MASTERARBEIT | MASTER'S THESIS

Titel | Title

Immunomodulatory effects of short-chain fatty acids on HDAC1 deficient CD4+ T cells

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Molecular Microbiology, Microbial Ecology and Immunobiology
Betreut von | Supervisor:	Clarissa Campbell Menezes BSc MSc PhD

ACKNOWLEDGEMENTS:

During the time of being a MSc student at the University of Vienna I had the pleasure of meeting a lot of interesting and kind personalities that shaped me to the scientist I am today. It was an amazing experience that I will always carry with me with pride and gratitude. This section is in honour of the people who supported me along this journey.

First of all, I would like to thank my amazing supervisor and Principal Investigator of the Campbell Laboratory group at CeMM **Prof. Dr. Clarissa Campbell**. She cheerfully introduced me to the world of immunology and parasitology and is a real role model for any aspiring scientist. I really appreciate that even though she was very busy at times, she always had an open ear for the questions and difficulties I encountered along the way, and it was only through her guidance that I was able to finish my research in a respectable manner.

I would like to thank Dr. Megan Teh, Dr. Tomas Brabec, Dr. Cristiano Fernandes, Dr. Maximilian Baumgartner, Elena Krstevska, Thet Khaing Nyein, Adrija Chakrabarty, Olivia Neuhofer, Dinah Dollack, Rodrigo Fernandes, Felix Grössing, Isabella Kraus and Franziska Schnaufer. They provided great company, help and scientific insights during my stay at the Campbell laboratory.

I would also like to express gratitude to the **SFB-70 program** and for providing the financial support for this work and additional guidance by its members Dr. Nicole Boucheron, Prof. Dr. Wilfried Ellmeier, Dr. Matarr Khan and Osamah Al-Rubaye.

I am also very grateful for the cooperation with **Dr. Joris Van Der Veeken** and his laboratory group at the IMP with special thanks to Cristina Jäger.

In the end, I would speak thanks to my family who taught me too many invaluable things to list them here, but which were crucial for me to become the man I am today. I am grateful for the opportunity to be able to follow this dream and for all the support they have given me.

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1.1 GRAPHICAL ABSTRACT:

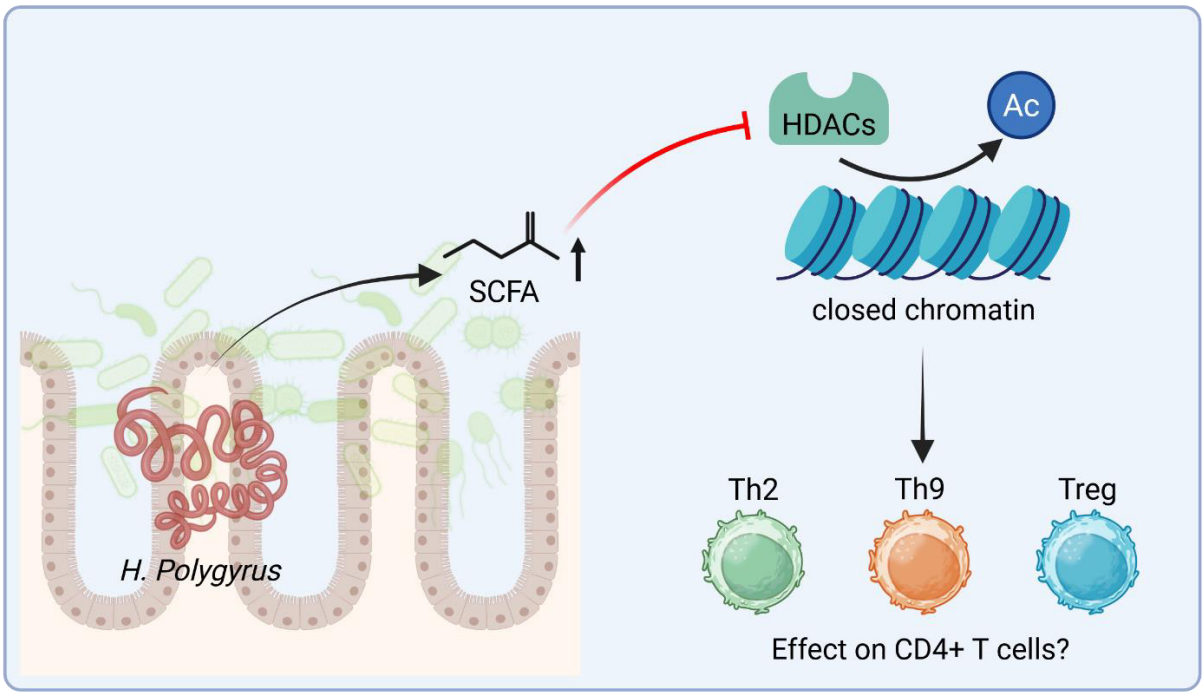

Figure 1: Graphical abstract of key components of this project. Parasitical nematodes such as *Heligmosomoides polygyrus* can affect the hosts metabolite pool including short-chain fatty acids (SCFA), which then act as pan-inhibitors of histone deacetylases (HDACs) in the host. Histone deacetylation on the position H3K27 leads to a more open and accessible chromatin which allows for distinct transcriptional processes that may alter hosts cell function. One of the major goals of this project is to investigate the SCFA-HDAC axis in the context of CD4+ T helper (Th) cell differentiation and function, focusing on Th2, Th9 and Treg cells. Created in https://BioRender.com

1.2 ABSTRACT (ENGLISH):

Around 2 billion people are infected with a soil-transmitted helminth (STH) worldwide, which can have detrimental effects on the body and even cause morbidity in extreme cases. In order to defend against this infection an immune response against this parasite is mounted by the innate and adaptive immune system of the host. CD4+ T helper cell subsets such as Th2, Th9 and regulatory T (Treg) cells are key contributors to this immune response and therefore have been the focus of several investigations in the field. Interestingly, various studies on report that the infection with an STH can also has beneficial effect in various diseases such as colitis, arthritis and COVID-19 related cytokine storm. Shifts in the microbiome composition caused by infection with a STH leads to in an increase of short-chain fatty acids (SCFAs) in the host. These SCFAs have been identified to possess immunomodulatory capabilities as they are able to inhibit histone deacetylases (HDACs), a key group of enzymes that regulate the acetylation status of histones as well as proteins. In general, HDAC inhibition results increases histone acetylation which allows for the chromatin to be in a more accessible structure and permissible for transcription. HDAC1 is a class I histone deacetylase and a key epigenetic regulator of the lineage-specific transcriptional programming in T helper cell subsets.

In this thesis the SCFA-HDAC1 axis and its implication in the expression of key transcription factors and cytokines of Th2, Th9 and Treg cells were investigated to gain more insight into the host-parasite dynamics and possible novel treatment avenues. Butyrate has been identified as the most potent driver of CD4+ T cell modulation compared to propionate and acetate. When administered to in vitro cell cultures, butyrate negatively impacts cell proliferation, promotes the expression of FOXP3 during Th9 polarization and increases IL-4 expression in Th2 cells. Together with these findings, the function of HDAC-1 as a T cell regulator and putative butyrate target was investigated through the use of $HDAC1^{flox}CD4^{Cre}$ mice, which possess a conditional knockout of the *hdac-1* allele in all conventional T cells. These effects were investigated both in naïve CD4+ T cells and on cultures of already committed/activated CD4+ T cells. Finally, a pilot experiment that investigates a possible mechanism by which butyrate downregulates Interleukin-9 (IL-9) expression in Th9 cells via induction of FOXP3 expression was conducted.

1.3 ABSTRACT (GERMAN):

Weltweit sind circa 2 Milliarden Menschen von einem durch Boden übertragenen und im Darm lebenden Helminth infiziert, wodurch sie viele schwerwiegende körperliche Schäden erleiden und in Extremfällen auch sterben können. Als Antwort auf eine Infektion mit diesem Parasiten mobilisiert der menschliche Körper das angeborene und erworbene Immunsystem, um die Infektion zu bekämpfen. Helfer T Zellen wie zum Beispiel Th2, Th9 oder Treg sind in diesem Unterfangen unentbehrlich, weshalb sie der Fokus von viele immunologischen Studien sind. Zur Überraschung vieler Forscher wurden weltweit eine Mehrzahl an Studien veröffentlicht, in welchen eine Infektion mit einem Darmparasiten positive Auswirkungen auf eine Vielzahl an Krankheiten wie zum Beispiel Colitis, Arthritis, Asthma und durch COVID-19 verursachten Zytokin-sturm hat. Einhergehend mit diesen Infektionen ist ein Wechsel in der Zusammensetzung des Microbioms des Hosts, wodurch erhöhte Konzentration von kurzen Fettsäuren im Darm erzeugt wird.

Diese Fettsäuren wirken als Immuno-modulatoren und sind fähig Histondeacetylasen zu inhibieren. Diese Histondeacetylasen sind Schlüsselenzyme in der Regulation des Acetylierungsgrades von Histonen und Proteinen. Eine Inhibierung von Histondeacetylasen führt zu einem höheren Acetylierungsgrades des Chromatins, wodurch es weniger kompakt und dadurch zugänglicher für Transkriptionsfaktoren ist. HDAC1 ist eine dieser Histondeacetylasen und ist essenziell für das Zellart spezifische Transkriptionsprogramm von T Helfer Zellen.

In dieser Thesis wurde untersucht, wie sich kurze Fettsäuren und HDAC1 auf die Expression von Transkriptionsfaktoren und Zytokinen von Th2, Th9 and Treg Zellen auswirkt, um dadurch mehr Einsicht in die komplexe Host-Parasit Dynamik zwischen Menschen und Darmparasiten zu erlangen. Butyrat, Propionat und Acetat wurden an in-vitro T Zell Kulturen getestet, wobei Butyrat als effektivste kurze Fettsäure in der Modulierung von diesen Zellen identifiziert wurde. Butyrate schwächte die Zellwachstumsrate von T Helfer Zellen, induzierte die Expression von FOXP3 in Th9 Zellen und erhöhte die IL-4 Expression in Th2 Zellen. Mit der Hilfe von $HDAC1^{flox}CD4^{Cre}$ Mäusen, welche einen konditionellen Knockout für das *hdac1* Allel besitzen, wurde zusätzlich der Einfluss von HDAC1 auf die durch kurze Fettsäuren erzeugten Effekte thematisiert. Diese Dynamik zwischen kurzen Fettsäuren und HDAC1 wurde untersucht in naiven sowie auch aktivierten CD4+ T Helfer Zellen. Abschließend wurde auch ein Initialexperiment ausgeführt, welches einen möglichen Mechanismus in dem FOXP3 auf dem *Il-9* Genlocus direkt bindet und dadurch dessen Expression inhibiert erforscht.

2. INTRODUCTION:

2.1 SOIL-TRANSMITTED HELMINTHS (STHS) WORLDWIDE:

Helminths can be categorized in two major phyla: the nematodes and platyhelminths. The nematode phyla, also known as roundworms, include soil-transmitted helminths and filarial worms which cause lymphatic filariasis and onchocerciasis. Platyhelminths are also known as flatworms and include flukes schistosomes and tapeworms which cause cysticercosis. Soil transmitted helminths (STHs) such as *Ancylostoma duodenale* (hookworm), *Trichuris trichiura* (whipworm) and *Ascaris lumbricoides* (roundworm) enter the mammalian body through contaminated soil containing the eggs of the parasite. They infect approximately 2 billion people worldwide with the highest infection prevalence being present in Papua New Guinea, Malaysia and the Philippines (as of 2020; [1]). The most important preventative measurement against helminth infection is proper hygiene while the treatment options are based on various drugs like mebendazole and albendazole. If these treatment options fail, an endoscopic and surgical therapy may be required [2]. The medical condition characterized by parasitic worm infestation termed helminthiasis, is the most frequent infectious disease in the world in terms of global disease burden [3]. Infection with these helminths put a great burden on the human body which can lead to impaired nutrient utilization, anaemia, developmental disturbances and morbidity [1,4].

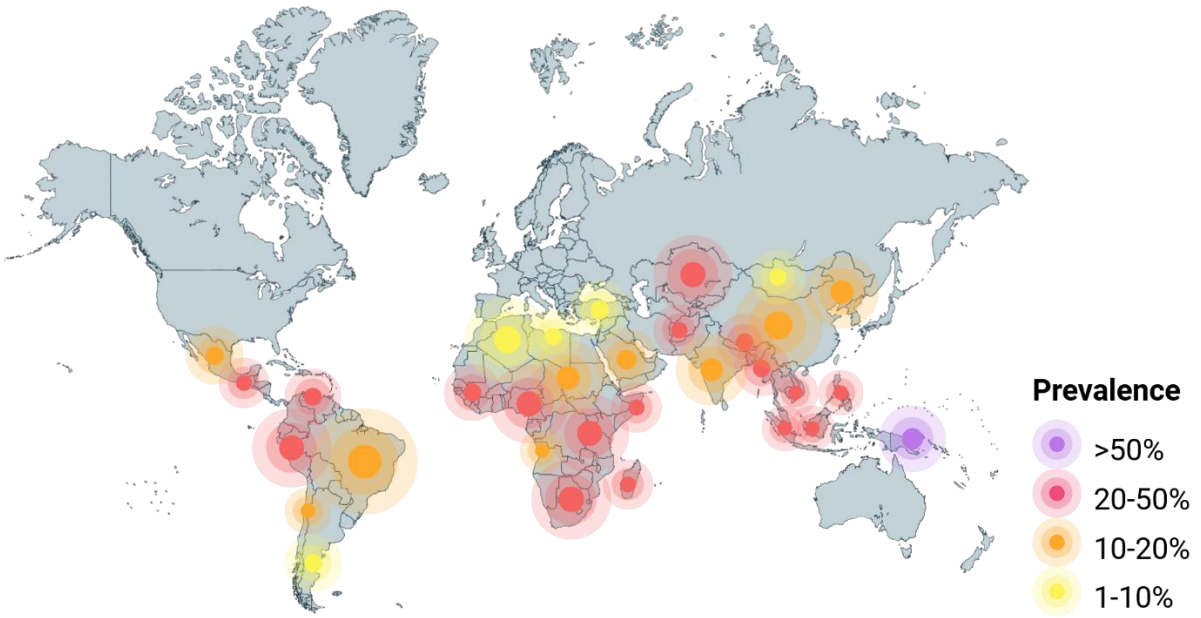

Figure 2: Depicted are global hotspots of soil-transmitted helminth (STH) infections and their prevalence as of 2020. This Figure compiles data from *Ancylostoma duodenale*, *Ascaris lumbricoides* and *Trichuris trichiura* infections. This figure has been adapted from [1]. Created in https://BioRender.com

After a successful infection of the mammalian body by the STH, the host immune system becomes activated and mounts an immune response against the parasite. The body's defence against the infection is rendered more difficult by immunomodulatory capabilities of the parasite, as it tries to facilitate its own survival while simultaneously protecting the host from excessive pathology and infection by other potential pathogens. Rodent-specific STHs like *Heligmosomoides polygyrus* and *Trichuris muris* are commonly used to study these immune responses. These studies typically rely on a single infection of an artificially high dose of parasites which is in contrast to natural occurring events where frequent low level exposure are a more realistic scenario. Even though there are specific immune responses depending on the species of parasite, many immune responses to the infection can be generalized. Typically, during the invasion of the host, the parasite will damage host tissue at the site of infection, leading to considerable remodelling of the environment and the release of alarmins such as interleukin (IL)-25 or IL-33. These alarmins will then stimulate an innate immune response as well as prime the initial adaptive immune response [5].

2.2 IMMUNE RESPONSES AGAINST HELMINTH INFECTIONS:

Helminthiasis-induced immune responses in a mammalian host are characterized by a prominent expression of IL-4, IL-5 and IL-13 through Th2 cells. Additionally, other T helper subsets such as Th9 cells provide additional help in the defence against the helminth through the production of IL-9 which promotes the induction of mastocytosis, increases the production of IgE and IgG1 and promotes the expulsion of the worms through facilitating increased intestinal contractility. IL-10 is another cytokine that is produced by Th2 and Th9 cells which, together with IL-9 induces a type 2 inflammatory with innate lymphocytes, mast cells, basophils and various other cells as key effector cells that try to reduce the parasitic burden [3]. TGF-β mimetics such as *Hp*-TGM, which is secreted by *H. polygyrus*, are actively secreted by the helminth in order to induce Treg cells which are beneficial to the helminths survival and also play role in preventing parasite-associated pathology. Alternatively to secreting these TGF-β mimetics, helminths can also induce Treg cells indirectly through interaction with bystander cells such as macrophages and dendritic cells (DCs)[6].

Infections with STHs have shown to induce various changes in the mammalian body and its associated microbial communities [7]. The term microbiome is referred to as the collection of microorganisms such as bacteria, fungi and viruses that naturally live in or on particular parts of the human body, such as the skin, the gastrointestinal tract and other mucosal sites. The human body hosts roughly as many microbial cells as mammalian cells. This highly complex and diverse system is dynamic and can change rapidly in response to environmental inputs experienced by the host. External factors that can lead to changes in the human microbiome besides infections with STHs are for example diet, exercise and

medication. The microbiome protects the host against pathogens and promotes a healthy development of the immune system. Environmental exposures can change or disrupt a healthy microbiome which could increase the incidence of neurological diseases, cardiovascular diseases, allergies, inflammatory bowel disease (IBD) and many more [8].

Intriguingly, plethora studies suggest that infections with certain intestinal parasites can have beneficial effects on the human body. The suppression of a range of inflammatory conditions like autoimmune disease and allergy have been associated with Treg cells arising during helminth infections [6,9,10]. During a study on multiple sclerosis (MS) patients that unintentionally became infected by gastrointestinal helminths of various species, it was found that TGF-β and IL-10 expression were increased, together with a more prominent Treg cell and regulatory B (Breg) cell activity. Breg cells are a diverse subset of B cells important for the regulation and suppression of immune responses and thereby help the body via maintaining immune homeostasis and preventing excessive inflammation [11,12]. In this study, significantly decreased disease exacerbations and fewer lesions were observable in infected over uninfected MS patients [13]. Another study from 2009 reported that rectal submucosal administration of *Trichinella spiralis* antigen reduces the severity of dinitrobenzenesulphonic acid (DNBS)-induced colitis [14]. Furthermore, a study from 2016 highlighted how an infection of *Nippostrongylus brasiliensis* in mice drives a Th2 and eosinophil response which supresses inflammatory arthritis [15]. A more recent study from 2024 showed that *Trichinella spiralis* infection of mice induces IL-9, which alleviated COVID-19 related cytokine storm [16]. Altogether, these studies show promising results showcasing how host-parasite interactions modulate immune responses in various disease models and could thereby help identifying novel treatment avenues.

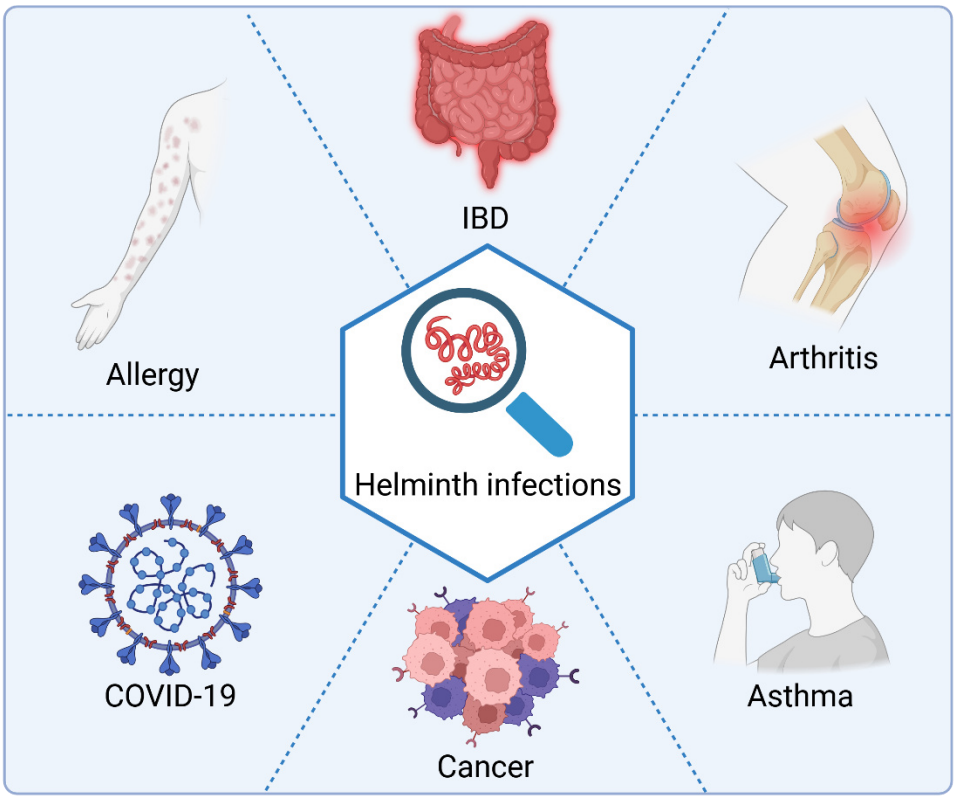

Figure 3: Inflammatory diseases shown to be ameliorated by helminth induced immunoregulatory processes caused. These diseases are potentially able to benefit from future research in the field of STH infections. Created in https://BioRender.com

2.2.1 INNATE IMMUNITY:

The essential mechanisms for rapid sensing and elimination of pathogens through recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) via pattern recognition receptors (PRR) as it occurs during a microbial infection is regulated by the innate immune system [17]. Innate lymphoid cells (ILC2s) are one of the earliest responders to STH infection. As they proliferate during the infection they act as early sources of IL-4, IL-5 and IL-13. This early cytokine production is important for the following adaptive immunity as a depletion of ILC2 resulted in a delay of Th2 responses [18]. Additionally, alternatively activated macrophages (M2) also play a key part in the innate immune response against STH. They are required for trapping and killing of larval stages of the parasites through the production of arginase-1 (Arg-1) [19,20]. Neutrophils, eosinophils, basophils and mast cells have also been identified to expand at the site of infection but their role in parasite expulsion is more difficult to define and, in some cases, might be species-specific. Fo example during a *T. muris* infection study depletion of basophils resulted in increased susceptibility to parasite infection while the same depletion had no impact on resistance to *H. polygyrus* [21,22]. Similar species-specific observations have been made regarding mast cells and eosinophils. Neutrophilia or the overproduction of neutrophils, has also been identified to either act to improve

worm expulsion via the release of neutrophil extracellular traps (NETs)[23] or function to repair tissue damage once the infection has been resolved [24].

