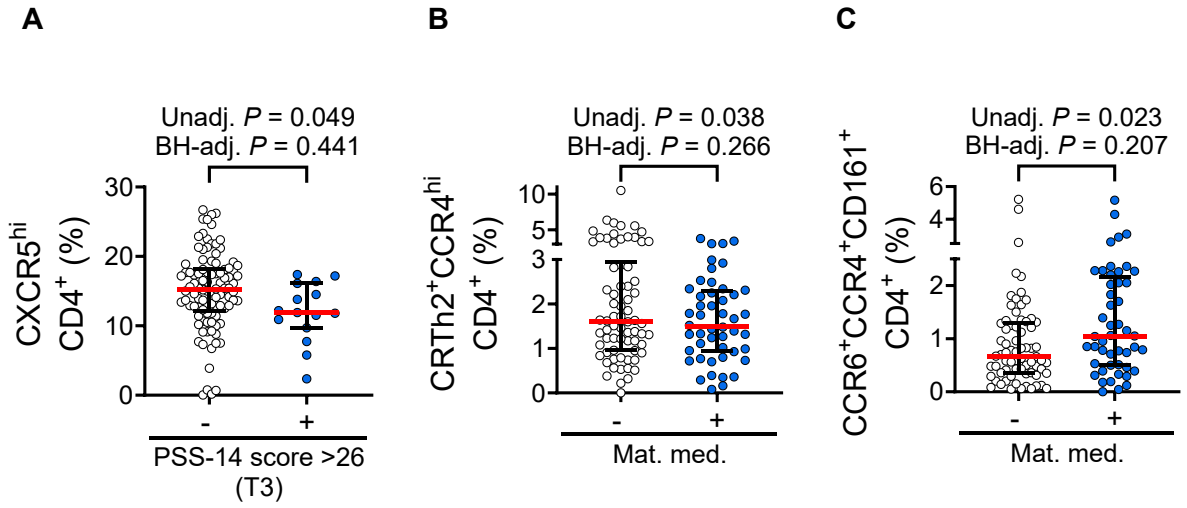

Figure S1: Association of single stressors on $CD4^+$ T cell subsets of 5-year-old children. (A) PSS-14 score >26 at third trimester on $CXCR5^{hi}$ $CD4^+$ T cells (FLTR: n = 98; 15). (B) Maternal medication use (Mat. med.) on $CRTh2^+CCR4^{hi}$ cells and $CCR6^+CCR4^+CD161^+$ $CD4^+$ T cells (FLTR: n = 70; 48). Data presented as median percentage (± IQR).

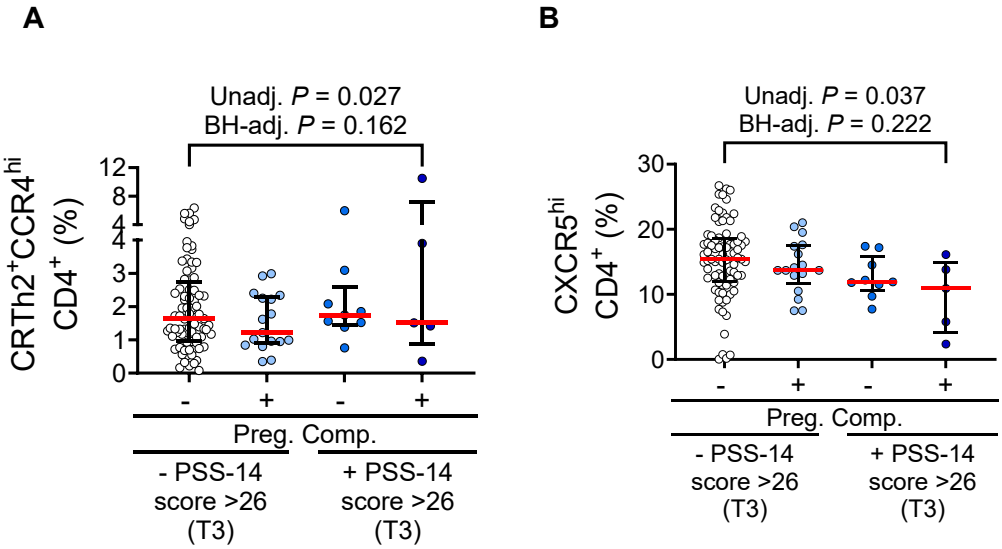

Figure S2: Association of pregnancy complications (Preg Comp.) and PSS-14 score >26 at third trimester (T3) with the frequencies of (A) $CRTh2^+CCR4^{hi}$ cells and (B) $CXCR5^{hi}$ cells of 5-year-old children (FLTR: n = 80; 17; 9; 5). Data presented as median percentage (± IQR).