2.2.2 ADAPTIVE IMMUNITY:

Adaptive immunity is the result of a gene locus duplications and subsequent differentiation events during early evolution of vertebrates and allows a more flexible immune response than the innate immune system can provide [25,26]. In contrast to innate immunity, which is already fully functional in the germline genome, the adaptive immune system depends on the random recombination of somatic gene segments followed by a selection process to remove cells recognizing self-antigens that arise by chance. T lymphocytes together with B lymphocytes form the cellular and humoral arms of the adaptive immune response, respectively. After the initial encounter with a pathogen, cells that express T or B cell receptors against its epitopes can persist in the host after the infection is cleared, allowing a fast secondary response in the case of re-exposure to the same pathogen in what is known as immunological memory [25].

T lymphocytes develop in the thymus and are exported to the periphery as naïve T cells, which are highly mobile and traffic between secondary lymphoid organs (SLO) including the spleen and lymph nodes. Dendritic cells engulf antigens from pathogens and bring them to SLO where they activate naïve T cells. T-helper cell differentiation is the instructive process in which a naïve T cell acquires its effector potential in response to various environmental signals such as the cytokine milieu present in the cell's vicinity [27]. After the lymphocytes have been activated, they emigrate from the SLO and travel to various sites in the body to exert their effector functions. Chemokine receptors and adhesion molecules regulate this trafficking directing the cells towards specific organs like the skin (via CLA-1 and CCR4) or the gastrointestinal tract (via the α4β7 integrin) [25].

Conventional T cells express αβ T cell receptors (TCRs), which recognizes antigen that is presented as a short peptide bound to MHC class I or class II molecules. Mature αβ T cells can be categorized in two distinct lineages according to the expression of their co-receptors. CD8+ cytotoxic T cells which recognize peptides presented on MHC-I molecules, and CD4+ T cells (including Th and Treg subsets) which detect peptides presented on MHC-II molecules [28]. All nucleated cells express MHC-I molecules and can therefore become the target of CD8+ T cell mediated killing in the event of a viral infection. MHC-II expression is restricted to certain cells of the immune system like dendritic cells, macrophages and, B cells (known as professional antigen presenting cells, or APCs) but is also found in monocytes and intestinal epithelial cells. The peptides presented by MHC-II are generally larger than those presented by MHC-I and are derived from extracellular proteins and from self-proteins that are degraded via endosomes. Dendritic cells are the most efficient APCs, also providing co-stimulation and

cytokines to activate and polarize naïve CD4+ T cells [29]. The now activated CD4+ T cell can provide soluble signals to other cells of the immune system as well as to non-immune cells. Autoreactive cells that escape negative selection in the thymus are kept in check by FOXP3 expressing regulatory T (Treg) cells, which also play a critical role in regulating the magnitude of immune reactions during infection. In general, each CD4+ T cell lineage is dependent on a complex network of cytokine signalling and transcription factors that impose unique, cell-type specific epigenetic landscapes [30].

TH2 CELLS:

Upon antigen recognition via TCR the naïve CD4+ T cells can undergo differentiation towards various cell types, including Th2 cells which expresses GATA-binding protein 3 (GATA3) as their lineage-defining transcription factor and are able to secrete the cytokines IL-4, IL-5 and IL-13. The differentiated Th2 cells then migrate to the site of inflammation where they reencounter their cognate antigen, resulting in the production of large amounts of cytokines. Th2 cells are important for protection from helminth infections, humoral immunity and tissue repair, but can also contribute to chronic inflammations such as asthma or allergy [31]. The Wnt pathway transcription factor 1 (TCF-1) expressed downstream of the Wnt-β-catenin signalling pathway was shown to be indispensable for initiation of the Th2 differentiation program in a GATA3 dependent process [32,33]. It has also been shown that TCF-1 simultaneously negatively regulate the Th1 formation, which is likely due to it negatively regulating IFN-γ production [33]. The IL-4 receptor is made up of a combination of the IL-4Rα chain together with the common cytokine receptor γ chain and responsible for binding extracellular IL-4 which mediates downstream signalling through a series of phosphorylation events carried out by receptor-associated kinases [34,35]. Signal Transducers and Activators of Transcription (STATs) are phosphorylated downstream of cytokine receptors and act as transcriptional regulators to drive cellular responses. For instance, IL-4 induces Th2 differentiation through STAT6, which upon phosphorylation induces a pool of genes including *Gata3*. Together with TCR-mediated ERK/MAPK pathway this results in high levels of GATA3 protein expression. STAT6 activation also results in suppression of T cell specific enhancers that would promote cell fates other than Th2. IL-2 also contributes to Th2 differentiation via activation of STAT5. GATA3 is not exclusively expressed in Th2 cells, as it also plays a role in early T cell progenitors during development. CD4 T cells that possess a deletion of the *Gata3* gene are prevented from developing into Th2 cells and instead become Th1 cells when activated [36]. GATA3 is also implicated in Th2 cell proliferation as shown through downregulation of bromodeoxyuridine (BrdU) incorporation upon loss of one GATA3 allele [37]. The *Il5* gene promotor region also contains a GATA3 binding site, suggesting that GATA3 is a direct trans activator of the *Il-5* gene. Taken together, GATA3 stands out as the master transcription factor for Th2 polarization [36]. IL-5 is a crucial cytokine for eosinophil activation, differentiation, proliferation,

survival and recruitment. This highlights the importance of IL-5 as a therapeutic target in the pathogenesis of eosinophilic asthma [38]. IL-13 has some overlapping functions with IL-4 but also possesses several unique effector functions like its dominant role in resistance to gastrointestinal nematodes and as central mediator in the lung for eosinophilic inflammation, mucus secretion and airway hyperresponsiveness [39].

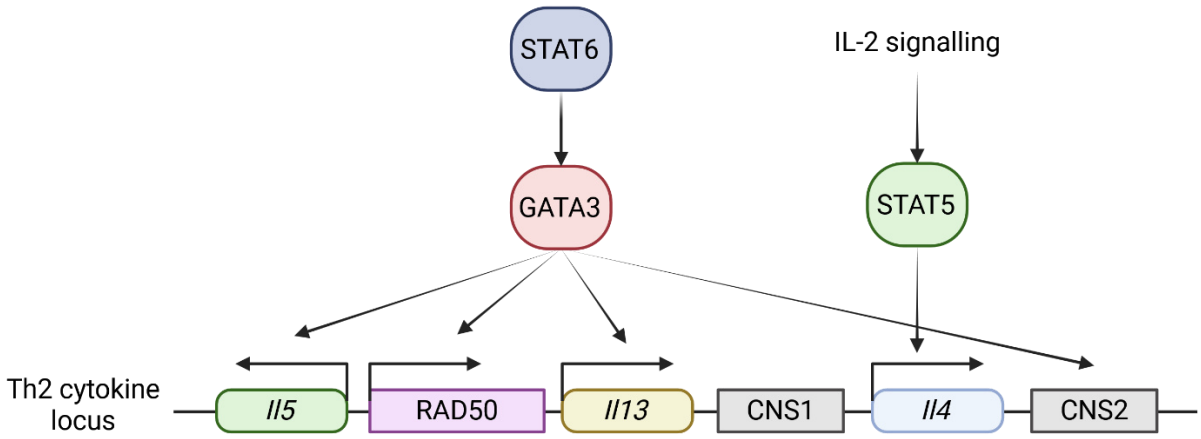

Figure 4: The Th2 cytokine locus showcasing GATA3 binding sites and STAT signalling. RAD50 is the locus control region of the Th2 cytokine genes consisting of four hypersensitive sites (RHS4, RHS5, RHS6 and RHS7). CNS1 and CNS2 are conserved non-coding sequences and function as gene enhancer regions. This figure has been adapted from [40,41]. Created in https://BioRender.com

TREG CELLS:

Regulatory T (Treg) cells are crucial for regulating peripheral immune tolerance and homeostasis through the wide range of environmental inputs they are able to detect and respond to [42]. Their suppressive capabilities are enacted through inhibitory cytokines (e.g., IL-10, IL-35 and TGFβ), cytolysis (e.g., via granzyme A/B or perforin), metabolic disruption (e.g., via IL-2 cytokine deprivation leading to apoptosis) and modulation of DC maturation and function (e.g., via LAG3) [43]. Based on their ontogeny and localization we can distinguish between two main Treg cell populations. The thymic derived Treg cells (tTreg; formerly known as natural Tregs or nTreg) arise during thymocyte development and are enriched for TCRs with a high affinity to self-peptides. In contrast, peripherally generated Treg cells (pTregs) that arise from naïve CD4 T cells activated by dietary or microbial antigens in the periphery. In the periphery these naïve CD4 T cells first start upregulating Treg signature gens such as CD25, CTLA4 and GITR becoming precursor Treg cells. Additional induction signals drive further pTreg cell differentiation, which can be divided in two distinct phases: the FOXP3-independent and the FOXP3-dependent phase [44]. In-vitro studies utilize induced Treg cells (iTregs) developed from naïve CD4+ T cells through stimulation with IL-2 and TGFβ in the presence of TCR signalling (2016) [45]. The X-chromosome linked FOXP3 acts as a lineage-defining transcription factor for Treg cells, being

important for their development and function [42,46]. Several regulatory elements that are specifically expressed at distinct stages of Treg cell development regulate FOXP3 gene expression, with many of the underlying mechanisms still not fully understood [44]. A study utilizing cell specific Smad2 conditional knockouts identified Smad2 and Smad3 as redundantly essential for TGFβ-mediated induction of FOXP3-expressing regulatory T cells as well as suppression of IFN-γ in CD4+ T cells [47]. FOXP3 mainly localizes to the nucleus where it can bind to over 700 genes that are involved in TCR signalling, transcriptional regulation and cell-cell communication. FOXP3 is able to exert its function by acting as a transcriptional activator as well as a repressor. FOXP3 either targets genes directly or with the help of other transcription factors [44]. Additionally, the NF-kB transcription factor family is crucial for the development and function of Treg cells. It consists of RELA (p65/RelA), c-REL (cRel), RELB (RelB), NFKB1 (p105/p50) and NFKB2 (p100/p52) who share identical but also non-redundant biological roles [48]. For tTreg but also peripherally induced Treg (pTreg) cells NF-kB signalling is essential for their suppressive function, homeostasis and in providing cell stability [48]. Furthermore, a study on primary Treg cells and Treg-like cells showcased how NFKB2, but not NFKB1, is required for maintaining FOXP3 expression [46]. In early thymic development T cell progenitors undergo TCR rearrangement which requires NF-kB signalling to provide survival signals to the thymocytes with a productively rearranged TCR beta chain. Following this, the TCR alpha chain is rearranged and cells become double positive (DP) thymocytes. Further downstream of T cell development, the CD4 single positive (SP) thymocytes are also dependent on NF-kB signalling for survival and maturation processes. In the generation of thymic Treg (tTreg) cells, moderately strong TCR-mediated NF-kB signalling is required for them to fully mature and achieve their suppressive capabilities [48]. The AP-1 proteins consisting of the four sub-families Jun, Fos, Maf and ATF-activating transcription factors are also important for Treg cell identity as they possess the ability to bind to the *foxp3* gene locus and promote the expression of FOXP3. These AP-1 proteins are regulated by the Mitogen Activated Protein Kinases (MAPK) induced signalling cascade which is a response to numerous environmental signals such as cytokines, growth factors and oncogenes. Active AP-1 components are able to form hetero-dimers with the NFAT transcription factor and through that this complex is able to control the transactivation of molecules involved in T cell responses like IL-2 by binding to composite DNA elements [49]. The development of various autoimmune diseases like Systemic Lupus Erythematosus (SLE) or Rheumatoid Arthritis (RA) is characterized by the breakdown of immunological tolerance which can often be due to aberrant regulatory T cells [50].

TH9 CELLS:

IL-9-secreting cells were first described 1994 [51] but have not been established as a distinct T helper cell subset until 2008 [52]. STAT6, which is induced through IL-4 signalling, has been shown to be crucial

for IL-9 production as absence of STAT6 leads to increased expression of transcription factors like FOXP3 and T-bet that repress IL-9 (see Figure 30). Additional activators of IL-9 production such as BATF and IRF4 also require STAT6 for their expression. PU.1 is another transcription factor that was identified to play a role in IL-9 production as it directly binds to the *Il9* promotor to regulate chromatin remodelling via recruitment of histone acetyltransferases (HATs) [53]. Furthermore, IL-2 signalling through STAT5 can also induce IL-9 production and has been shown to be modulated by a variety of other factors like nitric oxide, TL1A and IL-2-inducable T cell kinase (Itk) [54]. Transforming growth factor beta (TGF-β) is a key cytokine which promotes the reprogramming of Th2 cells to the Th9 cells and together with IRF4 and PU.1 characterizes the Th9 phenotype. The Th9 differentiation process starts with TGF-ß activating PU.1 which leads to downstream activation of the IL-4 receptor. Through this, STAT6 is induced followed by GATA3 and IRF4 activation. Together this signalling leads to the transcription and secretion of IL-9 [55]. *Smad2* and *Smad3* are not only important for developmental of regulatory T cells but also for Th9 cells. SMAD2/3 are recruited to the *Il9* promotor via TGFβ but are not sufficient to activate the promotor by themselves. Only with additional help via the IFN regulatory factor 4 (IRF4) can SMAD2/3 activate the *Il9* promotor [56]. IRF8 is another key transcription factor for Th9 cells, as it forms a complex with IRF4, PU.1 and BATF that is able to bind to DNA and boost *Il9* transcription [57]. Besides naïve T cells and Th2 cells, Treg cells as well as Th17 cells have also been shown to acquire an IL-9-secreting phenotype if specific pathways are being stimulated [58,59]. This highlights the cell plasticity potential of CD4+ T cells to differentiate towards a Th9 phenotype through reversible epigenetic changes as well as the complexity of underlying mechanisms. Th9 cells have been indicated to possess vast immunological potential through their IL-9 secretion. During helminth infection, IL-9 has a protective role by promoting the induction of mastocytosis (increased mast cell proliferation) and by increasing the production of IgG1 and IgE. Additionally, IL-9 facilitates increased contractility of the intestine leading to improved worm expulsion. A study working with experimental lung inflammation identified a role for Th9 cells in facilitating mast cell and eosinophil infiltration, mucus production and enhanced release of Th2 cytokines [60]. IL-9 also has been shown to induce innate and adaptive immune responses that favour anti-cancer immunity and tumor elimination [61].

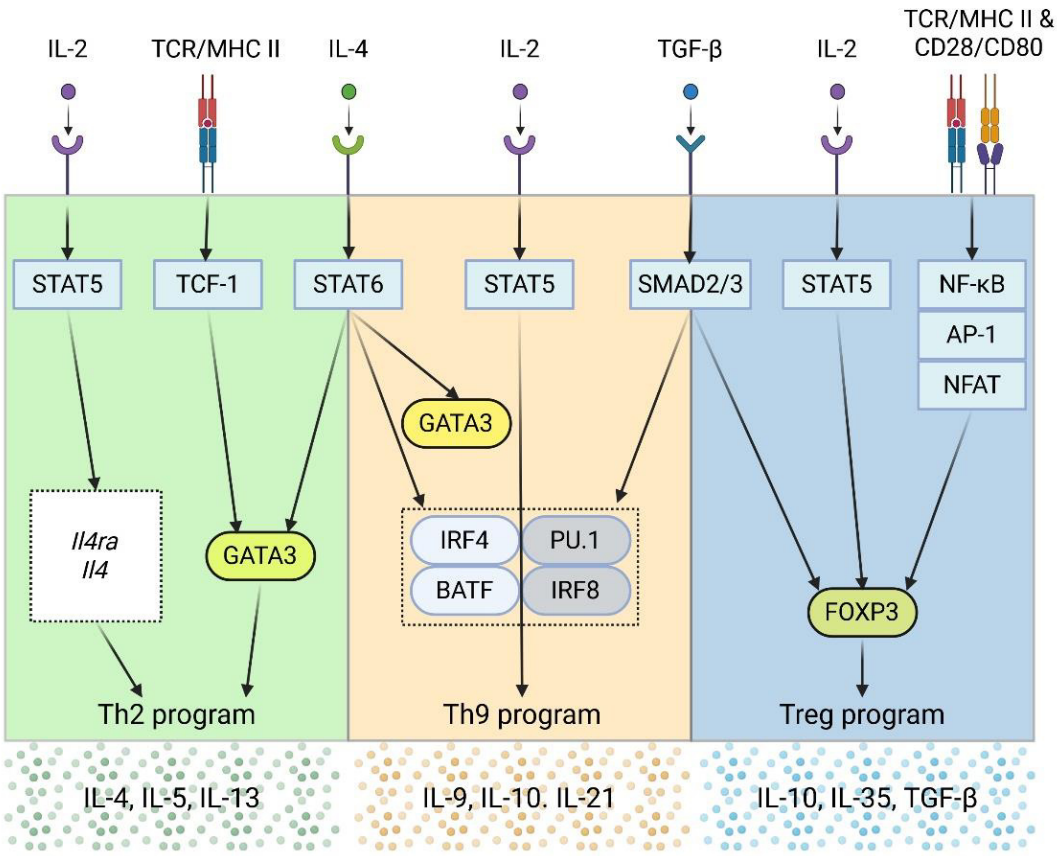

Figure 5: Depicted are simplified versions of the signalling pathways of Th2, Th9 and Treg cells. This figure has been adapted from [62]. Created in https://BioRender.com

T HELPER CELL PLASTICITY TOWARDS THE TH9-PHENOTYPE:

Lineage commitment requires T cells to maintain their transcriptional program despite changes in the surrounding cytokine environment following their initial activation. One can imagine that a Th1 cell specific to a viral epitope will become less useful in protecting the host against infection if it is not able to maintain its ability to produce IFN-γ upon exposure to IL-4 or other polarizing cytokines that would encourage other Th fates. To maintain their lineage commitment, genes required for the acquisition of different phenotypes are transcriptionally repressed via changes to the chromatin structure. Although not a pervasive feature, T helper cell subsets display some plasticity and may acquire other T cell fates. Interestingly, the Th9 cell subset seems to possess more plasticity compared to other T helper cell subsets. This lack of stability has made it challenging to identify key transcription factors that are important in preservation of Th9 phenotype. Many T helper subsets have been identified to adopt an IL-9 secreting phenotype under specific conditions. However it is unclear if those cells are only transiently producing IL-9 or bona fide Th9 cells. Th2 cells exposed to TGF-β can become Th9 cells with potential switch factors being PU.1 and the Smad proteins as they repress the Th2 cytokine program and STAT6 which promotes the production of these Th2 cytokines while inhibiting IL-9

[52,53,56,63]. GITR signalling in Treg cells can also imprint a Th9 phenotype through the activity of p50 and STAT6 [64]. As most of the transcription factors like BATF, IRF4 and the Smad proteins are active in Th9 but non-exclusive to this T helper subset, it remains unclear whether there is a lineage defining transcription factor for Th9 cells.

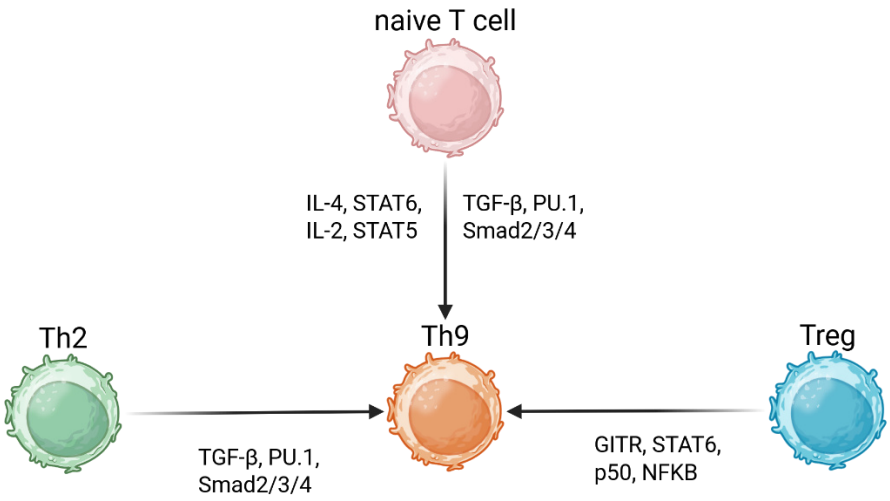

Figure 6: Key transcription factors, cytokines and signalling molecules involved in inducing a Th9 phenotype from naive T cells, Th2 cells and Treg cells. This figure has been adapted from [54]. Created in https://BioRender.com

2.3 HDACS IN T CELL IMMUNITY:

Histones are structural components of the nucleosome, which is composed of a histone octamer consisting of two molecules of histone proteins H2A, H2B, H3 and H4. The negatively charged eukaryotic DNA wraps around the positively charged histones in approximately two turns which results in a tightening of the DNA structure into chromatin [65,66]. The core of a histone shows a conserved structure consisting of a hydrophobic interior part called the histone fold, as well as N- and C- terminal protrusions. The histone N-terminus is the preferred target for various post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitination [67]. Neutralization of the positive charge of the lysine side chain of the histone N-terminus via acetylation by histone acetyl transferases (HATs) loosens up the tight nucleosomes, making the DNA more accessible for RNA polymerase II and thus leading to gene expression. Histone acetyl transferases (HDACs) remove histone acetylation which restores the compact chromatin structure, thereby rendering it less accessible for the RNA polymerase. This intricate interplay between HATs and HDACs play a great role in eukaryotic gene regulation through epigenetics. Aberrant gene expression through an imbalance of HATs and HDACs leads to instable chromatin structures and can be the cause for epigenetic diseases [66,68,69].

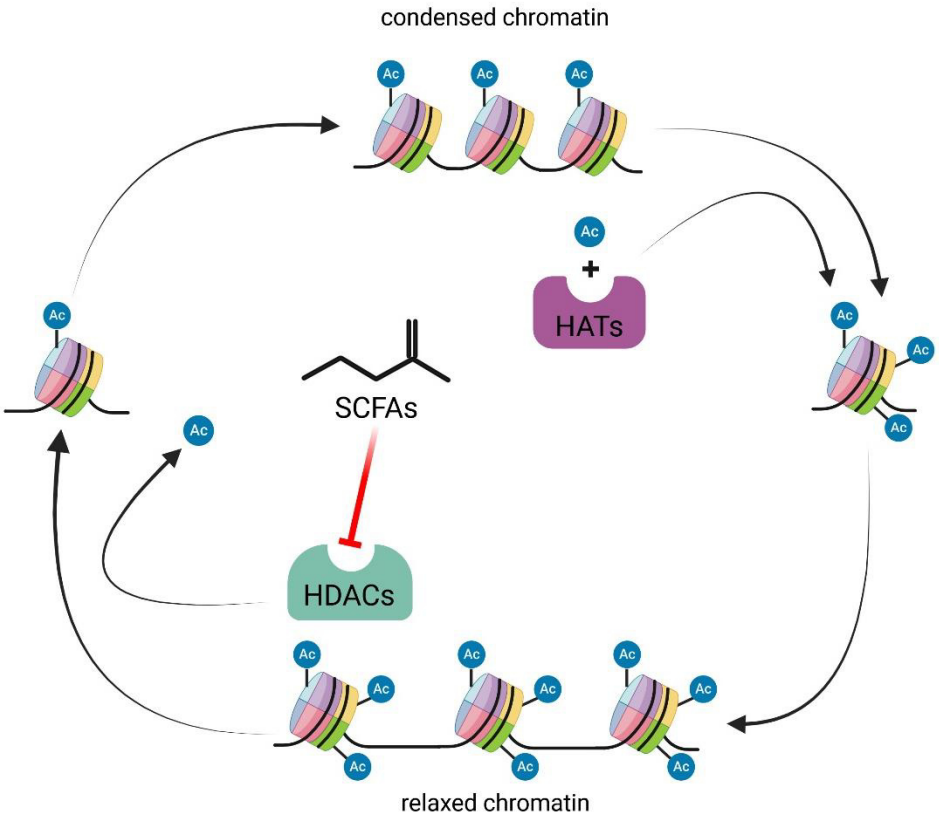

Figure 7: Illustration of the interplay between HATs, which add acetyl groups to histones resulting in transcriptional activation through positive charges opening up the chromatin structure and HDACs removing acetyl groups leading to transcriptional repression through heterochromatin formation. HDAC-inhibitors like SCFAs are highlighted as an interfering mechanism that blocks HDAC activity. This figure has been adapted from [70]. Created in https://BioRender.com

Based on the presence of a conserved deacetylase domain and on the dependence of certain cofactors, mammalian HDACs can be classified into two different families: zinc-dependent HDACs and the sirtuin family which requires nicotinamide adenine dinucleotide (NAD) as a cofactor for their catalytic activity. Furthermore, HDACs can be divided into five subclasses (I, IIa, IIb, III, IV) depending on their domain composition. Class I HDACs consisting of HDAC1, HDAC2, HDAC3 and HDAC8 are composed of an entirely conserved deacetylation domain which sets them apart from other classes. They are highly homologous to the yeast HDAC Rpd3 and are expressed and predominantly located in the nucleus showing strong activity towards histones [66].

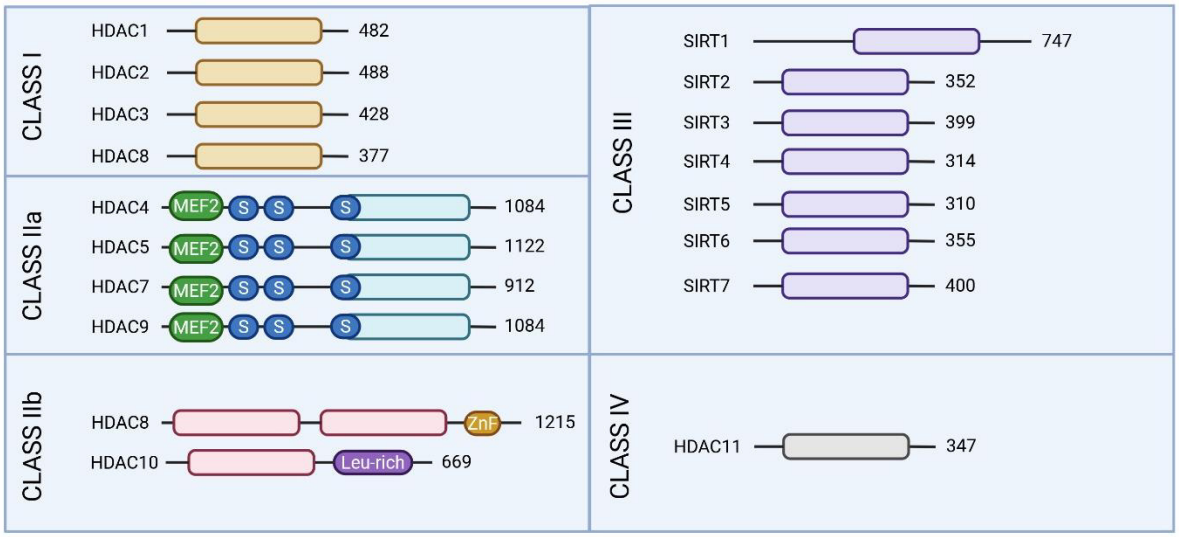

Figure 8: Classification and domain composition of human and yeast HDACs. The total number of amino residues in each HDAC is shown to the right of each protein. Myocyte enhancer factor-2 (MEF2)-binding motifs are depicted as short green cylinders, whereas 14-3-3 binding motifs are shown as short blue cylinders labelled with "S" (for serine). ZnF, ubiquitin-binding zinc finger. This figure has been adapted from [66]. Created in https://BioRender.com

HDAC1 and HDAC2 genes originated from a gene duplication event; the corresponding human and mouse proteins show 86% identical ammino acid sequences. HDAC1 was found to be involved in restriction of proliferation and cytokine expression in T cells whereas HDAC2 seems to have a specific anti-apoptotic function in cancer cells[71–73]. In recent studies that investigated HDAC1/HDAC2 knockdown and knockout genotypes certain redundancies and compensational effects between these HDACs have been observed. Multiple studies show increased HDAC2 levels upon HDAC1 knockout as well as HDAC1 upregulation upon HDAC2 deletion [71,74]. The increase of HDAC2 through HDAC1 deletion is reversible as reintroduction of HDAC1 in the knockout line resulted in reduction of HDAC2 levels. These findings point towards partially redundant functions of HDAC1 and HDAC2 [75].

In addition to histone acetylation, HATs and HDACs also regulate the acetylation status of various non-histone targets as mass spectrometry-based proteomics studies have shown [76]. This is an important regulatory mechanism affecting various characteristics and processes like protein stability, protein-protein interaction, enzymatic activity, localization and DNA binding [77,78]. FOXP3 can be used as a prime example of a protein target of acetylation/deacetylation from HATs/HDACs in a reversible process that modulates its biological activity in response to stimuli of internal or external origin [79]. Considering this broad regulation spectrum it makes HATs and HDACs also crucial in many cellular processes including cell survival, proliferation and apoptosis [77,78].

Many members of the HDAC family regulate crucial aspects of immune cell biology. Studies show that HDACs are implicated in T cell development, activation and differentiation. Specific HDACs are

important in the regulation of particular T cell subsets, as knockout/knockdown studies have revealed. Alteration in T cell function due to deletion or inhibition of HDACs is caused by chromatin remodelling and modulation of key regulatory factor activity. Furthermore, genome-wide mapping studies of HDAC1, HDAC2, HDAC3 and HDAC6 suggest that they function as modulator of gene expression rather than as repressors, as they did not bind to silent genes [80]. Interestingly, a double knockout of HDAC1 and HDAC2 resulted in the generation of MHC-class II-restricted CD4+ T cells with upregulated CD8+ T cell lineage genes. HDAC1 and HDAC2 has been identified to maintain CD4+ T cell lineage integrity through repression of RUNX3 expression resulting in downstream repression of CD8+ effector T cell-like programme [81]. These observations suggest that HDAC1/2 may regulate lineage commitment and limit T cell plasticity.

The main research question of this thesis lies on whether SCFA modulate T cell fate choice plasticity via HDAC1 inhibition. Particularly, we investigated the effects of butyrate and HDAC1 knockout on the differentiation of three CD4+ T cell subsets, T-helper 2 cells (Th2), T-helper 9 cells (Th9) and T-regulatory cells (Treg), which have interconnected developmental pathways. All these cell types play an important role during helminthiasis and experience high levels of SCFA in vivo. In the present study, we examined the effects of the SCFAs acetate, propionate and butyrate on naïve and activated Th2, Th9 and Treg cells, and identified butyrate as the most prominent regulator of naïve CD4+ T cell fate of the examined SCFAs whereas propionate showed increased regulatory capabilities in activated CD4+ T cells. Furthermore, first steps in unravelling the mechanism behind butyrate induced upregulation of FOXP3 expression in Th9 cells have been made that show a possible dependence on the presence of HDAC1.

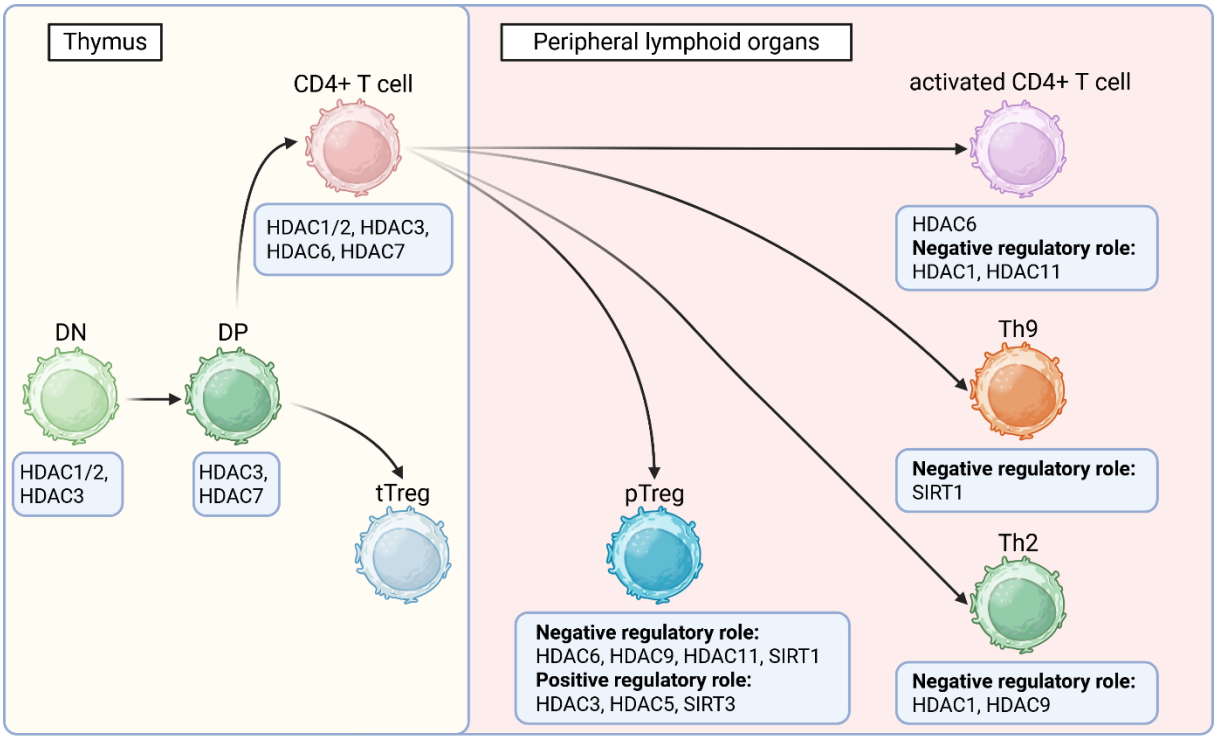

Figure 9: A simplified schematics of CD4+ T cell development and differentiation for the major thymocyte and peripheral T cell subsets. Depicting HDACs implicated in the regulation of specific T-helper subsets. Double negative (DN) cells transition to Double Positive (DP) cells after which the thymic Treg cell lineage (tTreg) and the CD4+ T cell lineage differentiate. The naïve CD4+ T cells then are able to differentiate into induced or peripheral Treg cells (pTreg), Th9 cells or Th2 cells. This depiction does not include all cell lineages branches, rather only the ones relevant for this thesis. This figure has been adapted from Ellmeier & Seiser (2018)[80]. Created in https://BioRender.com

2.4 SCFA AS HDAC INHIBITORS:

Throughout the many helminth-induced immunomodulatory effects a mouse sustains during the infection, the focal point of this study lays in the ability of STHs to change the short-chain fatty acid (SCFA) composition by altering the microbiota of its host. SCFA are a subset of saturated fatty acids that contain six or less carbon molecules which includes hexanoic acid (6C), pentatonic acid (5C), butyrate (4C), propionate (3C) and acetate (2C). The human SCFA pool mainly consists of acetate, propionate, butyrate and a smaller fraction of valerate. A study done on fecal samples revealed that mice and humans possess similar concentrations of propionate and butyrate, with acetate being higher in human samples relative to murine [82]. These SCFAs are produced by the microbiota that populate the mammalian gut through fermentation of dietary fibres and resistant starches [83]. Fiber fermentation requires the presence of specific bacteria that populate the colon which explains the absence of SCFA in germ-free mice. The major site of their production is the colon, with highest concentrations (70-140 mM) in the proximal part. Concentrations and molar ratios of SCFA can vary depending on factors like microbiota composition, diet and the host. Following their production in the

colon, SCFA are absorbed into the systemic circulation and thus reach various organs, allowing them to affect various processes throughout the body. During an *H. polygyrus* infection a significant increase in *Lactobacillus* can be observed [84–87]. Lactobacilli are part of the categorization "probiotic", which refers to live microorganisms that confer health benefits to the host, when administered in large quantities. Recently it has been proposed that these health benefits are at least partly mediated through the restoration of the microbiota and through that the SCFA levels [88]. Acetate, propionate and butyrate have important roles in regulating the immune and inflammatory responses. They do that via two key mechanisms: activation of G-protein coupled receptors like GPR41 or GPR43 and through inhibition of HDACs. GPR43, which can be found on innate immune cells like neutrophils, is able to bind acetate and through that is able to facilitate anti-inflammatory signals highlighting the role of SCFAs in modulating disease susceptibility and severity [89]. Cell-free assays suggest that the HDAC inhibition (HDACi) of SCFAs show some specificity towards different HDAC isoforms, but the general consensus is that SCFA have a broad spectrum of HDAC inhibition. For example, butyrate is able to inhibit most zinc-dependent HDACs except class IIa HDACs. This allows SCFAs like to affect different steps of the inflammation process as well as the regulation and modulation of various immune cells [89–91]. Taken together, an infection with a STH facilitates the growth of certain microbiota which increases the SCFA concentration in the host. These SCFA act as immunomodulatory molecules by inhibiting HDACs and favouring anti-inflammatory immune responses.

A.

SCFAs	SCFAs receptors	Human SCFA Proportion *
Acetate	FFAR2 (GPR43) FFAR3 (GPR41)	~60%
Propionate	FFAR2 (GPR43) FFAR3 (GPR41)	~20%
Butyrate	FFAR2 (GPR43) FFAR3 (GPR41) GPR109A	~20%

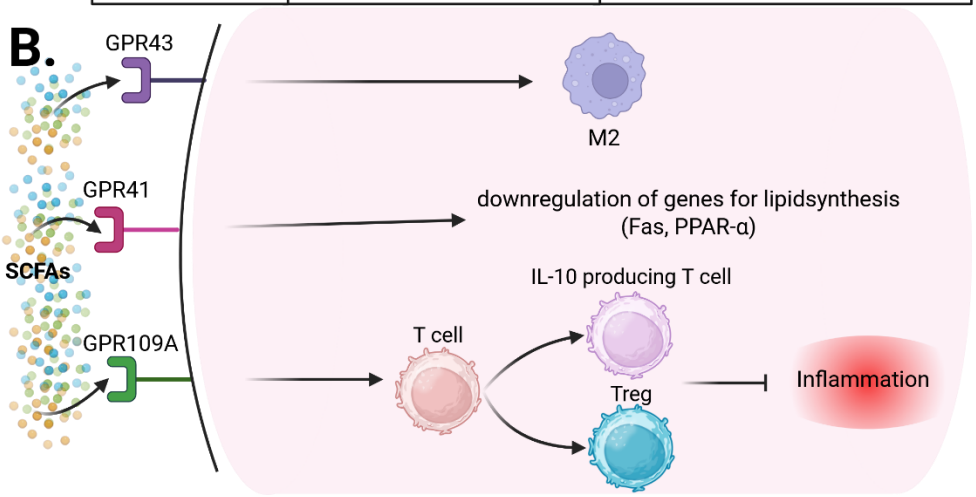

Figure 10: SCFAs, their cellular targets, relative fecal abundance in humans and respective signalling mechanisms. The G protein-coupled receptors (GPRs) are synonymous for their Free Fatty acid Receptors (FFARs). B. SCFAs binding to different GPRs results in immunoregulation. SCFA signalling through GPR43 promotes M2 macrophage activation, whereas SCFAs that bind to GPR41 trigger the downregulation of the genes Fas and PPAR-α which are responsible for lipid synthesis. Lastly, SCFAs that bind to GPR109A induce T cell differentiation to Treg cells and IL-10 producing cells which alleviate inflammation. This figure has been adapted from [92] and [93]. Created in https://BioRender.com

2.5 SCFA IN HELMINTHIASIS:

A healthy diet includes dietary fibres which upon ingestion are processed to SCFAs via the microbiota. These SCFA are able to promote various beneficial effects including the reduction of chronic inflammation, enhancement of mucosal barrier integrity as well as facilitating the growth of beneficial bacteria such as *Bifidobacterium*. An infection with an intestinal parasite can lead to serious health problems and even death but paradoxically can also confer certain health benefits as various studies suggest. During helminthiasis the parasite is able to modulate the hosts microbiota composition which results in a shift in microbiota derived metabolites including the SCFA pool. Through this, the parasite is able to alter the hosts immune response towards a modified Th2/regulatory state which is able to protect against allergic inflammation [94]. Past studies suggest that the infection by a STH can

ameliorate experimentally induced diseases such as encephalomyelitis, diabetes and thyroiditis[95]. Oral administration of *Trichuris suis* eggs in patients suffering from Crohn's disease has been shown to successfully ameliorate symptoms and suggests that natural exposure to helminths affords protection from similar immunological diseases [96]. Anti-inflammatory cytokine secretion induced by the introduction of a *H. polygyrus* modified microbiome has been shown to be sufficient in mediating protection against allergic asthma in a GPR-41 dependent manner [97].

These studies highlight the importance of parasite derived bio-modulatory molecules, such as SCFAs, that are able to change the hosts microbiome and immune response in various ways and thereby creating a favourable environment for the parasite to thrive in.

2.6 AIMS OF THIS STUDY:

One of the main research topics my host laboratory is understanding how a *H. polygyrus* infection drives microbiome changes and through that affects molecular pathways that control host T cell immunity. This research question is split into two major parts: one part that investigates how *H. polygyrus* infection affects mice that are deficient of HDAC-1 and their microbiome, and another part that investigates these effects on CD4+ T cells isolated from uninfected mice that are exposed to SCFAs in order to assess immunological changes from a more mechanistic point of view. During this thesis I was working on in-vitro cell culture experiments of cells isolated from HDAC-1 KO mice and investigated how they behave and change in a setting that mimics a worm infection through SCFA exposure. The exposure of the SCFAs of acetate, propionate and butyrate exerts effects on the specific CD4+ T cell subsets (Th2, Treg and Th9 cells) allowing me to look at the immune response to an infection in a more targeted fashion. Although this is a somewhat artificial setting, these results can be used as proof of principle study which then can be supported through the in-vivo part of this project.

- Specific aim 1: Assess the effect of SCFAs on the de novo generation of Th2, Th9 and Treg cells from naïve CD4 T cells obtained from HDAC1^{flox}CD4^{Cre} mice

- Specific aim 2: Assess the effect that SCFAs have on already committed Th2, Th9 and Treg cells generated from HDAC1^{flox}CD4^{Cre} mice

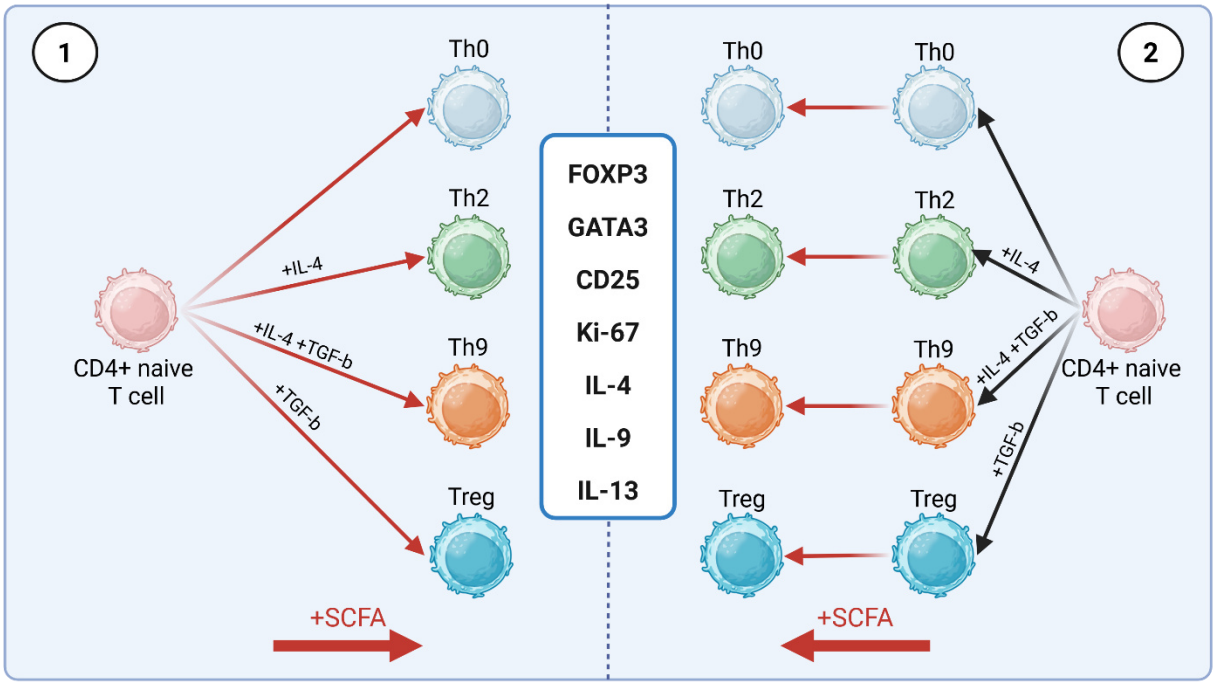

Figure 11: Graphical illustration of the specific aims of this thesis. 1) Effect of SCFAs on Th2, Treg and Th9 cells generated from HDAC1^{flox}CD4^{Cre} mice during initial cell fate commitment 2) Effect of SCFAs on already committed Th2, Th9, Treg cells generated from HDAC1^{flox}CD4^{Cre} mice. Created in https://BioRender.com

3. MATERIALS:

3.1 MOUSE STRAINS:

3.1.1 C57BL/6J:

C57BL/6J has its genome fully sequenced and is he most commonly used mouse inbred strain. They are used as all-purpose strains and background strains for generation of transgenics. They are long-lived, breed well and show low susceptibility to tumors and are used in many research areas such as immunology, developmental biology, genetics and neurobiology [98]. In this thesis they were used to optimize protocols and conditions, flow cytometric staining and for training purposes.