Table S2: Benjamini-Hochberg-adjusted *P*-values of univariable linear regression analyses of prenatal and early-life stressors with $CD4^+$ T cell subsets in children included in the PRINCE study at 5 years of age (Table 4A and 4B).

	Chronic diseases	Maternal BMI >29.9 at 1st trimester (12.-14. GW)	PSS-14 score >26			Maternal medication use	Maternal infections	Pregnancy complications	Antibiotic use in the first year of life
			1st trimester (12.-14. GW)	2nd trimester (24. GW)	3rd trimester (34.-36. GW)				
	N=20	*N=9*	*N=14*	*N=20*	*N=15*	*N=48*	*N=68*	*N=22*	*N=31*
Total N:	*Total N:*	*N=118*	*N=104*	*N=113*	*N=113*	*N=118*	*N=118*	*N=116*	*N=101*
Baseline:	*No*	*BMI <30*	*PSS-14 score <27*			*No*	*No*	*No*	*No*
	BH-adjusted P-value	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	0.974	0.702	0.702	0.702	0.702	0.702	0.974	0.974	0.974
• $CD4^+CD25^-$ memory cells	0.996	0.722	0.722	0.722	0.722	0.722	0.996	0.996	0.996
• $CXCR3^+CCR6^-$ cells	0.870	0.707	0.870	0.884	0.884	0.707	0.870	0.884	0.975
• $CRTh2^+CCR4^{hi}$ cells	0.895	0.895	0.895	0.453	0.266	0.266	0.978	0.978	0.895
• $CCR6^+CCR4^+CD161^+$ cells	0.569	0.945	0.945	0.569	0.569	0.207	0.669	0.569	0.569
• $CCR6^+CCR4^+CD161^-$ cells	0.679	0.612	0.919	0.612	0.612	0.612	0.919	0.612	0.679
• $CXCR5^{int}$ cells	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964
• $CXCR5^{hi}$ cells	0.726	0.726	0.931	0.726	0.441	0.726	0.907	0.726	0.726
• $CD25^+CCR4^-$ $CD45RA^+$ cells	0.902	0.729	0.902	0.949	0.949	0.949	0.949	0.949	0.729
• $CD25^+CCR4^+CD45RA^-$ cells	0.862	0.459	0.862	0.862	0.459	0.862	0.862	0.862	0.459

Table S3: Multivariable linear regression analyses of abdominal symptoms/headaches and maternal L-thyroxine use with $CD4^+$ T cell subsets of in children included in the PRINCE study at 5 years of age. Adjusted model for sex of the child (female) and mode of delivery (C-section).

	Abdominal symptoms/headaches		
	Maternal L-thyroxine use		
Baseline: No L-thyroxine use and no abdominal symptoms (N=63)	*N=5*		
	Coeff.	*P-value*	*BH-adjusted P-value*
• naive $CD4^+CD25^-$ cells	-5.380	0.129	0.387
• $CD4^+CD25^-$ memory cells	5.415	0.128	0.384
• $CXCR3^+CCR6^-$ cells	-2.364	0.519	0.900
• $CRTh2^+CCR4^{hi}$ cells	-0.928	0.220	0.330
• $CCR6^+CCR4^+CD161^+$ cells	*1.259***	*0.005*	*0.010*
• $CCR6^+CCR4^+CD161^-$ cells	*1.997**	*0.041*	0.104
• $CXCR5^{int}$ cells	0.686	0.692	0.869
• $CXCR5^{hi}$ cells	0.452	0.860	0.986
• $CD25^+CCR4^-$ $CD45RA^+$ cells	-8.669	0.074	0.111
• $CD25^+CCR4^+CD45RA^-$ cells	1.503	0.708	0.708