3.1.2 HDAC1^{FLOX}CD4^{CRE}:

Complete disruption of HDAC1 results in severe developmental defects such as growth retardation and is sufficient to cause death before day 10.5 of gestation. This can be circumvented through conditional *hdac1* knockouts like the HDAC1^{flox}CD4^{Cre} mice utilized throughout this thesis [99]. The Cre/*loxP* system is a powerful tool in mouse genetics and is frequently used to generate tissue-specific expression or knockout of genes. This specific mouse strain is homozygous for an *hdac-1* allele that is flanked by two *loxP* sites with the Cre-recombinase being constitutively expressed on the *cd4* locus. In CD4+ cells, the

Cre recombinase is translated and removes the *hdac-1* sequence resulting in *hdac-1* knockout cells. These mice are housed with Cre negative littermates that are used as negative controls since their *hdac-1* allele is intact due to not having the Cre recombinase [100].

3.1.3 FOXP3^{HCD2}IL-9^{GFP}:

These mice express the human *cd2* gene under the same promotor as the *foxp3* gene allowing us to detect foxp3 expression extracellularly, through staining of hcd2 which is transported to the outer membrane of the cell [101]. Additionally this mouse strain co-expresses a green fluorescence protein (GFP) upon IL-9 expression. Together this mouse can be used to investigate FOXP3 and IL-9 expression in the same sample without extracellular fixation or intracellular staining.

3.1.4 FOXP3$^{AID\text{-}DTR\text{-}GFP}$RS26$^{TIR1(F74G)}$:

This mouse strain utilizes auxin-inducible degron (AID) technology to degrade the FOXP3 protein upon exposure to the plant hormone auxin. TIR1(F74G) under the constant expression of the Rosa26 gene locus promotor serves as E3 ligase complex which is stabilized through the binding of auxin. In the presence of auxin hormone indole-3-acetic acid (IAA) the AID tagged proteins (like the FOXP3 in this mouse strain) are targeted by the TIR1-SCF E3 ligase complex for degradation. A point mutation in the TIR1 gene from F74A to F74G results in an improved version of this system called “second-generation AID” or “AID2”. The AID2 system is characterized through less leakiness and 1000-fold lower IAA concentrations required to perform compared to the original AID system [102–104]. Upon expression of FOXP3 a GFP protein fused to the diphtheria toxin receptor (DTR) is co-expressed. This allows for targeted depletion of cells expressing FOXP3 through the exposure to the diphtheria toxin [104,105].

This mouse strain was kindly provided by the laboratory group of Dr. Joris Van Der Veeken.

3.2 HELIGMOSOMOIDES POLYGYRUS INFECTION IN MICE:

For the purpose to investigate STH infections, this thesis utilized the closely related laboratory model of the nematode *Heligmosomoides polygyrus*, which is called *Heligmosomoides polygyrus bakeri*. In contrast to *H. polygyrus*, *H. polygyrus bakeri* is able to infect laboratory mice (*Mus musculus*) without the administration of powerful immunosuppressants [106,107].

H. polygyrus is a parasitic living intestinal nematode that can be found in wild European wood mice (*Apodemus sylvaticus*). It is phylogenetically placed in the Order of *Strongylida*, which it shares with the human hookworm parasites *Ancylostoma duodenale* and *Necator americanus*. *H. polygyrus* is used to study chronic helminthiasis, a disease directly related to the intensity of the infection that can lead

to iron-deficiency, malnutrition, impaired cognitive performance, anemia and morbidity [20,108]. Furthermore, *H. polygyrus* is widely used to study the effects of a helminth infection on the hosts immune system in the context of various disease models such as experimental auto immune encephalomyelitis (EAE) and the T cell transfer model of colitis [109,110]. *H. polygyrus* exerts widespread immunomodulatory effects via so called *H. polygyrus* excretory-secretory antigen (HES) which include a TGF-beta-like ligand that is able to induce de novo regulatory T cells [111].

In order to infect mice in an experimental setting, the infective L3 larvae of *H. polygyrus* are introduced orally via gavaging. After 24 h past ingestion, the larvae have penetrated the submucosa of the small intestine where they undergo two developmental molts after which they emerge as adult worms back into the lumen. In the lumen they will feed on host tissue, mate and produce eggs. The eggs ware excreted by the feces of the mouse into the external environment. Finally, the eggs hatch and undergo two molts resulting in infective L3 larvae which continue the lifecycle [20].

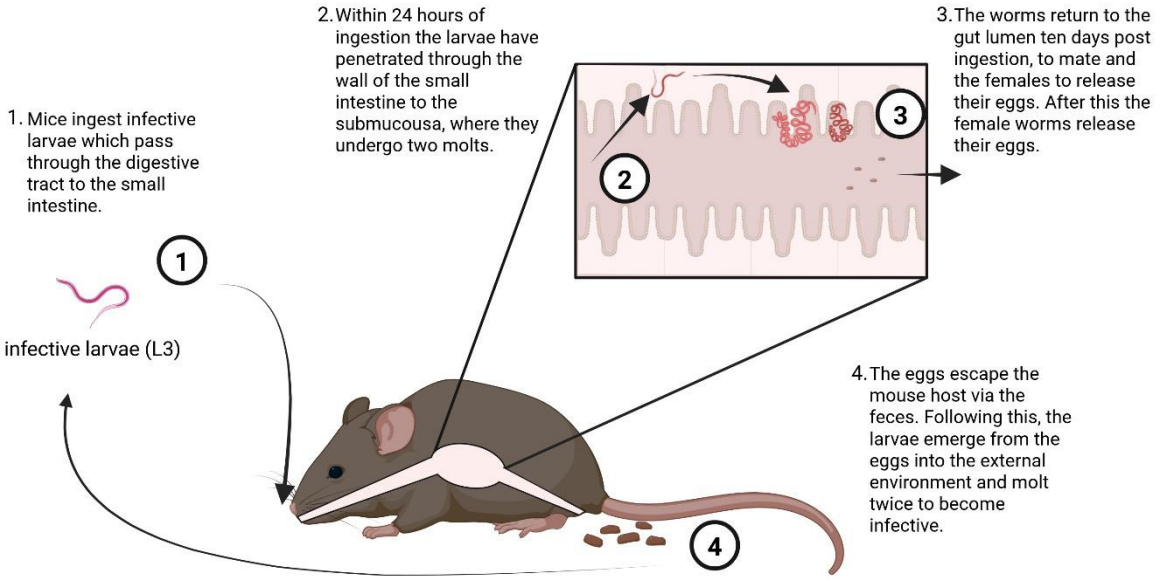

Figure 12: Lifecycle of Heligmosomoides polygyrus in mice. This figure has been adapted from [20]. Created in https://BioRender.com

3.3 MEDIA AND BUFFERS:

T cell culture medium (complete RPMI):

500 mL base RPMI-1640 (Sigma-Aldrich) supplied with:

- 50 mL heat inactivated Fetal Bovine Serum (FBS; Sigma Aldrich)
- 5 mL Sodium pyruvate solution (1 mM; Sigma Aldrich)
- 10 mL Minimal essential amino acids (MEM; Sigma Aldrich)

- 5 mL 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 10 mM; Sigma Aldrich),
- 5 mL L-Glutamine (200 mM; Sigma Aldrich)
- 5 mL Penicillin-Streptomycin (Sigma Aldrich)
- 500 µL Beta-mercaptoethanol (BME; Fisher Scientific)

MACS buffer:

- 500mL 1x phosphate buffered saline (PBS; Sigma Aldrich)
- 2% Fetal Bovine Serum (FBS; Sigma Aldrich)
- 2mM ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich)

SCFAs (Sigma-Aldrich):

Sodium-Acetate (S5636)

Sodium-Propionate (P5436)

Sodium-Butyrate (303410)

The Biotin-antibody Cocktail, Anti-Biotin MicroBeads and CD44 MicroBeads used throughout the CD4+ naïve T-cell enrichment process are all part of the "Naïve CD4+ T cell Isolation Kit" from Miltenyi Biotec (cat. number: 130-104-453).

Restimulation Medium:

T cell culture medium (complete RPMI) supplied with:

- 0,05 µg/mL phorbol myristate acetate (PMA; Sigma Aldrich)
- 0,5 µg/mL Ionomycin (Sigma Aldrich)
- 1 µg/mL Brefeldin A (Sigma Aldrich)
- 2 µM Monensin (Sigma Aldrich)

Transcription factor staining media (eBioscience Foxp3 / Transcription Factor Staining Buffer Set):

- Extracellular stain: Antibodies and live/dead stain diluted in 100% PBS
- Fixation: 75% eBioscience Fixation/Perm Diluent mixed with 25% eBioscience Fixation/Permeabilization concentrate
- Intracellular stain: Antibodies diluted in 10% MQ H_2O (water produced through the Milli-Q System; ion-free, pyrogen-free and ultrapure) together with 90% eBioscience Permeabilization Buffer.

Cytokine staining media (BD Cytofix/Cytoperm Fixation/Permeabilization Kit):

- Extracellular stain: Antibodies and live/dead stain diluted in 100% PBS
- Fixation: 100% BD Fixation/Permeabilization solution
- Intracellular stain: Antibodies diluted in 10% MQ H_2O together with 90% BD-Perm/Wash Buffer

H. polygyrus propagation media:

- Autoclaved MQ H_2O
- 10000 U/mL Nystatin (Sigma Aldrich)

3.4 ENTINOSTAT:

Entinostat, also known as MS275 is a synthetic benzamide derivate able to inhibit class I and IV HDACs and thereby promoting histone hyperacetylation and transcriptional activation of certain genes resulting in inhibition of cell proliferation, terminal differentiation and apoptosis. It is currently under clinical trials and has received the "breakthrough designation" status for the US FDA in combination with exemestane for advanced breast cancer management [112]. The narrow-spectrum HDAC inhibitory effect of Entinostat is highest against HDAC1 and significantly less against HDAC2 and HDAC3, with limited activity against HDAC8 [113]. This allows Entinostat to function as a viable positive control for HDAC1 inhibitory effects with practical relevance as it is being heavily investigate in the cancer research field. The Entinostat used throughout this thesis is supplied by Biozol Diagnostica.

3.5 ANTIBODY LIST:

Antigen / Target	Channel	Dilution	Provider
Live/Dead	APC-Cy7	1:1000	
CD4	AF700	1:400	BD Biosciences (557956)
GITR	APC	1:500	ThermoFisher (17-5874-81)
CD62L	PE-Cy7	1:400	ThermoFisher (25-0621-82)
CD44	BV650	1:400	BioLegend (105331)
GATA3	PE	1:50	BioLegend (103106=
FOXP3	FITC	1:400	ThermoFisher (11-7321-82)
hCD2 (FOXP3)	APC	1:200	BioLegend (300214)
Ki67	Per-CP-Cy5.5	1:1000	ThermoFisher (46-5698)

CD25	BV605	1:400	BioLegend (103106)
IL-4	FITC	1:50	Miltenyi Biotec (130-131-900)
IL-9	APC	1:100	Miltenyi Biotec (130-102-407)
IL-9	BV421	1:100	BioLegend (514109)
IL-13	PE	1:200	ThermoFisher (12-7133-82)

4. METHODS:

4.1 THE CRE-LOXP SYSTEM:

The main mouse genotype used in the bulk of the experiments throughout this thesis is the HDAC1^{flox}CD4$^{Cre+/-}$ strain which utilizes the Cre-loxP system. This system is a well-established tool for mammalian genetics and cell biology due to its simplicity and adaptability in assessing various biological questions [114]. The principal idea behind the Cre-loxP system is that the Cre recombinase, a tyrosine site-specific recombinase, recognizes a unique 34 bp long DNA fragment called loxP. After recognition the Cre recombinase facilitates a site-specific deletion of a DNA sequence flanked by two loxP sites. Terminology commonly used in science refers to a DNA sequence that is flanked by two loxP sites as "floxed" [115]. In the specific case of the HDAC1^{flox}CD4$^{Cre+/-}$ strain the HDAC-1 DNA sequence is floxed in both "WT" and "KO" genotype with the only difference being that the KO genotype also expresses the Cre recombinase in the *cd4* gene loci while the WT genotype is negative for *cre*. The end result of a KO genotype is that only cells which express CD4 also express the Cre recombinase resulting in the excision of the *hdac-1* gene. The WT genotype being negative for *cre* serves as a "clean" background to the KO genotype as it possesses the floxed *hdac-1* background which stays functional. While this system allows for a conditional knockout of *hdac-1*, it does not prevent the KO cells from compensating the loss in HDAC-1 expression by overexpressing HDAC-2 which has been shown to have unique but also redundant functions to HDAC-1. In order to circumvent this compensation effects a catalytically dead HDAC-1 strain can be used, as it is still transcribed at normal rates but not functional.

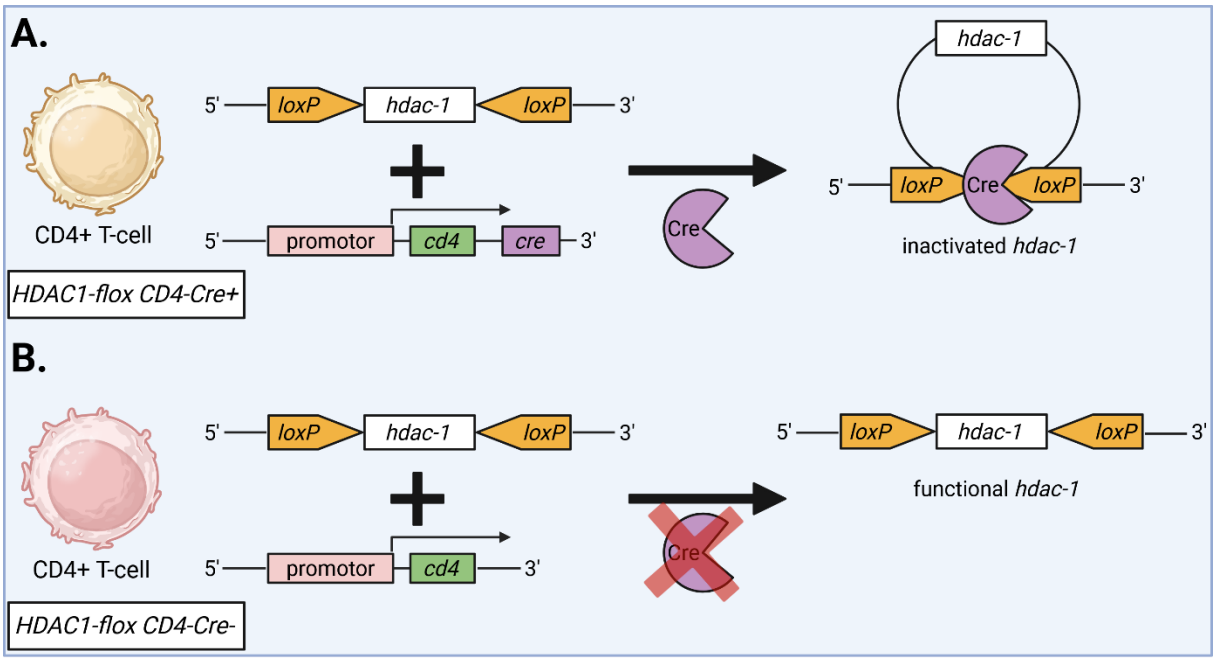

Figure 13: Graphical illustration of the Cre-loxP system on the example of a HDAC1^{flox}CD4$^{Cre+/-}$ mouse strain. A. The CD4+ T cell expresses Cre under control of the CD4 promotor region resulting in the targeted deletion of the hdac-1 gene which is flanked by two loxP sites. B. The CD4+ T cell lacking the Cre recombinase in the cd4-loci fails to delete hdac-1 resulting in a functional allele. Created in https://BioRender.com

4.2 IN-VITRO T CELL POLARIZATION:

In-vitro T cell polarization assays allow the cultivation of a specific cell type or a co-culture consisting of two or more cell types together under controlled conditions. While in a co-culture experiment the T cells are able obtain their activation signals via antigen bound to APCs, it is also possible to stimulate them through monoclonal antibodies to the T cell receptor (TCR; αCD3) together with antibodies specific for the co-stimulatory receptor CD28 mimicking the APC/T cell interaction [116,117]. Through these assays scientists are able to elucidate molecular mechanisms underlying specific T cell subset differentiation into as well as how various factors such as cytokines and microbial products affect them. In consortium with in-vivo approaches the in-vitro CD4+ T cell polarization systems serves as a valuable set of tools to gain more insight into adaptive immune response and its regulatory mechanisms that govern T helper cell differentiation [118]. During the practical part of this thesis the CD4+ T cell polarization system was a key tool used to investigate the effects that SCFA as well as HDAC1 knockout have on naïve or committed Th2, Th9 and Treg cells.

The standard in-vitro CD4+ T -cell culture procedure for the practical part of this thesis starts by harvesting a spleen from a mouse, as it harbours roughly 4-6 10^6 of naïve CD4+ T cells. For bigger experimental layouts requiring a greater number of CD4+ naïve T cells the lymph nodes can also be isolated. Right after the organ harvest, a 96-well flatbottom cell culture plate is coated with 100 µL

/well aCD3 (2 ng/mL) and aCD28 (2 ng/mL) plate bound antibodies diluted in PBS for 4 hours at 37 C°. Next the isolated organs are mashed through a 70 µM strainer into a 50 mL Falcon tube using the end of a plastic syringe. After spinning down the Falcon tube at 1700 rpm for 4 minutes the supernatant is discarded and the pellet suspended in 1 mL complete RPMI. Depending on the amount of CD4+ T cells needed for the seeding of the experiment a specific volume of this 1 mL cell suspension is taken and enriched via a Miltenyi CD4+ T cell enrichment kit following the ratio of 1 µL of spleen suspension requiring 0.0125x the base protocol steps. Previous in vitro cell culture experiments showed that this small deviation from the standard Miltenyi protocol resulted in overall improved results of CD4+ enrichment. As an example I will explain the enrichment for 800 µL of spleen suspension utilizing 10x Miltenyi protocol. Firstly the 800 µL cell suspension is centrifuged at 1700 rpm 4 minutes after which the supernatant is discarded, and the pellet resuspended in 400 µL of MACS buffer. To this suspension add 100 µL of Biotin-Antibody Cocktail, mix and incubate the Falcon tube for 10 minutes at 4 C°. Then add 200 µL of MACS, 200 µL of Anti-Biotin MicroBeads and 100 µL of CD44 MicroBeads to the cell suspension and incubate it for 20 minutes at 4 C°. After this step the LS column are placed in a magnetic separator and rinsed using MACS. Following this, the cell suspension is added to the column and the flow through is collected in a new 15 mL Falcon tube. Then the columns are rinsed again with 3 mL of MACS and the flowthrough collected in the same 15 mL Falcon tube. Lastly the 15 mL Falcon tube is centrifuged at 1700 rpm for 4 minutes, the supernatant discarded and the cell pellet containing the now enriched naïve CD4+ T cells resuspended in on 1 mL of complete RPMI. This cell suspension is then stored at 4 °C until required again for the cell-seeding of the culture plate. To ensure the quality of the enrichment step the purity of our CD4+ naïve T cell suspension is checked via flow cytometry. For this, 20-30 µL of the cell suspension is stained for Live/Dead, CD4, CD25, CD44, CD62L and GITR via antibodies. Whereas the Live/Dead stain together with the CD4 antibody allows us to identify the live CD4+ part of our population, we expect our enriched naive CD4+ cells to be low in CD44 and CD25 expression, high in CD62L expression and negative for GITR (Figure 15). Only Tregs from secondary lymphoid organs are GITR positive, this can act as a surrogate marker. After ensuring the quality of our enrichment is good, the cells are counted using a Neubauer counting chamber and trypan blue staining. Finally the cells are seeded in the 96-well flatbottom cell culture plate that has the activation antibodies washed using PBS. Depending on the desired cell type the specific polarization conditions are added. For Th2 polarization 20 ng/mL IL-4 are added per well, while for Treg polarization 2ng/mL TGF-β are added per well. In order to polarize the cells toward Th9 lineage both, 20 ng/mL IL-4 and 2ng/mL TGF-β, are added per well. Additionally, an artificial cell type termed “Th0” is kept in each culture plate as a control and for gating purposes during flow cytometric analysis. This cell type is generated through the absence of polarization conditions IL-4 and TGF-β. Lastly the treatment of either 250 µM sodium acetate (Sigma-Aldrich, S5636), 250 µM sodium propionate (Sigma-Aldrich, P5436, 250

µM sodium butyrate (Sigma-Aldrich, 303410), equal volume PBS or 250 nM Entinostat (Selleckchem, S1053) is added and the final volume of each well filled up to 200 µL using complete RPMI. The outer wells of the plate are filled up with PBS in order to minimize evaporation of the wells while the plate is in the incubator. Finally the plate is then placed in an incubator at 37 °C for three days, after which the Th9 and Treg cells can be analysed and the Th2 cells will be reseeded into a new plate with new media containing treatment and polarization conditions. After an additional three days the Th2 are also ready to be analysed using flow cytometry and staining antibodies. While working with the cells a sterile atmosphere is required in order to prevent contamination of the culture plate.

This layout is adapted for the experiment line that wants to investigate the effect of SCFA on already committed CD4+ T cells with a difference being that the cells will be incubated for three days prior to SCFA exposure. Additionally, all cell types are then kept in cell culture for another three days until being analysed using flow cytometry.