*Note. *P-value<0.05; L-thyroxine= levothyroxine*

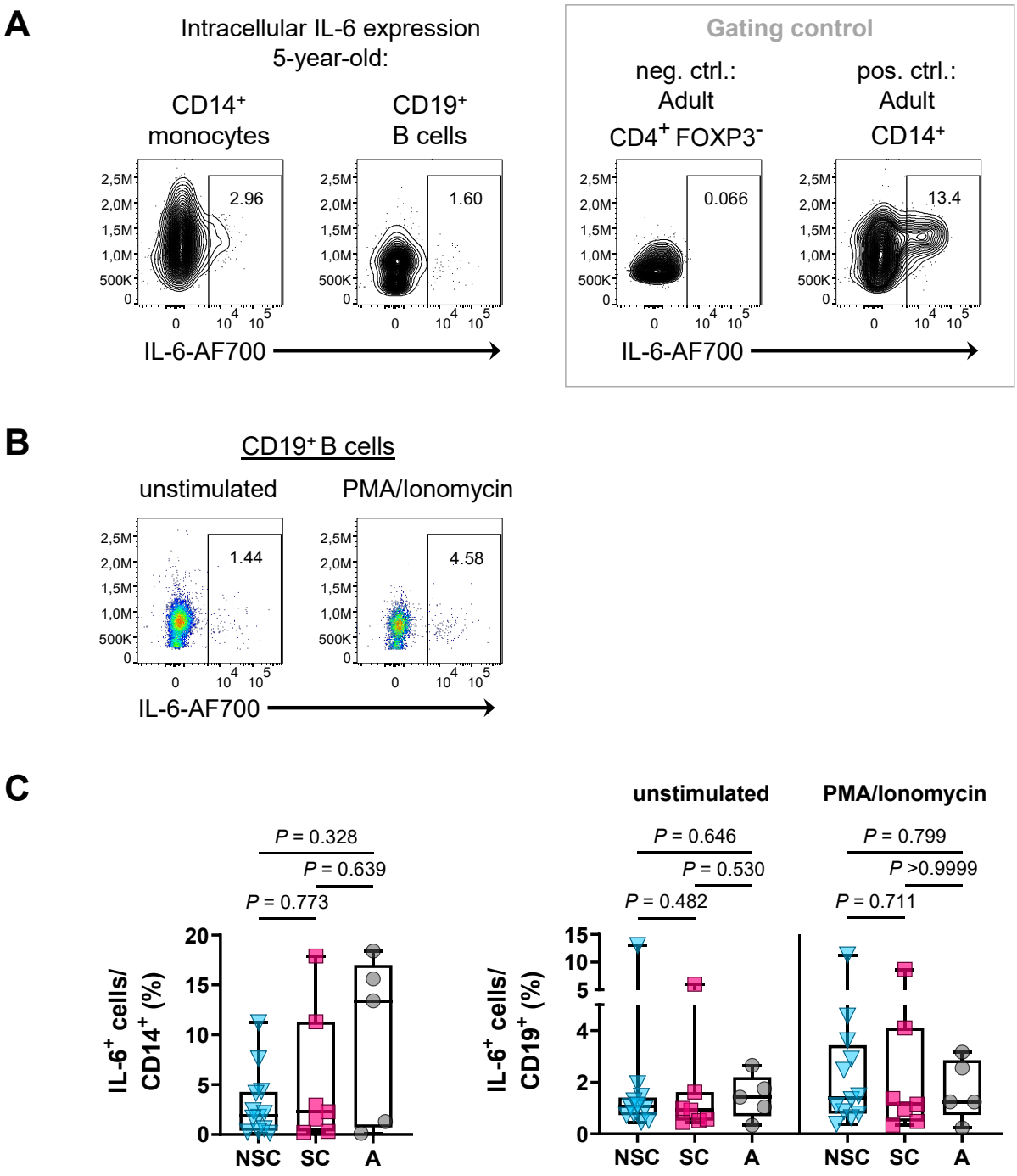

Figure S3: Intracellular IL-6 expression of $CD14^{+}CD19^{-}$ monocytes and $CD19^{+}CD14^{-}$ B cells from 5-year-old children with abdominal symptoms/headaches and exposed to early-life stressors, including prenatal maternal medication use and/or early life antibiotic use (symptomatic children (SC)). IL-6 production of $CD14^{+}CD19^{-}$ monocytes and $CD19^{+}CD14^{-}$ B cells from 5-year-olds who were not exposed to stressors and did not show abdominal symptoms/headaches (non-symptomatic children (NSC)), and from adults (A) were used as comparisons. **A** Representative flow cytometric plots of the gating strategies to identify IL-6-positive $CD14^{+}CD19^{-}$ monocytes and $CD19^{+}CD14^{-}$ B cells. **B** Flow cytometric plots of IL-6-production by $CD19^{+}CD14^{-}$ B cells from 5-year-olds 18-hour stimulation with 0.05 µg/ml PMA and 1 µg/ml ionomycin or unstimulated. **C** Frequencies (%) of IL-6-producing $CD14^{+}CD19^{-}$ monocytes and $CD19^{+}CD14^{-}$ B cells. Data are presented as boxplots where midlines indicate medians, boxes indicate interquartile range and whiskers indicate minimum/maximum range. Differences between groups were evaluated by two-sided Mann–Whitney U-tests, where *P*-values < 0.05 were used to identify a significant result.

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Hamburg, den 17.03.2025 Unterschrift

D. Sandfort

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