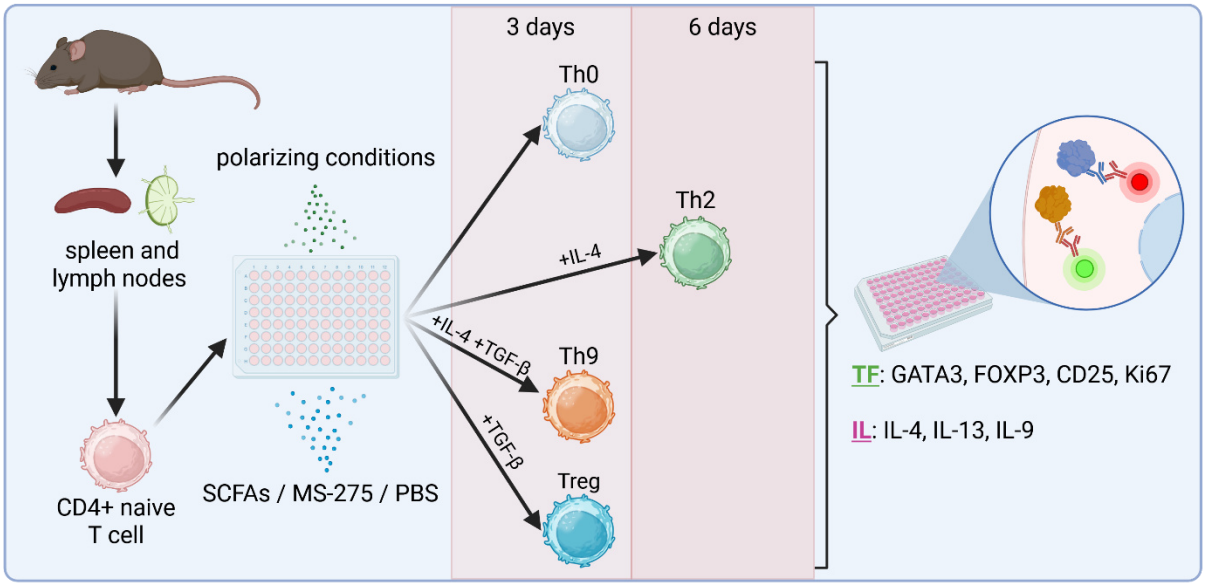

Figure 14: General experiment workflow for an in-vitro polarization assays working with naïve CD4+ T cells. Initially compartments of the immune system of the mouse (e.g. spleen or lymph nodes) are physically isolated. Following this is an enrichment step for CD4+ naïve T cells. The cells are then seeded on a 96-well flat bottom plate together with different polarization conditions. The SCFA treatment together with the PBS and Entinostat (MS-275) controls is either given immediately when seeding or after 3 days of culture. Finally one half of the cultured cells are stained for transcription factors while the other half is first restimulated using Ionomycin, PMA, Monensin and Brefeldin A and then stained for cytokine expression. After the staining process is completed, the cells are analysed using flow cytometry. Created in https://BioRender.com

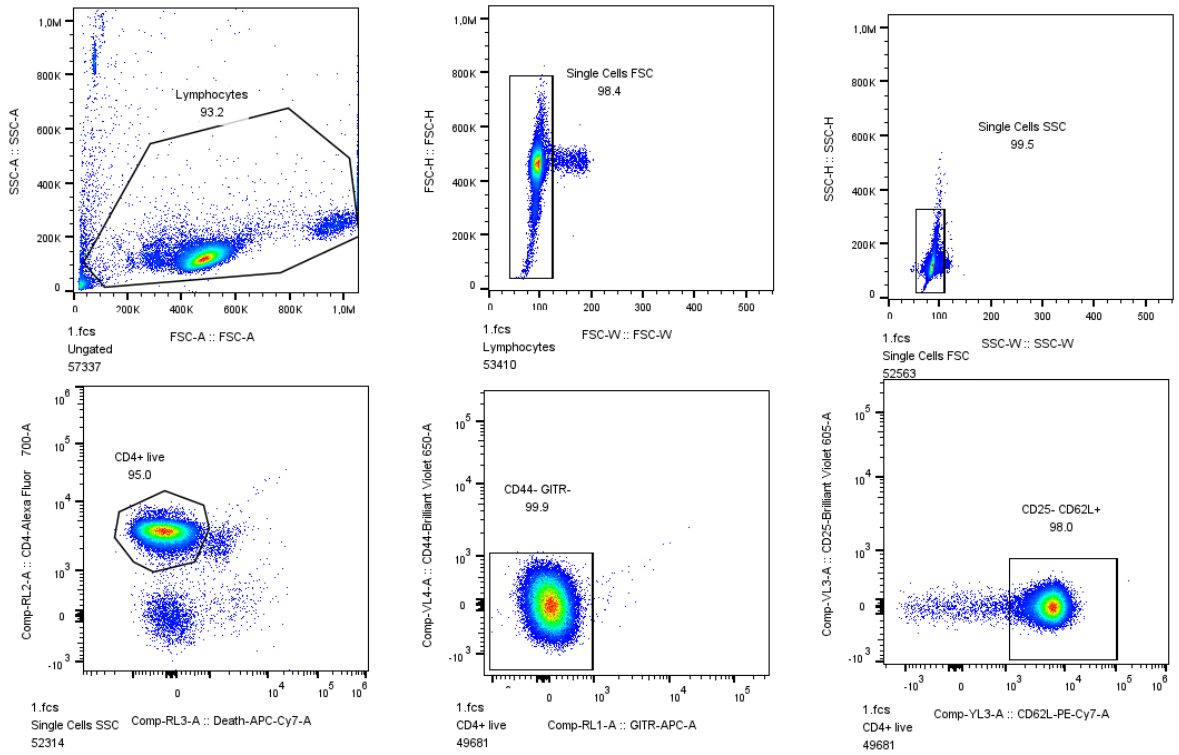

Figure 15: Depicted here is an example of a purity check. Purity checks are done before seeding cells to ensure the enrichment step was successful. Through this quality assurance check the comparability between different experiments is improved.

4.3 TRANSCRIPTION FACTOR AND CYTOKINE STAINING:

Standard procedure for immunofluorescent staining typically starts by staining the extracellular antigens after which a fixation step follows that is usually done with formaldehyde which stabilizes the cell membrane. Finally a detergent or alcohol is added to permeabilize the cell membrane allowing intracellular antibodies to bind to their antigen. The antibodies are conjugated to fluorochromes which allows their specific detection through flow cytometry. Depending on the localization of the target, different protocols and buffer systems are used to obtain optimal results. Phosphorylated signalling proteins require also a specific buffer system that utilizes a fixation/methanol protocol [119].

The staining of CD4+ T cells during this thesis was done by utilizing the protocols and buffer of two staining kits, the “eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set [120]” used for transcription factor staining and the “BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit [121]” for cytokine staining. The staining antibodies and their fluorochrome conjugates against the marker molecules investigated during this thesis are listed in the antibody list on pages 23-24. The staining process was done at 4 °C in the dark in order to protect the stains from bleaching through the light, as recommended in the protocols. The kits both use antibodies diluted in PBS for the extracellular stain but differ in the fixation solutions and the solution in which the intracellular staining antibodies are

diluted (see 3.3 Media and Buffers). The transcription factor stain consists of a 20 minute extracellular stain followed by a 30 minute fixation step and finally a minimum of 1-2 hour intracellular staining step. For the cytokine stain the extracellular staining step is also 20 minutes but the fixation step is only 20 minutes. Again the intracellular staining step was done for a minimum of 1-2 hours. Alternatively, the intracellular staining step can be done overnight which results in a minimal improvement of the staining process. After the intracellular staining was competed the samples are ready to be analysed via flow cytometry.

4.4 FLOW-CYTOMETRY:

Flow cytometry measures the various physical characteristics of single cells such as size, granularity and other features that are detectable through changes in the scattering of light. As the light from the laser beam hits the cell it is deflected into forward scatter (FSC) which passes through the cell and side scatter (SSC) which is collected at approximately 90 ° to the laser beam. While FSC is proportional to the cell-surface are or size the SSC reflects the cells granularity or internal complexity. Flow cytometry is often used to detect dyes or monoclonal antibodies which are bound to specific molecules of the cell that can be found extracellular or intracellular. The main components of a flow cytometer are fluidics which direct the liquid containing he particles a focused light source from the lasers. The excitation optic focuses the light on the cells while the collection optics simultaneously transmit the scattering of the light or fluorescence to an electronic network. This network detects the signal and converts it into digital data proportional to the light intensity. Finally this data can be analysed via the computer. In principle there are two types of flow cytometry, sorting a non-sorting. Additional to the ability to perform light scattering and fluorescence emission, a sorting flow cytometry allows the user to sort the detected cells/particles via e.g. droplet deflection. A fluorescence activated cell sorter (FACS) is a flow cytometer that is able to sort fluorescent-labelled cells from a mixed population. Fluorochromes are commonly used in complexation to antibodies that bind to the cellular target of choice. Following the often termed “staining-process” the cells can be analysed via the flow cytometer which emits a laser specific to the excitation range of he fluorochrome. The now excited fluorochrome emits light at a specific wavelength which can be detected by the flow cytometer via photomultiplier tubes (PMTs). PMTs possess greater sensitivity compared to photodiodes which are commonly used to detect strong light signals such as FCS. It is possible to detect many fluorochromes in a single cell but with increasing complexity additional fluorescence compensation becomes more relevant. The compensation tries to correct for spectral overlap of fluorochromes, an event in which the emissions of two fluorochromes are overlapping to some extent and by that clouding the analysation process giving for example false positive signals. Flow cytometry a helpful tool in detection of various

membrane, cytoplasmic or nuclear antigens and is therefore commonly used in the fields of cellular biology and immunology [122].

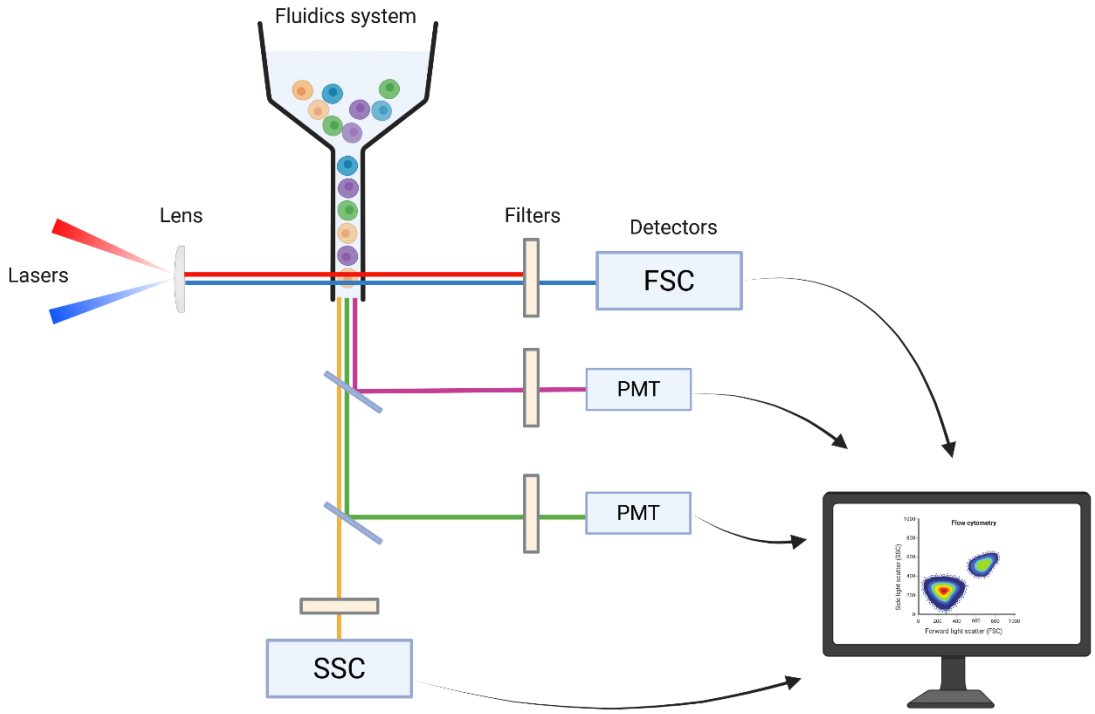

Figure 16: An illustration of the basic principle of flowcytometry. Fluidics bring the sample into a single cell suspension, after which the lasers are focused on the cell which scatters the light. The scattered light is then directed through filters into detectors such as photodiodes or photomultiplier tubes (PMTs). These signals are then directed to the computer for analysis. This figure has been adapted from [122]. Created in https://BioRender.com

The flow cytometer used throughout this thesis was the "Attune CytPix Flow Cytometer" from ThermoFisher which was connected to a plate reader in order to sample directly from a 96-well V-bottom culture plate. This flow cytometer combines acoustic focusing with a high-speed brightfield camera which allows for simultaneous high throughput flow cytometry and high resolution brightfield imaging [123]. This machine also possesses an internal cytometric software which was used for an initial analysis of the samples, while FlowJo was used for the final analysis.

Figure 17: The Attune CytPix Flow Cytometer used throughout this thesis to acquire flow cytometric data from various cell cultures. Figure taken directly from the ThermoFisher homepage https://www.thermofisher.com/at/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-nxt-flow-cytometer/models/cytpix.html

4.5 ANALYSIS OF FACS DATA USING FLOWJO:

The flow cytometric data acquired is analysed using cytometric data analysis software such as FlowJo which provide an analysis environment and help to facilitate the analysis of big datasets. Standard flow cytometric analysis is done via successive gating steps. Gating is a method in which a gate-box is drawn over data that is collected from one or more channels of the dot-plot. This gate contains a subpopulation that is highlighted in other plots that display information from alternate channels [124]. During this thesis the gating method was used to first exclude debris from the data obtained of the sample, after which FCS height to width followed by SSC height to width dot plots were utilized to exclude cell doublets. After obtaining the single cell population a gate was drawn in the CD4 to live/dead dot plot which includes only live and CD4+ T cells. This subpopulation was then used to further analyse transcription factor (Figure 18) or cytokine expression (Figure 19) of the flow cytometric sample dataset. After the analysis of one sample is concluded the successive gating process was copied over to all other flow cytometric sample datasets in order to ensure comparability between the acquired datasets. Additionally the compensation matrix used during the data acquisition process of the samples can be modified again during data analysis in FlowJo in order to fine tune the separation between each channel further.

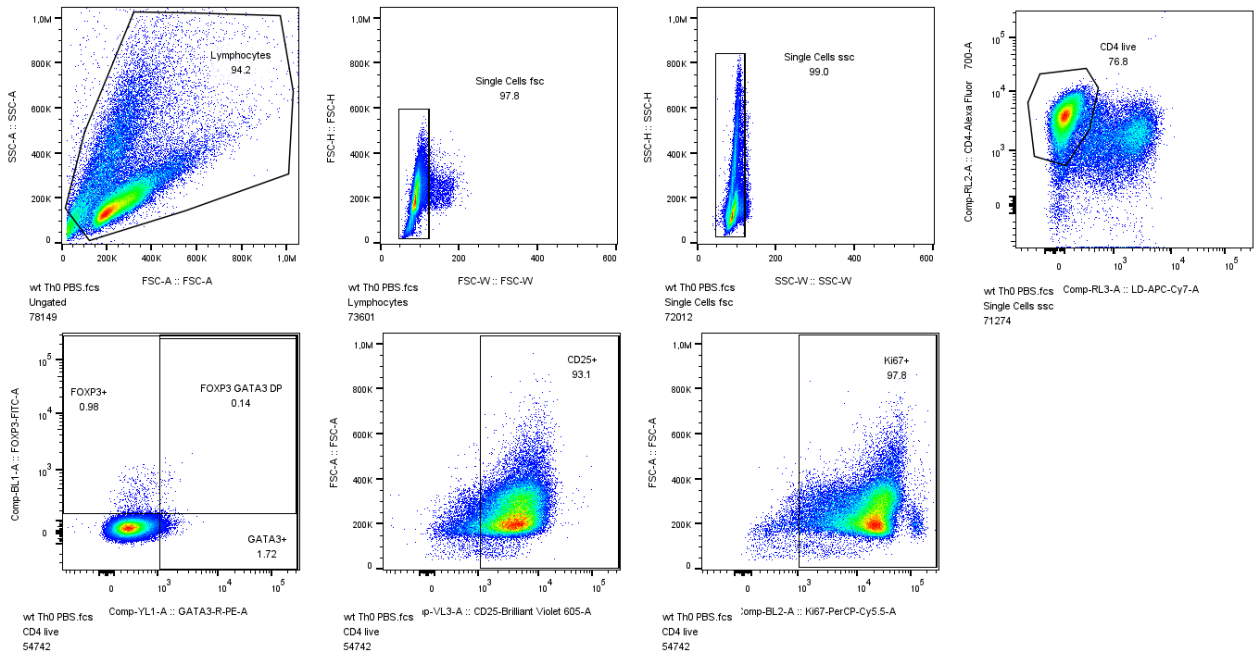

Figure 18: Standard gating process for transcription factor analysis of in-vitro cultured CD4+ T cells using FlowJo on the example of Th0.

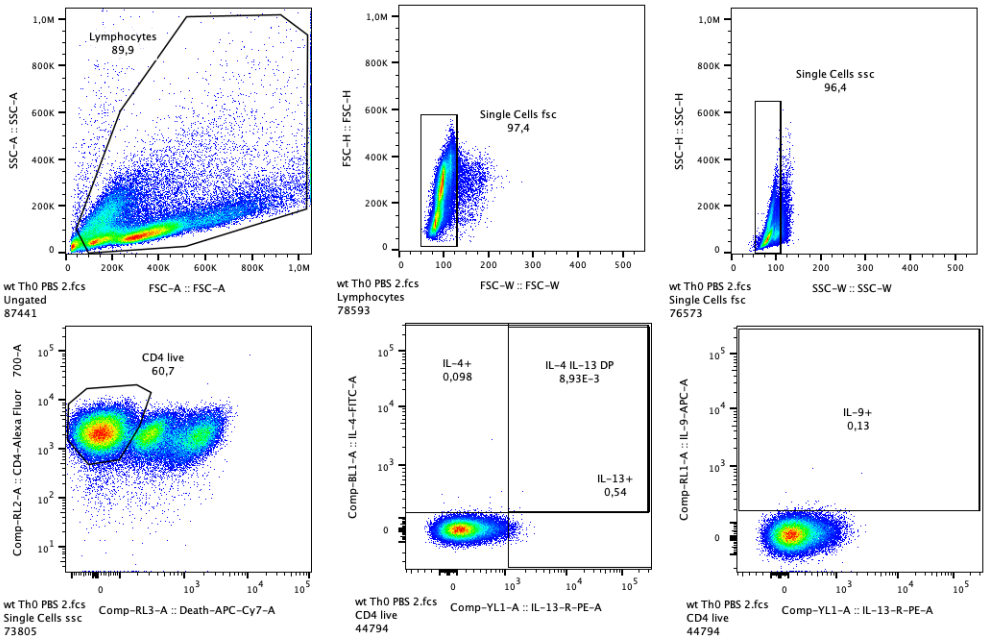

Figure 19: Standard gating process for cytokine analysis of in-vitro cultured CD4+ T cells using FlowJo on the example of Th0.

4.6 *H. POLYGYRUS* PROPAGATION:

Prior to infecting the mice the infective L3 stage larvae are rested at room temperature for 30-60 minutes to recover from the 4 °C storage conditions, after which they are washed for 15 minutes in PBS. Following this the worms are counted in order to allow a precise infection of 250-300 larvae per 6-8 week old, male, C57Bl6/J mouse. In order to infect the mice 250-200 µL of larvae suspension are

orally gavaged. *H. polygyrus* starts to produce eggs after 10-14 days of infection which allows to start the stool collection from day 10-12 past the initial infection step. The collection can be carried out for 6-8 weeks. The infected mice are put into a clean cage without bedding and with wire bottoms for a maximum of 12h/day to collect the feces from them. Exceeding this 12h/day time-limit is stressful for mice and should be avoided. The feces are then collected into a 50 mL Falcon tube after which the ex-vivo propagation steps continue.

The stool containing the eggs is homogenized by filling up the falcon tube to the 45 mL marking with autoclaved MQ H2O and mixed via vortexing the Falcon tube briefly. Then the Falcon tube is centrifuged at 1500 rpm for 2 minutes after which the supernatant is carefully discarded. Following this a small amount of MQ H2O is added to the tube allowing to wash away the slurry deposit on the top of the content trough gentle stirring of the tube. After discarding the supernatant the flacon tube is again filled up with autoclaved MQ H2O and homogenized through mixing via vortexing. Then the centrifugation step is repeated, supernatant discarded, and slurry deposit washed away through gentle stirring. After this step, 2 mL of 10000 U/mL Nystatin are added to the falcon tube and to two Whatman paper filter discs in order to prevent fungal infection of the samples. The falcon tube is filled up one last time to the 45 mL marking with MQ H2O, homogenized and centrifuged. After the final supernatant has been discarded, the stool is transferred to a glass petri dish in order to mix it roughly 1:1 with autoclaved charcoal to mimic the natural soil conditions for *H. polygyrus*. Ensure that the mixture is moist but not runny by utilizing a Nystatin spraying solution of 100 U/mL on dry stool-charcoal mixtures. The mixture is then transferred from the glass petri dish to a small plastic petri dish in which a Nystatin drenched Whatman paper disc is placed. This small petri dish is then placed in a bigger petri dish filled with water to create a humidification chamber. Finally this chamber is the placed into a 25 °C incubator to facilitate the growth of new L3 larvae.

After one week in the incubator the L3 larvae can be retrieved from the stool-charcoal mixture through sedimentation. A sedimentation apparatus is prepared by connecting a glass funnel to a glass conical through a rubber segment. After ensuring the system is leakproof, warm tap water is filled up to 1/4 of the funnel. Then the interior of the funnel is lined up with a tissue paper filter and the stool-charcoal mixture is placed on top of the filter. Repeat this process in order to also collect L3 larvae from the Whatman paper itself. After overnight sedimentation the supernatant of the conical can be removed through a vacuum pump connected to a Pasteur pipette and add autoclaved MQ H2O to the rim of the tube to wash particles that may have decanted with larvae. Following this, the L3 are sedimented again for 30 minutes afterwards this step is repeated two more times until the supernatant is clear. Lastly, the L3 are resuspended in MQ H2O and transferred into a small tissue culture flask. The MQ H2O larvae

mixtures of different collection days are pooled and stored at 4 °C until further use for a new mouse infection.

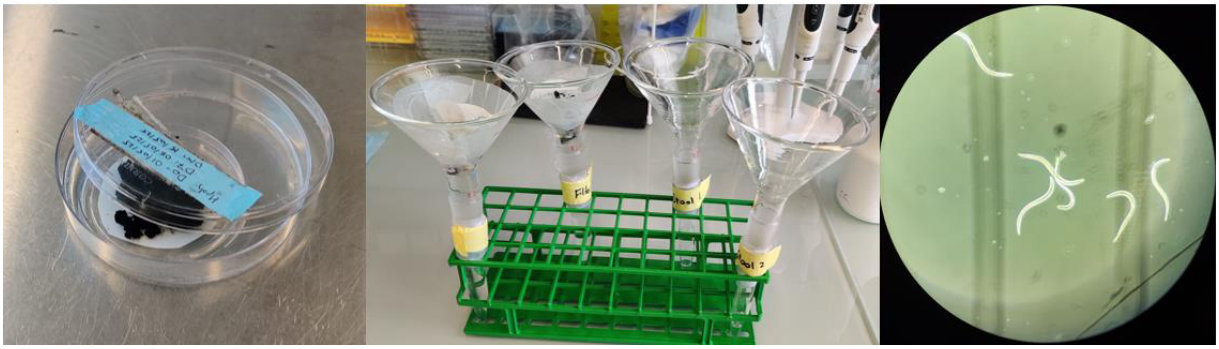

Figure 20: Depicted are the humidification chamber on the left which provides optimal humidity condition for the larvae to hatch. The picture in the center shows the sedimentation apparatus to collect the hatched larvae. The picture on the right shows the collected infective L3 larvae of *H. polygyrus*.

5. RESULTS:

5.1 OPTIMIZING CULTURE CONDITIONS FOR TH9 POLARIZATION:

Previous work by a former master student, already provided the baseline conditions for Th2, Treg and Th9 in-vitro cell culture. However, for Th9 cells these conditions were not optimal since they failed to produce IL-9, a key effector molecule of this cell type (Figure 21, left plot).

In order to identify optimal IL-9 production conditions for the Th9 cells, an in-vitro cell culture experiment was conducted that investigated varying strength of CD4+ T cell activation via aCD3/aCD28 stimulation, as well as titration TGF-ß which has been shown to stimulate the Th9 cell phenotype. Finally, the addition of IL-2 has also been investigated but did not show any significant effect on IL-9 expression. This in-vitro experiment was performed utilizing naïve CD4+ T cells from a HDAC1^{flox}CD4^{Cre} and a *C57BL/6J* mouse following standard in-vitro culture procedure. After three days of culture the cells have been stained for IL-9 and analysed using FlowJo.

The newly optimized condition for IL-9 expression utilizes 2 µg/mL of aCD3/aCD28, 2 ng/mL TGF- ß and 50 U/mL IL-2 together with unchanged IL-4 conditions of 20 ng/mL and aIFN-γ of 10 µg/mL (Figure 21, center plot). Even though a stronger activation via 5 µg/mL of aCD3/aCD28 yielded a higher IL-9 expression (Figure 21, right plot) we settled on the medium amount of IL-9 expression, in order to preserve potential increases or decreases via additional treatment in future experiments. Between both mice genotypes similar IL-9 stimulation patterns have been observed so we adapted this optimization for both, the HDAC-1 KO and *C57BL/6J* mouse model.

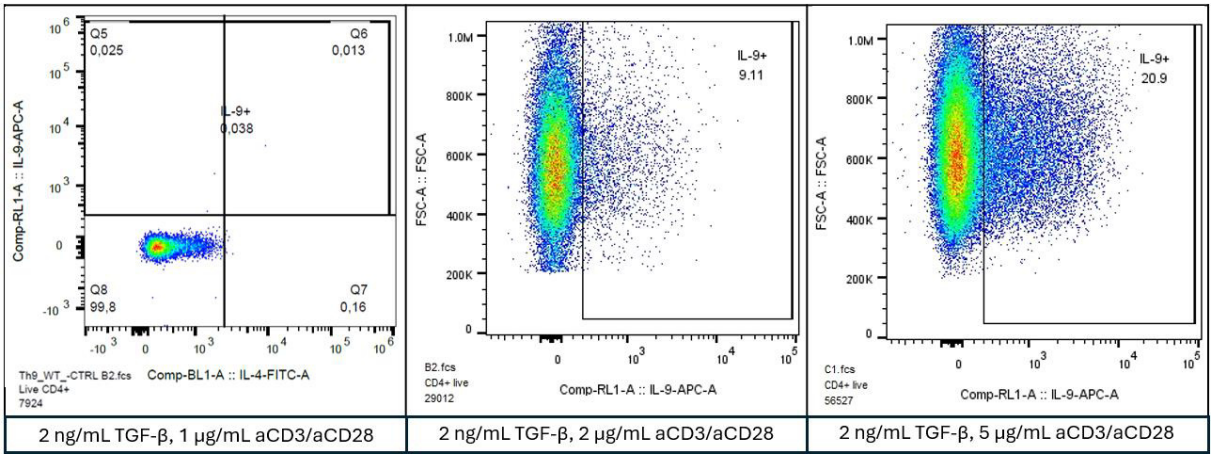

Figure 21: Varying strength of T cell activation via aCD3 and aCD28 result in different IL-9 expression. The plot on the left shows previously used conditions for Th9 cell culture that failed to stimulate IL-9 expression. Optimal IL-9+ expression can be seen in the center plot which was produced by 2 µg/ml aCD3/aCD28 together with 2 ng/mL TGF-ß. The plot on the right side depicts the conditions which achieved the highest IL-9 expression through 5 µg/ml aCD3/aCD28 together with 2 ng/mL TGF-ß.

5.2 EFFECTS OF SCFA ON WT AND HDAC1-DEFICIENT NAÏVE CD4 T CELLS:

Previous findings of the Campbell laboratory group show that the levels of acetate, propionate and butyrate are changed during an *H. polygyrus* infection in mice (Figure 22). Propionate shows the most significant increase during *H. polygyrus* infection, followed by butyrate and then acetate. To gain more insight into the effects these SCFAs have on CD4+ T cells they have been selected to be investigated in the in-vitro cell culture part of this project, where they have been added to Th9, Th2 and Treg cell cultures.

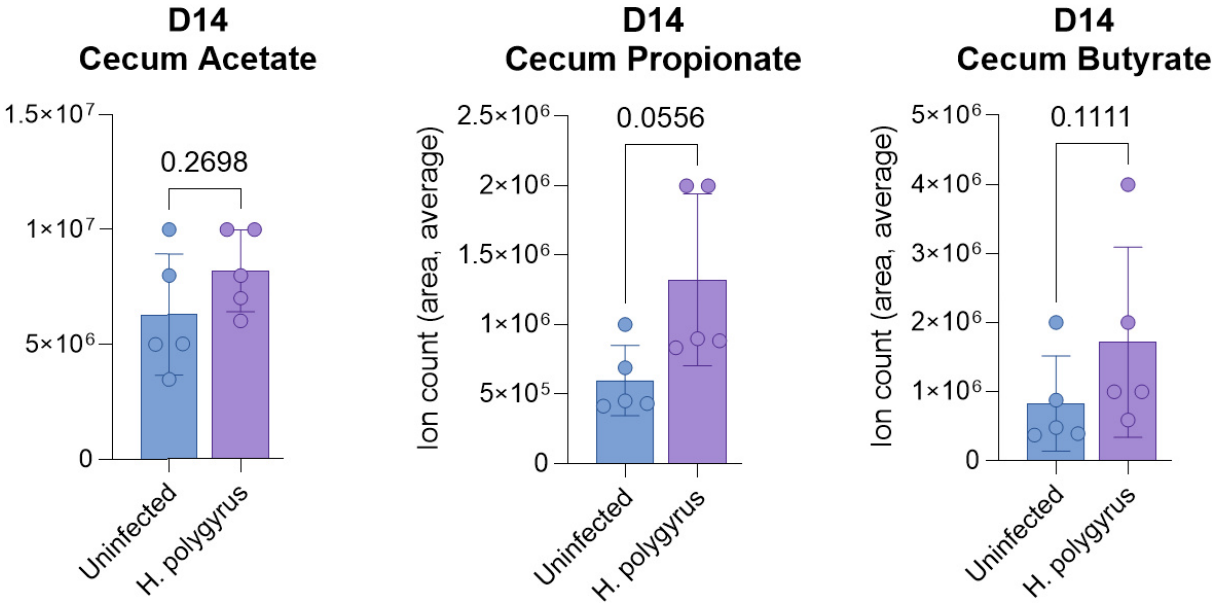

Figure 22: Mice infected with *H. polygyrus* show a trend for increased levels of acetate, propionate and butyrate in the cecal contents 14 days post infection. This figure was kindly provided by Adrija Chakrabarty, a PhD student of the Campbell laboratory group.

In order to investigate how SCFAs like butyrate, propionate and acetate affect naïve CD4+ T cell subsets an in-vitro experiment was carried out. Furthermore, to analyse the impact that an HDAC-1 KO has on these cells in conjunction with the SCFA addition, immune cells from HDAC1^{flox}CD4^{Cre+} *and a* HDAC1^{flox}CD4^{Cre-} mice have been used in parallel.

Splenocytes from a HDAC1^{flox}CD4^{Cre+} and a HDAC1^{flox}CD4^{Cre-} mouse have been isolated and enriched for CD4+ naïve cells using a Miltenyi mouse naïve CD4+ enrichment kit (cat. number: 130-104-453). After ensuring the purity of each enriched cell population the naïve CD4+ T cells were seeded into a 96 well plate containing the polarization conditions for Th2, Th9 and Treg cells with and without the addition of 250 µM butyrate, propionate or acetate. As negative control PBS was added in the same volume as the SCFA while as positive control 250 nM Entinostat was added in the control wells. The Entinostat was kept in culture for only 24h after which the media was replenished without Entinostat. Following three days of culture, half of the Th9 and Treg cell population was stained for their transcription factors

expression, while the other half was restimulated using Ionomycin, Brefeldin A, Monensin and PMA after which the cytokines were stained using respective antibodies. The Th2 cells were reseeded after three days on a freshly coated new 96-well plate with new media containing the polarization conditions, SCFAs and controls excluding Entinostat. After an additional three days of cell culture the Th2 cells were stained and analysed like the Th9 and Treg cells.

The analysis revealed that butyrate is the most potent driver of naïve CD4+ T cell modulation. While the addition of SCFAs to naïve Th2, Th9 and Treg cells did only show minimal change in their viability, a very prominent decrease in number was observed in Th9 and Treg cells. The addition of butyrate resulted in the greatest decrease of CD4+ number followed by propionate and lastly acetate which showed little to no effect on number. For Th2 cells the number and viability of CD4+ T cells were less affected by the addition of SCFA. For all cell types there seems to be a trend in which the HDAC-1 KO exacerbates this decrease of CD4+ number and frequency. This effect is most prevalent and significant in Th9 and Treg cells, while Th2 cells just show a weak trend (Figure 23).

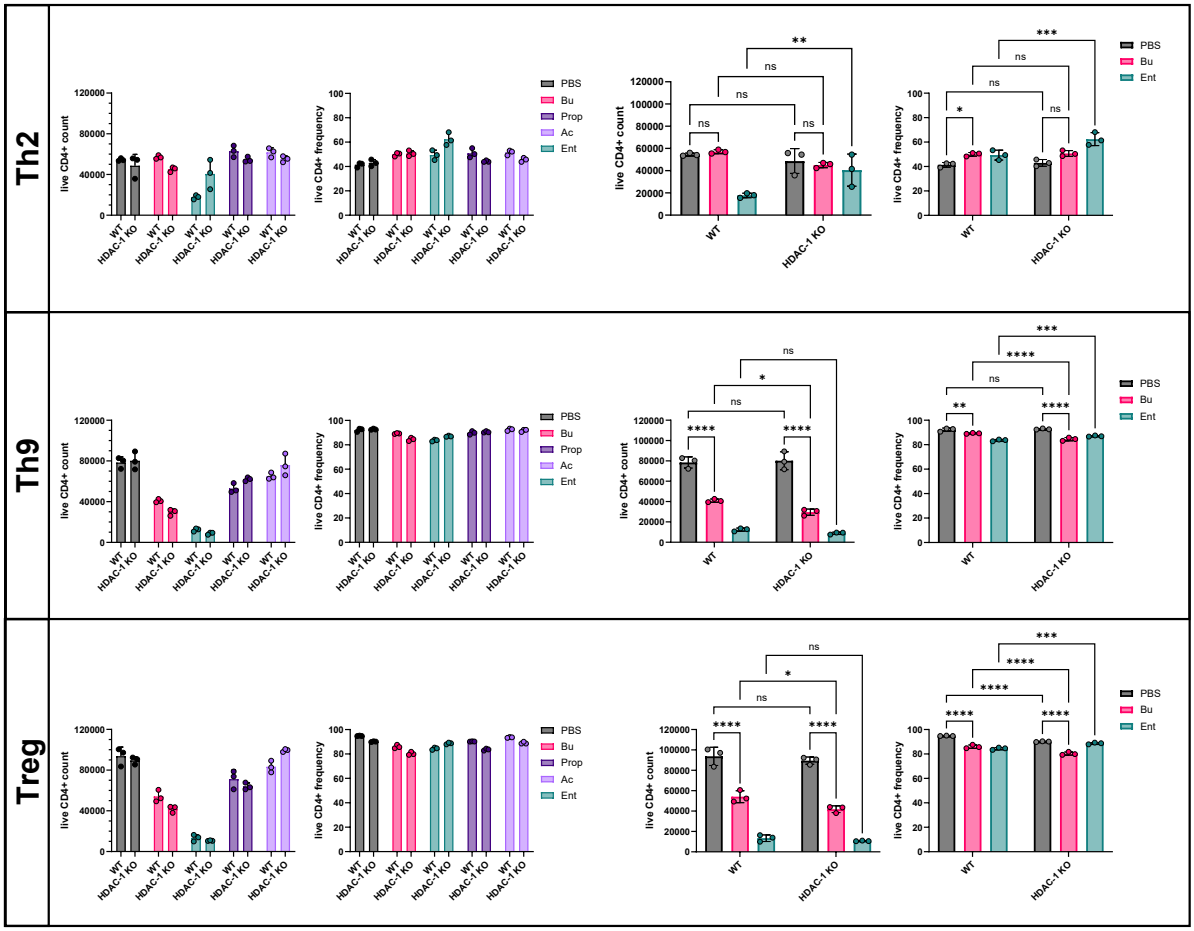

Figure 23: The effect of the SCFAs butyrate (Bu), propionate (Prop), acetate (Ac) on number and viability of naïve Th2, Th9 and Treg cells generated from CD4+ T cell that have been isolated from HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT) are shown on first two graphs of each row. Entinostat (Ent) was used as a positive control for HDAC inhibition while phosphate buffered saline (PBS) was used as

a negative control. The following two graphs show representative experiments highlighting the effect of butyrate on CD4+ number and viability isolated and in HDAC-1 context. The statistical analysis was done via Graph Pad Prism utilizing Two-Way ANOVA with P-values of ns > 0.05, * ≤ 0.05. ** ≤ 0.01, * ≤ 0.001, **** < 0.0001.**

The addition of butyrate to Th2 cells did not result in a significant change of GATA3 expression. Furthermore, HDAC-1 also does not seem to affect GATA3 expression in these cells. Analysis of the IL-4 expression indicates an upregulation in WT Th2 cells through the addition of butyrate. This effect is also visible in the HDAC-1 KO Th2 cells but less prominent. Contrary to this finding the IL-13 expression is decreased through the addition of butyrate. Even though the HDAC-1 KO Th2 cells indicate a higher IL-13 baseline the downregulation through butyrate is again more prominent in the HDAC-1 deficient cells (Figure 24, first row). Interestingly, the addition of butyrate resulted in a significant upregulation of FOXP3 in both WT and HDAC-1 KO in Th9 cells. In general the baseline expression of FOXP3 was lower in the KO compared to WT samples. Decreased levels of IL-9 expression have been identified through butyrate treatment. Again, this effect was more prominent in the HDAC-1 deficient Th9 cells compared to the WT cells (Figure 24, second row). The FOXP3 expression of WT Treg cells was not influenced by the addition of butyrate, while the KO Treg cells show a trend towards upregulation of FOXP3 through butyrate. Furthermore, the baseline level of FOXP3 expression was significantly lower in HDAC-1 deficient cells. Butyrate treatment resulted in a significant upregulation of GATA3 in both KO and WT regulatory T cells. Finally the IL-13 expression in Treg was increased through butyrate but while the WT cells show only a trend, the KO cells indicate a significant increase (Figure 24, third row).

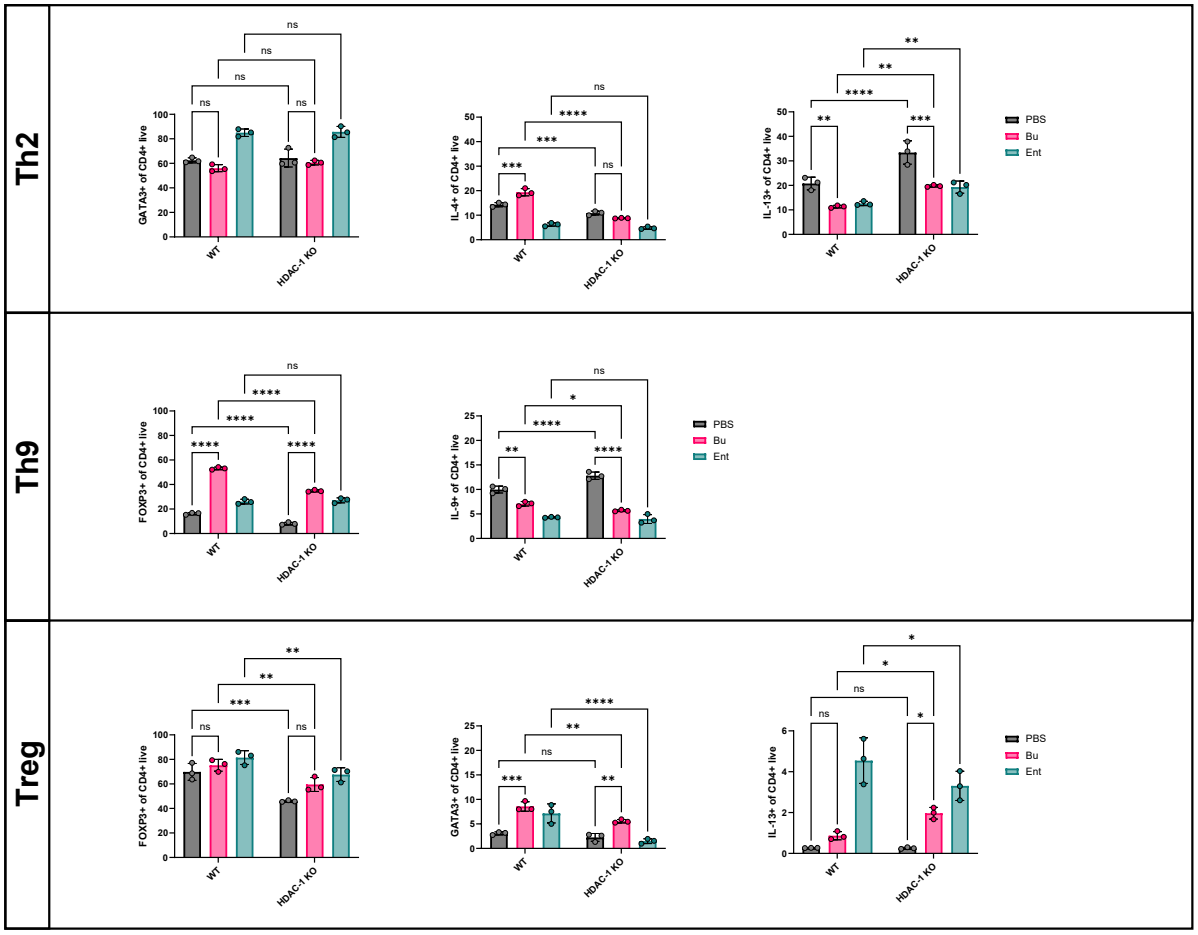

Figure 24: The effect of butyrate (Bu) on key transcription factors (GATA3, FOXP3) and cytokines (IL-4, IL-13, IL-9) of naïve CD4+ T cells from HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT) polarized towards Th2, Th9 and Treg cells. Showing each cell type in its respective row. Entinostat (Ent) was used as a positive control for HDAC inhibition while phosphate buffered saline (PBS) was used as a negative control. All polarization were carried out with the same batch of naïve CD4+ T cells. The statistical analysis was done via Graph Pad Prism utilizing Two-Way ANOVA with P-values of ns > 0.05, * ≤ 0.05. ** ≤ 0.01, * ≤ 0.001, **** < 0.0001.**

This experiment was repeated four times in order to verify the consistency of the before observed effects. Following this, the fold change of butyrate treatment over the PBS control for both HDAC-1 KO and WT of the observed effects were calculated. Taken together this fold change calculation supports most of the observed trends but also highlights the variability between the individual experiments. The increase of FOXP3 through the addition of butyrate in Th9 cells is the most prominently conserved pattern observed as it is the only fold change calculation that show significantly higher FOXP3 upregulation in the HDAC-1 KO Th9 cells (Figure 25, second row).

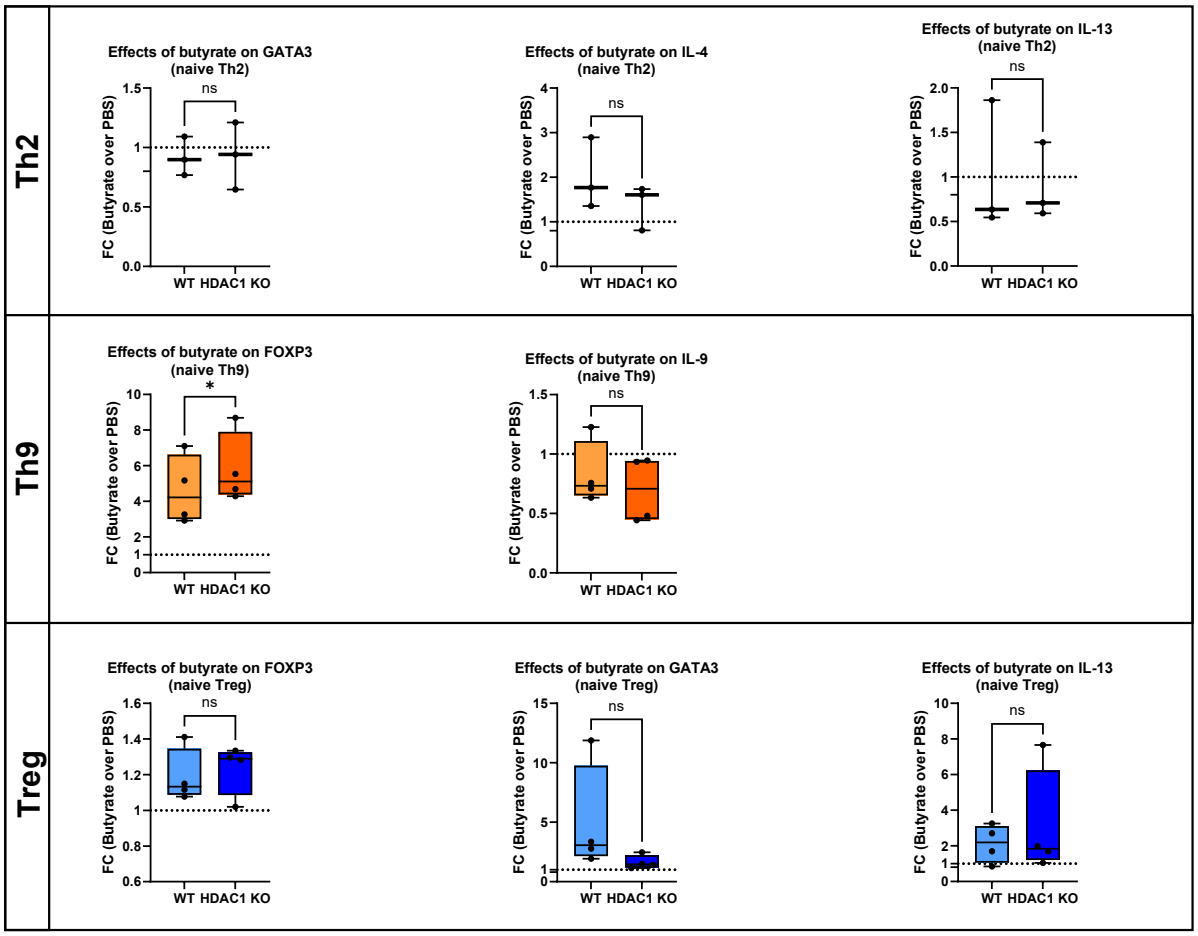

Figure 25: Depicted are butyrate over pbs fold-change calculations for the expression (% of parent) of key transcription factors and cytokines of HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT) CD4+ T cells. Each dot represents a separate experiment in which an arithmetical middle of transcription factor or cytokine of interest has been calculated through technical replicates. The statistical analysis was done via Graph Pad Prism utilizing Students-T test with P-values ns > 0.05, * P ≤ 0.05.

5.3 EFFECTS OF SCFA ON ACTIVATED CD4 T CELLS:

To gain insight on how SCFAs like butyrate, propionate and acetate affect already activated CD4+ T cell committed to a specific subset (Th2, Th9, Treg) another set of in-vitro experiments was carried out. Additionally, the impact that HDAC-1 KO has on these cells in conjunction with the SCFA addition was also investigated. Together with the previous set of experiments on naïve CD4+ T cells, this experimental setup on activated CD4+ T cells should provide an additional layer of understanding on the SCFA-HDAC1-T cell axis. For this experiment immune cells from HDAC1^{flox}CD4^{Cre+} *and a* HDAC1^{flox}CD4^{Cre}-mice have been used in parallel.

Splenocytes from a HDAC1^{flox}CD4^{Cre+} and a HDAC1^{flox}CD4^{Cre-} mouse have been isolated and enriched for CD4+ naïve cells using a Miltenyi CD4+ enrichment kit. After ensuring the purity of each enriched cell population the naïve CD4+ T cells were seeded into a 96-well plate containing only the polarization

conditions for Th2, Th9 and Treg cells. After three days of culture all cell types have been reseeded into a new plate containing polarizing conditions together with butyrate, propionate, acetate and the controls. As negative control PBS was added in the same volume as the SCFA while as positive control 250 nM Entinostat was added in the control wells. The Entinostat was kept in culture for only 24h after which the media was replenished without Entinostat. Following three more days of culture, all cell types have been analysed using flow cytometry. For cytokine expression analysis half of the cell population have been restimulated with Ionomycin, Brefeldin A, Monensin and PMA after which they were stained using antibodies. Important to note here is that Th9 and Treg cells will be kept in culture for a total of six days compared to the naïve CD4+ T cell setup in which they only stayed three days in culture.

In contrast to naïve CD4+ T cells, a trend in which propionate- but not butyrate- affects the number and viability of activated CD4+ T cells can be seen. However, this set of experiments showed greater variability in comparison to the naïve CD4+ T cell setup. In activated Th2 cells the addition of propionate resulted in the most prominent decrease of cell viability and count. Butyrate treatment produced interesting results, as it decreased count but not viability of CD4+ T cells in the HDAC-1 KO cells, while in WT cells it increased CD4+ viability as well as cell count (Figure 26, first row). In activated Th9 cells propionate also decreased CD4+ viability and number to a greater extent compared to butyrate. Still, butyrate was able to significantly reduce CD4+ T cell count and viability in the HDAC-1 KO cells. In the WT Th9 cells count was low, with statistical analysis indicating a significant reduction in viability but only pointing towards a trend of CD4+ number decrease (Figure 26, second row). Propionate also decreased count and viability of Treg more strongly than butyrate. The addition of butyrate did not significantly change the number or frequency of CD4+ T cells of regulatory T cells generated from WT mice strains. In Treg cells generated from HDAC-1 KO cells. However, a significant decrease of CD4+ number and viability was observed (Figure 26, third row).

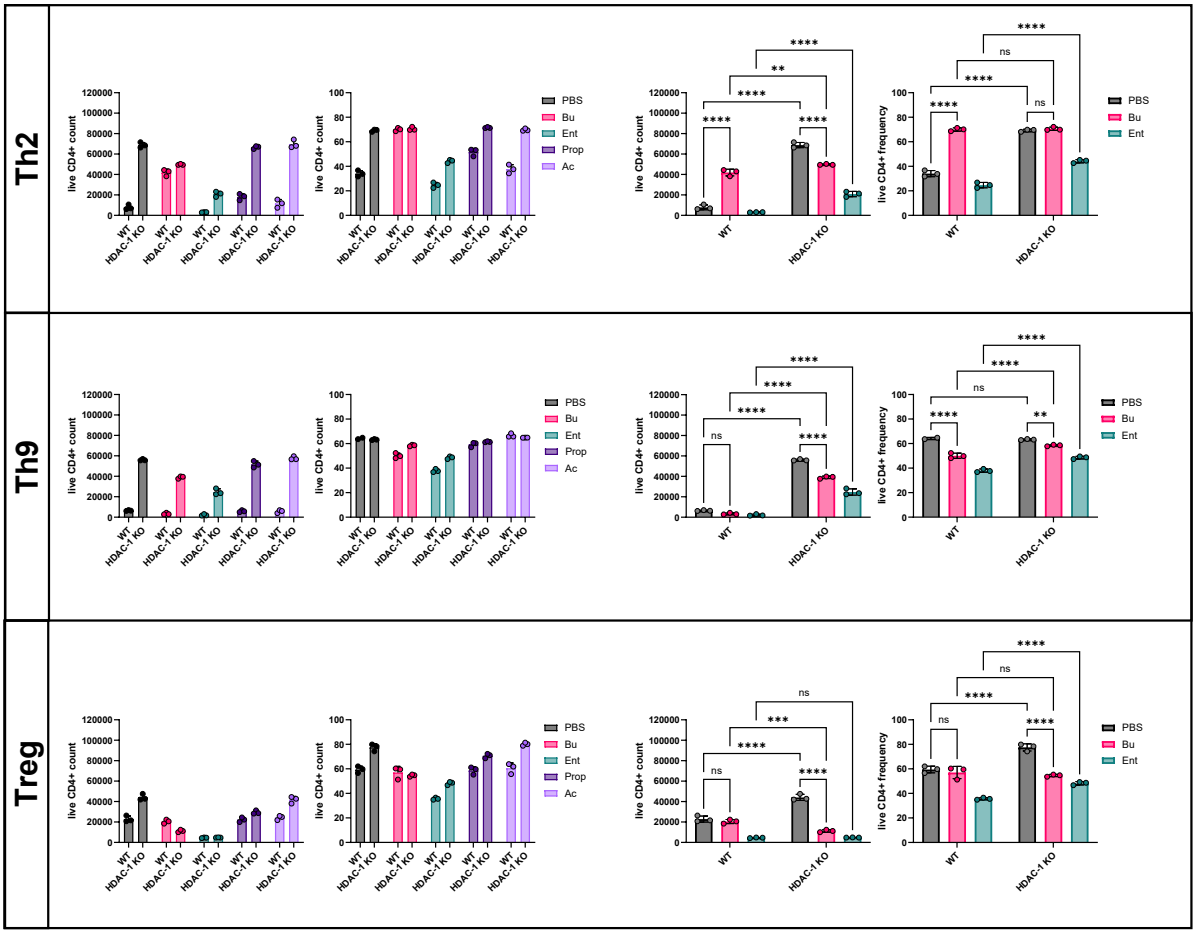

Figure 26: The effect of the SCFAs butyrate (Bu), propionate (Prop), acetate (Ac) on number and viability of activated Th2, Th9 and Treg cells generated from CD4+ T cell that have been isolated from HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT) and kept in culture for three days under polarizing conditions are shown on first two graphs of each row. Entinostat (Ent) was used as a positive control for HDAC inhibition while phosphate buffered saline (PBS) was used as a negative control. The following two graphs show representative experiments highlighting the effect of butyrate on CD4+ number and viability isolated and in HDAC-1 context. The statistical analysis was done via Graph Pad Prism utilizing Two-Way ANOVA with P-values of ns > 0.05, * ≤ 0.05. ** ≤ 0.01, * ≤ 0.001, **** < 0.0001.**

Activated Th2 cells from both WT and HDAC-1 KO mouse strains had a trend towards increased IL-4 expression upon treatment with butyrate with the effect being significant in the WT cells where the baseline expression also was higher than in the HDAC-1 KO. A similar patter can be observed in the IL-13 expression increase through the addition of butyrate. In Th9 cells the addition of butyrate resulted in upregulation of FOXP3 similar to the effect observed in the naïve CD4+ T cell experiments (Figure 27, first row). The WT Th9 cells showed stronger upregulation of FOXP3 through butyrate than the HDAC-1 deficient Th9 cells as well as higher baseline levels of FOXP3. Interestingly, a significant increase in IL-4 and IL-13 expression had been observed in both WT and HDAC-1 KO Th9 polarized CD4+ T cells. This was reflected with increased GATA3 expression that was present in both WT and KO, but stronger in the WT Th9 polarized cells (Figure 27, second row). Contrary to the naïve regulatory T cells, activated Treg cells exposed to butyrate showed a trend towards downregulation of FOXP3 expression.

Except a trend towards upregulation of IL-13 expression in WT Treg cells, this cell type remained largely unaffected by the exposure to butyrate (Figure 27, third row).

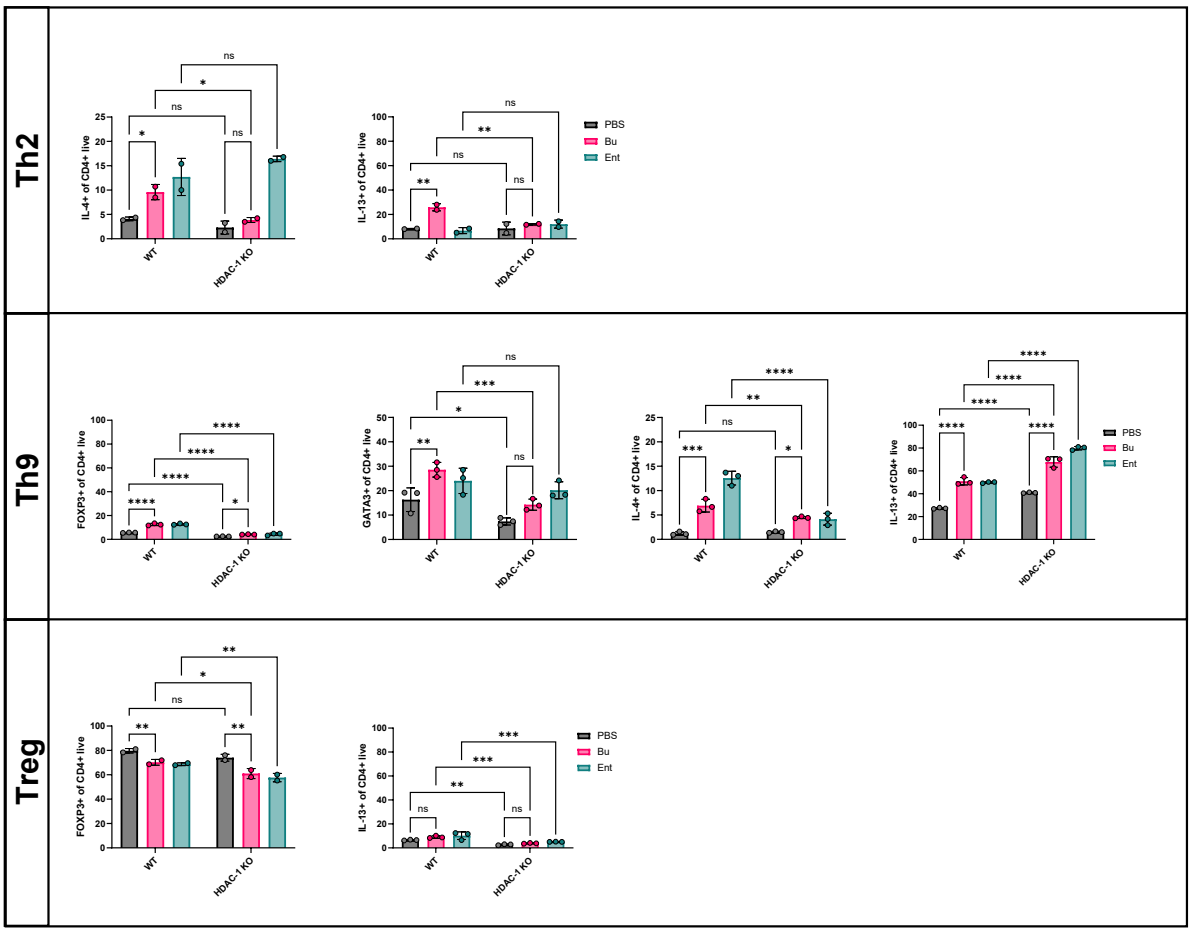

Figure 27: The effect of butyrate (Bu) on key transcription factors (GATA3, FOXP3) and cytokines (IL-4, IL-13, IL-9) of activated Th2, Th9 and Treg cells generated from HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT), that have been kept in culture under polarizing conditions for three days, are shown in graphs of their respective row. Entinostat (Ent) was used as a positive control for HDAC inhibition while phosphate buffered saline (PBS) was used as a negative control. Data was generated from single live CD4+ T cells of a representative experiment. The statistical analysis was done via Graph Pad Prism utilizing Two-Way ANOVA with P-values of ns > 0.05, * ≤ 0.05. ** ≤ 0.01, * ≤ 0.001, **** < 0.0001.**

This experiment was repeated three times in order to verify the consistency of the observed effects, after which the fold change of butyrate treatment over the PBS control for both HDAC-1 KO and WT cell were calculated. These fold change calculations support the main effects observed in the plots of the chosen representative experiment (Figure 27) but also depict the variance of many parameters. The most consistently observed effect is the increase of GATA3 in Th9 cells and its exacerbation in HDAC-1 T cells (Figure 28, second row, second graph). The effect of butyrate in upregulation of FOXP3 was less pronounced in the experiments conducted on activated and committed CD4+ Th9 cells compared to the naïve cells.

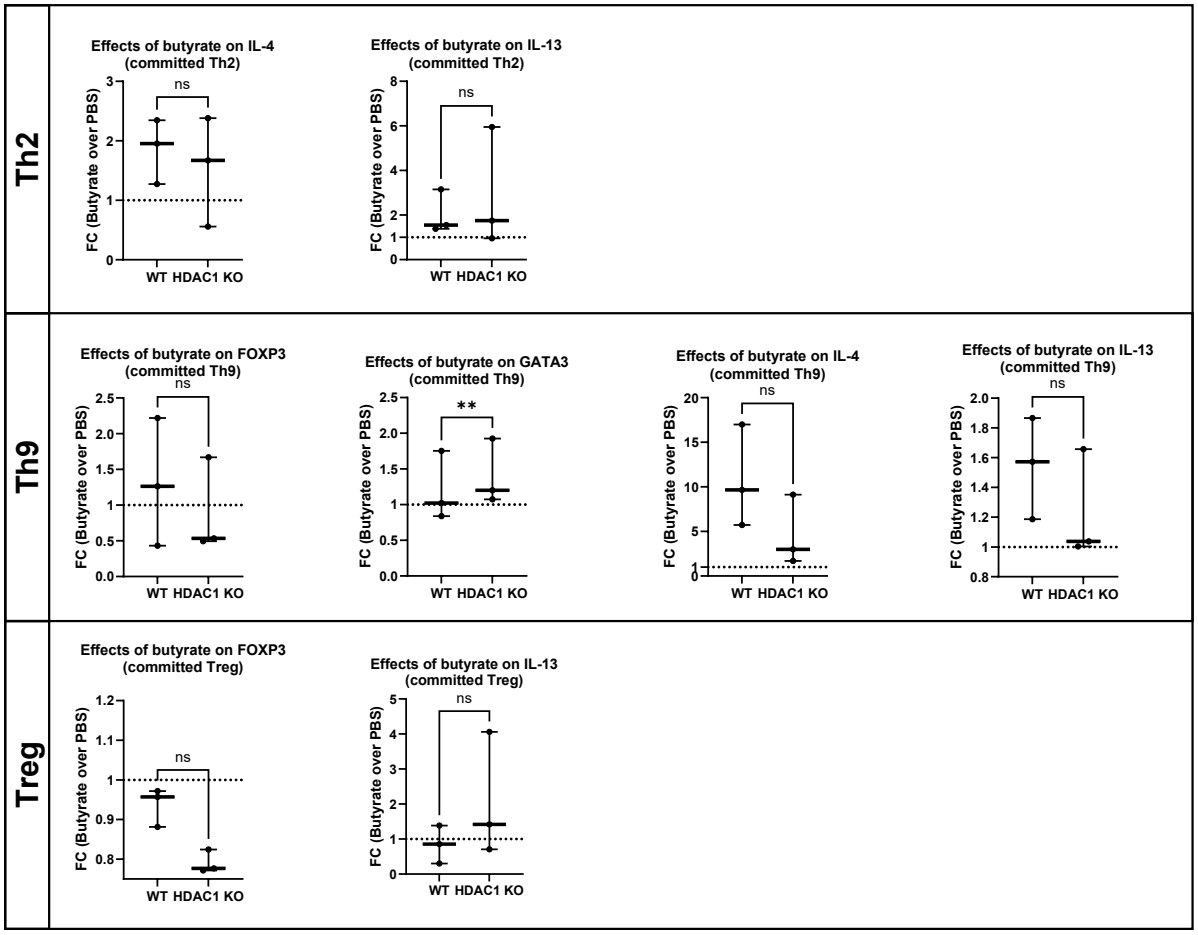

Figure 28: Depicted are butyrate over pbs fold-change calculations for expression (% of parent) key transcription factors and cytokines of activated CD4+ T cells generated from HDAC1^{flox}CD4^{Cre+} (HDAC1 KO) and HDAC1^{flox}CD4^{Cre-} (WT) mice. Each dot represents a separate experiment in which an arithmetical middle of transcription factor or cytokine of interest has been calculated through technical replicates. The statistical analysis was done via Graph Pad Prism utilizing Students-T test with P-values ns > 0.05, * ≤ 0.05, ** ≤ 0.01.

5.4 ANALYSIS OF FOXP3 AND IL-9 CO-EXPRESSION USING FOXP3^{HCD2}IL-9^{GFP} REPORTER CELLS:

Next mechanistic experiments were performed in order to gain further insights into the observed effect in Th9 cells that started to express increased levels of FOXP3 and decreased levels of IL-9 when exposed to butyrate. One possibility is that butyrate favours the expression of FOXP3 over IL-9 inside a given cell (cis effect). Another possibility is that butyrate increases the proportion of FOXP3+ in culture, and that these cells supress the production of IL-9 by other cells (trans effect). To test the first hypothesis, an experiment utilizing FOXP3^{hCD2}IL-9^{GFP} reporter mice was conducted. Cells from these mice allowed us to measure FOXP3 expression together with IL-9 expression in the same cell. FOXP3 protein co-expressed hCD2 which was transported to the outer membrane of the cell where it was possible to be stained using antibodies, while IL-9 expression was observable due to the expression of a GFP reporter protein. After three days of culture, in which naïve CD4+ T cells from this mouse strain

have been polarized using Th9 polarization conditions with butyrate, PBS or Entinostat, cells were restimulated using Ionomycin, Brefeldin A, Monensin and PMA to boost cytokine production. Following this, cells were stained for FOXP3 utilizing an anti-hCD2 antibody. This experiment again showed the upregulation of FOXP3 together with the downregulation of IL-9 in Th9 cells exposed to butyrate (Figure 29, top row plots) confirming our previous findings. Furthermore, the results of this experiment hints towards a trans effect of butyrate on FOXP3 and IL-9 expression since FOXP3+ cells as well as FOXP3- cells show a significant decrease of IL-9 expression when compared to Th9 cells that have been treated with PBS. Therefore, the hypothesis of a cis-effect in which butyrate favours FOXP3 expression over IL-9 has been proven wrong.

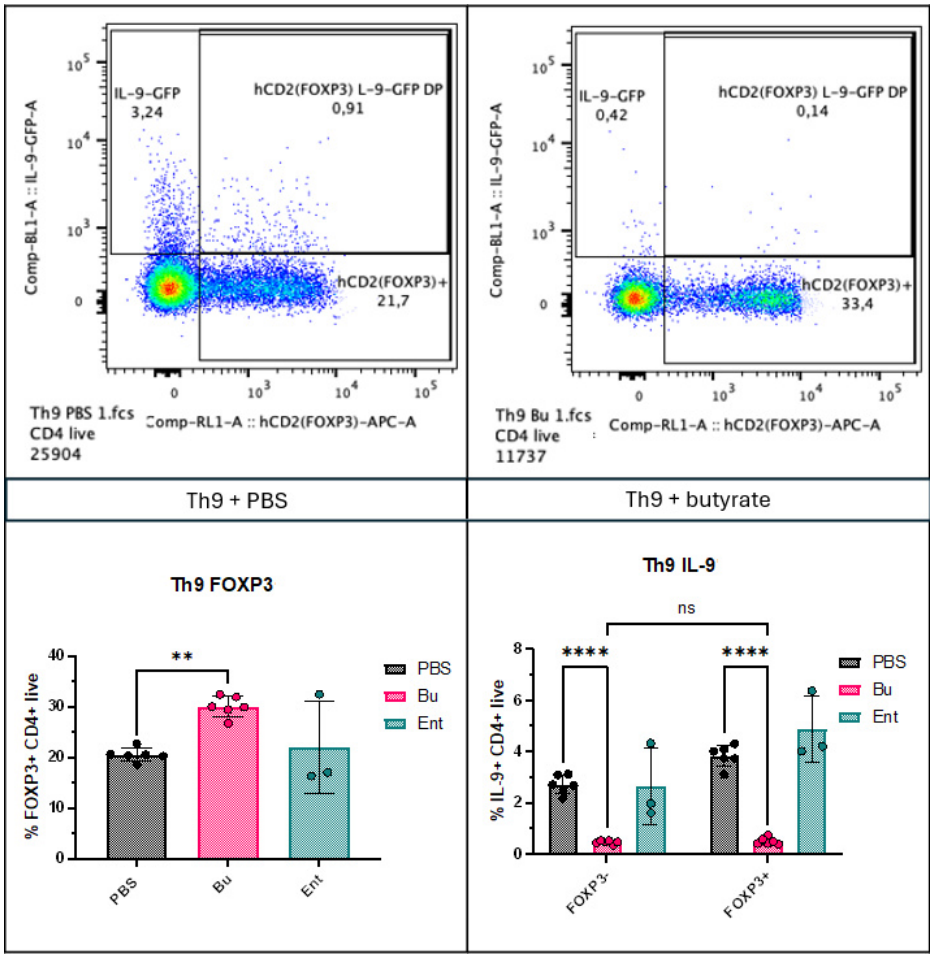

Figure 29: Depicted is the flowcytometric analysis of FOXP3 and IL-9 expression in Th9 polarized cells the using Foxp3-hCD2 IL-9-GFP reporter mouse strain. The plot on the left depicts expression of said parameters after exposure to the negative control PBS, while the center plot shows Th9 that have been exposed to butyrate. The graph on the right shows the FOXP3 expression of the live CD4+ population followed by IL-9 expression of FOXP3+ population. The statistical analysis was done via Graph Pad Prism utilizing One-Way ANOVA coupled with Dunnett's multiple comparisons test and P-values of ns > 0.05, * ≤ 0.05. ** ≤ 0.01, * ≤ 0.001, **** < 0.0001.**

5.5 EFFECTS OF ACUTE FOXP3 PROTEIN DEGRADATION ON IL-9 PRODUCTION:

To gain further insight into the mechanistical dynamics of FOXP3 and IL-9 regulations in Th9 cells the contents of two particular studies were used to build a hypothetical model explaining regulation of the *Il-9* gene locus. These two studies are Xiao et al. (2015) [64] and Son et al. (2024) [125]. Together these articles show how Glucocorticoid-induced TNFR-related protein (GITR) is able to upregulate p50 resulting in the recruitment of HDACs to the *foxp3* locus to produce an inaccessible chromatin structure. Additionally, GITR is able to activate STAT6 which binds to the *Il-9* locus accessible through recruitment of a HAT called p300 [64]. Furthermore, FOXP3 has been identified to be able to bind to CNS1 of the *Il-9* locus and thereby inhibit *Il-9* transcription [125].

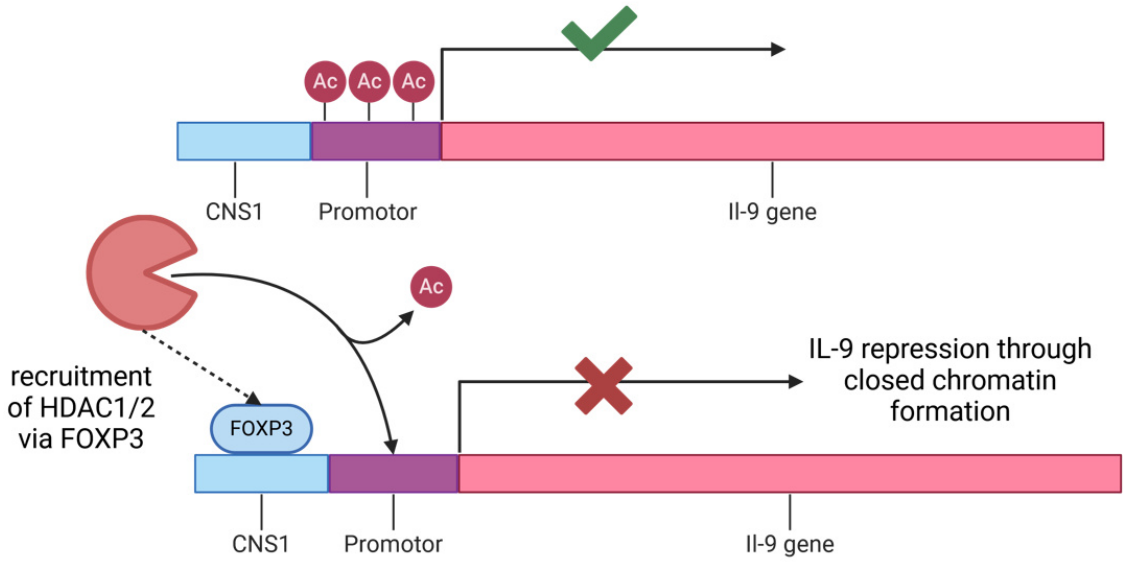

Figure 30: Hypothetical mechanism of the recruitment of HDAC1/2 to CNS1 of the *Il-9* gene locus resulting in deacetylation of its promotor region. This deacetylation results in a more compact chromatin state leading to repression of IL-9 expression. Figure adapted from [64,125] and created in Microsoft PowerPoint.

The increase of FOXP3 expression together with a decrease in IL-9 expression observable in our Th9 cell cultures that have been exposed to butyrate raised the question of whether these two effects are dependent on each other.

To investigate if functional FOXP3 protein is required for this IL-9 downregulation we utilized FOXP3$^{AID\text{-}DTR\text{-}GFP}$Rs26$^{TIR1(F74G)}$ in which FOXP3 can be degraded through the addition of auxin to the cell culture. After isolation and enrichment of CD4+ naïve T cells from a FOXP3$^{AID\text{-}DTR\text{-}GFP}$Rs26$^{TIR1(F74G)}$ mouse 45000 cells have been seeded in a 96-well flat bottom culture plate containing Th9 polarizing conditions and media. Additionally, Th9 cells were exposed to butyrate or PBS and half of the butyrate exposed Th9 cells were also exposed to auxin in order to degrade FOXP3. After three days of culture the cells have been stained for transcription factor and cytokine expression in order to determine FOXP3 and IL-9 expression. The results of this experiment again highlight the upregulation of FOXP3 in butyrate

treated Th9 cells and also indicate that the auxin degradation of FOXP3 was successful (Figure 31, left plot). Furthermore, the auxin degradation of FOXP3 resulted in IL-9 expression levels that were similar to the control samples indicating the rescue of IL-9 expression through absence of FOXP3 protein (Figure 31, right plot).

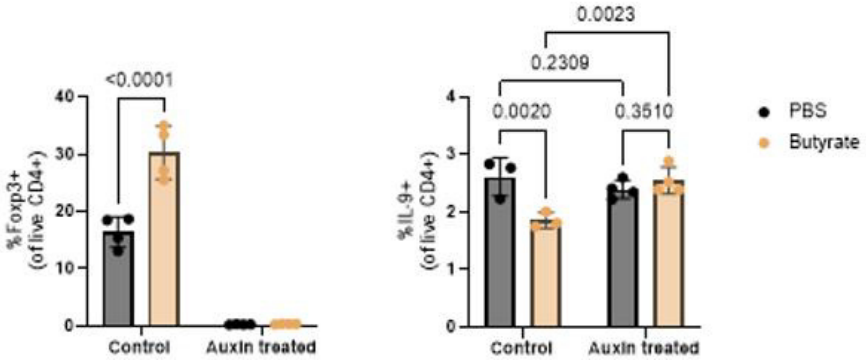

Figure 31: Depicted are the FOXP3 and IL-9 expression of Th9 polarized CD4+ T cells isolated from FOXP3$^{AID\text{-}DTR}$-GFP$^{RS26TIR1(F74G)}$ mice that have either been exposed to butyrate or PBS with and without auxin treatment to degrade FOXP3. The statistical analysis was done via Graph Pad Prism utilizing Two-Way ANOVA.

6. DISCUSSION:

6.1 BUTYRATE AS THE MOST ACTIVE SCFA IN MODULATING T CELL FATE:

The general consensus of many studies points to an overall SCFA concentration increase during an infection with a STH, the trends of specific SCFA increases show a greater variety [84–86]. Since the microbiota is the main source of SCFA, changes in microbiota composition between different mouse keeping facilities can be one reason for the observable differences. Another factor is the time of day, at which the sample for SCFA concentration measurement is taken as well as the sample method used [126]. The SCFA pool also fluctuates throughout the day and is also influenced by the eating behaviour of the host [127,128]. The complexity of the microbiome makes it difficult to conduct comparable studies and highlights the importance of in-vitro cell culture studies, which allow for a more artificial but controlled setting for investigation of various kinds of effects specific SCFA assert on the host. The in-vivo part of this project showed a prominent increase in butyrate and propionate upon *H. polygyrus* infection (Figure 22) highlighting the relevance of these SCFA. Additionally, the cell culture experiments conducted during this thesis support this further as addition butyrate and propionate also prominent effects on Th2, Th9 and Treg cells.

Throughout the experiments conducted in this study, butyrate has been identified as the most impactful SCFA on CD4+ T cell number, viability and cytokine/transcription factor expression modulation. The exposure of butyrate to naïve CD4+ T cells polarized in Th9 and Treg conditions

resulted in a reduction of CD4+ number but and a trend towards viability reduction. This finding is supported by previous findings of Fontenelle and Gilbert (2012)[129]. The negative effect of butyrate on T cell proliferation and viability was not observed in naïve CD4+ cells polarized in Th2 conditions. Activated CD4+ T cells that have been exposed to butyrate after three days of culture show similar trends, with the exception of Th2 in which butyrate seemed to increase count and viability but only in CD4+ T cells from the HDAC1^{flox}CD4^{Cre-} mouse strain. Additionally, the SCFA summary plots indicate that propionate is more impactful on already committed CD4+ T cells compared to naïve CD4+ T cells as.

Butyrate was also identified as the most active modulator of T cell fate and effector function in comparison to propionate and acetate. In naïve CD4+ T cells that are polarized in Th2 conditions, butyrate exposure resulted in a prominent upregulation of IL-4 and a trend towards downregulation of IL-13, while GATA3 was less affected as the fold change calculations indicate. Activated Th2 cells also increased their IL-4 expression upon butyrate exposure but contrary to the naïve Th2 exposed to butyrate they also showed a trend towards IL-13 increase. Interestingly, the increase of IL-4 through butyrate seemed to be more prominent in Th2 cells polarized from the HDAC1^{flox}CD4^{Cre-} mouse strain compared to the HDAC1^{flox}CD4^{Cre+} strain.

IL-13 increase upon butyrate exposure was also observable in naïve Treg cells, while in activated Treg cells this effect was less prominent. In naïve Treg cells this IL-13 increase was more consistent than in naïve or activated Th2, which could be due to the fact that Th2 cells express a higher baseline of IL-13 compared to Treg cells that could possibly mask the effect of butyrate in Th2 cells. In line with this, the naïve CD4+ T cells polarized in Treg conditions also had consistent increased expression of GATA3 through butyrate compared to Th2 cells and additionally increased their FOXP3 expression. Activated Treg cells however, showed a consistent decreased FOXP3 expression and IL-13 expression which remained unchanged upon butyrate exposure.

Finally, in naïve CD4+ T cells that were polarized in Th9 conditions the addition of butyrate resulted in a significant upregulation of FOXP3 as well as a consistent downregulation of IL-9 expression. This effect was also reported in the study of Vieira et al. (2019)[60] in which butyrate was identified to attenuate lung inflammation through modulating Th9 cells. However, in activated Th9 cells exposed to butyrate the upregulation of FOXP3 together with and its effect on IL-9 downregulation was less consistent. This points towards a possible downregulation of IL-9 that is dependent on increased FOXP3 expression driven by butyrate. This thesis is supported by findings of Dardalhon et al. (2008) in which blocking of FOXP3+ Treg cell generation resulted in the induction of T helper cells that produce IL-9 and IL-10 [130]. Furthermore FOXP3 has been shown to bind the *Il9* locus and actively repressing *Il9* transcription via an incompletely understood mechanism involving GITR and STAT6 [54,64].

Furthermore, in activated Th9 cells exposed to butyrate the GATA3 expression together with IL-4 and IL-13 expression was consistently upregulated. This was not observable in naïve Th9 cells.

Taken together, out of the tested SCFAs butyrate is the most active modulator of CD4+ T cells. Butyrate reduces proliferation rate and viability in Th9 and Treg cells and is able to increases type II cytokine expression in a variety of cell types. There are also observable trends in upregulation of GATA3 as well as a significant upregulation of FOXP3 in Th9 cells. However, to solidify the hypothesis that the increase in FOXP3 through butyrate directly induces the downregulation of IL-9 additional experiments need to be conducted. Furthermore the Th2 titration experiment highlights the impact that the strength of T cell activation has on in-vitro cell culture, as the cytokine expression of Th2 shifts greatly depending on the concentration of aCD3 and aCD28 used.

6.2 ROLE OF HDAC1 IN T CELL FATE CHOICE AND PLASTICITY UPON TREATMENT WITH SCFA:

The $HDAC1^{flox}CD4^{Cre}$ mouse strains allowed us to contextualize many of the observed effects that butyrate has on CD4+ T cells to reduced deacetylation via deficiency of HDAC1. Important to note here is that this mouse strain used throughout this thesis is a conditional knockout strain only expressed in CD4+ cells that results in a Cre facilitated excision of hdac1. This could result in a transcriptional upregulation of HDAC2 and through that an increase in compensatory effects through the overlap in some functions of HDAC1 and HDAC2. To circumvent this additional layer of complexity a catalytically dead HDAC1 strain could be used.

The HDAC1 KO resulted in a slight reduction of CD4+ count and viability in naïve as well as activated Th2, Th9 and Treg in-vitro cell culture experiments. The before mentioned effects of butyrate on count and viability were also observed to similar strength in the HDAC1 KO.

In naïve Th2 cells exposure to butyrate resulted in an increase of IL-4 together which was less prominent in the HDAC1 deficient CD4+ T cells. The decrease of IL-13 in naïve Th2 cells was observed in the HDAC1 knockout to similar strength as in the WT as the fold change calculations revealed. The GATA3 expression also was similarly affected by butyrate in both KO and WT naïve Th2 cells. Activated Th2 cells exposed to butyrate showed a similar pattern, but the expression measured varied greater than in the naïve Th2 cells. One possible explanation for less prominent effects observable in HDAC1 knockout cells compared to wildtype cells is the possibility of HDAC2 compensating the regulatory function of HDAC1. Butyrate acting as a pan HDAC inhibitor together with the additional loss of HDAC1 resulting in more HDAC2 being targeted by the inhibitory effects of butyrate.

Contrary to this, the upregulation of FOXP3 in naïve Th9 through butyrate was observable to a significant stronger extent in the HDAC1 KO compared to the WT pointing towards a possible non-redundant function of HDAC1 to HDAC2 in regulation of this mechanism. The additional inhibitory effect that butyrate has on HDACs together with the loss of HDAC1 resulted in a more prominent upregulation of FOXP3 possibly because of HDAC1 having a higher affinity to the foxp3 locus than HDAC2 thereby resulting in an increased acetylation status of this locus making it more accessible for transcription. Statistical analysis comparing IL-9 expression of HDAC1 knockout compared to WT Th9 cells exposed to butyrate did not show any significant results. However a trend was still observable in which HDAC1 knockout facilitates the downregulation of IL-9 expression. In line with this the upregulation of FOXP3 through butyrate in activated Th9 was also less prominent in activated Th9 deficient in HDAC1 compared to naïve Th9. The greater variability of transcription factor and cytokine expression observed in the experiments on activated CD4+ T cells that were exposed to butyrate masked a lot of trends that were observable in single experiment analysis but still preserved the increased upregulation of GATA3 through butyrate in HDAC1 deficient Th9 cells.

In line with this observation, the upregulation of FOXP3 through butyrate in naïve Treg cells was also pointing towards a trend of increased strength in the HDAC1 KO. In activated Treg cells exposed to butyrate the HDAC1 knockout resulted in a more prominent downregulation of FOXP3 compared to the WT Treg cells. Furthermore, the upregulation in of IL-13 through butyrate was more consistent in naïve HDAC1 knockout Treg than in activated HDAC1 deficient Treg cells.

To summarize these findings, loss of HDAC1 results in an overall decrease of CD4+ T cell count and viability and seems to be particularly important for regulating the expression of FOXP3 and IL-9 in Th9 cells as. Loss of HDAC1 in Th9 cells exacerbated the butyrate induced increase of FOXP3 in consortium with downregulation of IL-9 expression. Together these findings point towards HDAC1's importance in safeguarding cell fate of Th9 and Treg cells. Furthermore, HDAC1 seems to play a role in regulating the expression of IL-4 and to some extent IL-13 in Th2 cells. On activated CD4+ T cells the variability of measured transcription factors and cytokines varied greatly, masking some of the effects observable in single experiments. However, the butyrate induced increase of GATA3 in was consistently stronger in HDAC1 deficient Th9 cells compared to the WT Th9 cells. This points towards HDAC1 being potentially relevant for specific deacetylation of the gata3 locus.

6.3 BUTYRATE REPRESSES IL-9 VIA FOXP3 UPREGULATION IN TH9 CELLS:

The general consensus of the experiments conducted throughout this thesis is that there is a connection between FOXP3 and IL-9 which can be influenced through HDAC1 and exacerbated via

butyrate exposure. Literature mentioned throughout this thesis supports this hypothesis. The pilot experiment in which utilizes FOXP3$^{AID\text{-}DTR\text{-}GFP}$Rs26$^{TIR1(F74G)}$ provided further insight into this field. Again, we were able to observe the butyrate induced upregulation of FOXP3 together with downregulation of IL-9 expression in Th9 cells polarized from this mouse strain. But more importantly, we were also able to observe the rescue of IL-9 expression through auxin induced degradation of FOXP3 protein which provided further insight into the mechanism of this interaction. This experiment supports the hypothesis that the FOXP3 protein is specifically able downregulate IL-9 expression in Th9 cell cultures. Together with the results from the experiment utilizing Th9 cells generated from the FOXP3^{hCD2}IL-9^{GFP} mouse strain these two experiments point towards a trans effect of butyrate in which it upregulates the proportion of FOXP3+ cell in culture, and that these cells supress the production of IL-9 by other cells. These two experiments do not support the proposed hypothetical model in which FOXP3 binds to the *Il-9* gene locus an downregulates its expression possibly via recruitment of HDAC1 or HDAC2 (Figure 30). Together with the data generated from the in-vitro cell culture experiments on naïve Th9 cells it is possible that the loss of HDAC1, which possesses greater affinity to the *foxp3* locus, facilitates the upregulation of FOXP3 expression.

7. FUTURE PERSPECTIVES:

Looking into the next steps this project could take, one of the first topic I would deconvolute is the compensatory aspect between HDAC1 and HDAC2. For each effect we observed during this thesis it is not clear if there is and to what extend HDAC2 can compensate for the loss of HDAC1. This problem could be assessed via a catalytically dead HDAC1 mouse strain, in which HDAC1 is transcribed and translated normally but does not possess catalytical activity rendering it unable to deacetylase its targets. This approach would allow HDAC1 and HDAC2 to be expressed in normal quantities instead of HDAC2 transcription being possibly upregulated through the absence of HDAC1 as it is in the HDAC1^{flox}CD4^{Cre}.

Furthermore, the downregulation of IL-9 in consortium with an upregulation in FOXP3 observable in our Th9 cells that have been exposed to butyrate is an interesting aspect that should be investigated more. Additionally, the experiment in which auxin was used to degrade FOXP3 suggests that the IL-9 downregulation is dependent on functional FOXP3 being present in the cell. As the FOXP3 auxin degradation experiment was just a pilot experiment as it was the last experiment conducted on this topic, I would first repeat it to solidify the observed effect is true. The dynamic of Th9 cells adapting a more regulatory T cell like phenotype is quite fascinating. To investigate this aspect more thoroughly the acetylation status of Th9 cells exposed to butyrate could be investigated and compared to Th9 cells without butyrate exposure. Besides *foxp3* being acetylated in the Th9 cells exposed to butyrate, I

would also expect the gene loci of NFκB to be acetylated while GITR, STAT6 and p50 to be less acetylated in the butyrate treated Th9 cells. These loci are of specific interest as they have been identified to be key turning points for Treg cells that adapt an IL-9 expressing phenotype. Even though, butyrate has been identified as a pan-HDAC inhibitor it could be possible that the resulting reduced HDAC activity of Th9 cells exposed to butyrate is effectively directed towards key gene loci such as the *Il-9* locus through FOXP3 where they can downregulate IL-9 expression and through that facilitate the acquisition of a more regulatory-like T cell phenotype. Flow cytometry could be used for an initial quantification of acetylation status in these cells of interest after which Chromatin Immunoprecipitation (ChIP) would allow to further identify DNA regions associated with histone acetylation. Additionally to this effect we observed in Th9 cells exposed to butyrate, other effects observed in Th2 and Treg cells should also be analysed in their acetylation status.

Finally, as the experiments in this study were exclusively conducted on mice CD4+ T cells it would be important to verify these effects in human cell lines such as Jurkat T lymphocyte cells or other immortalized human T cell lines.

8. LIST OF FIGURES:

9. ABBREVIATIONS:

aCD3	anti-CD3
aCD28	anti-CD28
AID	Auxin-inducible degron
AP1	Activator protein-1
APC	Antigen presenting cell
Arg-1	Arginase-1
ATF	Activating transcription factor
BATF	Basic leucine zipper ATF-like transcription factor
BrdU	Bromodeoxyuridine
CCR4	Chemokine receptor 4
CD62L	Cluster of differentiation 62L; L-selectin
ChIP	Chromatin immunoprecipitation
CLA-1	Cutaneous lymphocyte antigen
CNS	Conserved non-coding sequence
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid

DNBS	Dinitrobenzensulphonic acid
DTR	Diphtheria toxin receptor
EAE	Experimental Auto Immune Encephalomyelitis
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FFAR	Free fatty acid receptor
FOS	FOS family of transcription factors; AP-1 transcription factor subunit
FOXP3	Forkhead box protein P3
FSC	Forward scatter
GATA3	Gata-binding protein 3
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GPR	G-protein coupled receptor
HAT	Histone acetyltransferase
hCD2	Human CD2 gene
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibition
HES	*H. polygyrus* excretory-secretory antigen
HP-TGM	Heligmosomoides polygyrus TGF-β Mimic
IAA	Indole-3-acetic acid
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
IL	Interleukin

IL-4R	Interleukin 4 receptor
ILC	Innate lymphoid cell
IRF	Interferon regulatory factor
Itk	Inducible T-cell kinase
iTreg	Induced regulatory T-cell
Jun	Jun family of transcription factors; AP-1 transcription factor subunit
KO	Knockout
LAG3	Lymphocyte activation gene 3
M2	Alternatively activated macrophage
MACS	Magnetic-activated cell sorting
MAF	MAF family of transcription factors; AP-1 transcription factor subunit
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MQ	Milli-Q
MS275	Entinostat
MS	Multiple Sclerosis
NET	Neutrophil extracellular trap
NFAT	Nuclear factor of activated T-cells
NFKB	Nuclear factor kappa B family
p50	NFKB1; member of NFKB family
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate

PMT	Photomultiplier tubes
PRR	Pattern recognition receptors
pTreg	Peripherally generated regulatory T-cell
PU.1	Transcription factor of the ETS family
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
ROSA26 (Rs26)	Reverse orientated splice acceptor, clone 26
Rpd3	Reduced potassium dependency 3
SCFA	Short-chain fatty acid
SCF	Skp1-Cullin-F-box
SLE	Systemic Lupus Erythematosus
SIRT	Sirtuin
SLO	Secondary lymphoid organ
SMAD	SMA and MAD related proteins
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STH	Soil-transmitted helminth
T-bet	Member of T-box protein family
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Th	T-helper
TIR1(F74G)	Transport inhibitor response 1 ;F74G point mutation
TL1A	Tumor necrosis factor (TNF)-like cytokine 1A

tTreg	Thymic derived regulatory T-cell
WT	Wildtype

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