UNIVERZITA KARLOVA

Přírodovědecká fakulta

Studijní program: Biologie

Studijní obor: Imunologie (NIMUN)

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Role antigenní prezentace lymfoidních buněk vrozené imunity 3. typu v ustanovení periferní tolerance

Role of type 3 innate lymphoid cells mediated antigen presentation in peripheral tolerance establishment

DIPLOMOVÁ PRÁCE

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Praha, 2022

Prohlášení

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V Praze, 11.8. 2022 David Machač

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Poděkování

Chtěl bych velmi poděkovat svému školiteli RNDr. Dominiku Filippovi, CSc., za poskytnutí skvělého odborného zázemí, cenné rady a za nikdy nekončící podporu při vypracovávání této práce. Dále děkuji za neobyčejně obětavý přístup Mgr. Jiřímu Březinovi při technické, experimentální a odborné pomoci. Nemohu opomenout Mgr. Matouše Vobořila, PhD., Mgr. Tomáše Brabce PhD. a Mgr. Jana Dobeše, PhD. kteří vždy pomohli s interpretací výsledků, a rádi se podělili o své bohaté zkušenosti.

Abstract

Mutual tolerance between organisms is a key evolutionary mechanism for the emergence of commensal and mutualistic relationships. In case of the immune system, the creation of tolerogenic mechanisms towards our own tissues, but also commensal-mutualistic organisms and food antigens, together with defensive components of immunity and their balanced action, is a necessary condition for our survival. To better defend against pathogens, adaptive immunity with randomly assembled TCR and BCR receptors of lymphocytes has evolved. T lymphocytes capable of recognizing the body's own antigens are either deleted (recessive tolerance) or converted to regulatory T cells (Tregs) (dominant tolerance) in the thymic medulla by mechanisms which is collectivelly referred to as central tolerance. Compared to the establishment of central tolerance, the establishment of peripheral tolerance is a more demanding process in terms of the enormous variability of microorganisms living on our mucosal surfaces and skin along with the exposure to a wide variety of food antigens during ontogenesis. It has been shown that innate lymphoid cells expressing the RORγt transcription factor have the ability to present antigens derived from intestinal microorganisms and thus perform the deletion of T lymphocytes recognizing these antigens. These cells act in an analogous fashion as the epithelial cells in the thymic medulla, yet the question concerning their capacity to generate Tregs remains unanswered . Using tissue-specific KO mouse models and advanced flow cytometry analysis, we identified "Lti like cells" expressing the PD-L1 costimulatory molecule that are capable to perform the deletion of T lymphocytes to prevent the development of colitis. Importantly, we also observed that under these conditions, the Lti like cells can induce Tregs specific to intestinal microbiota. By depleting the intestinal microbiota, we also verified the dependence of RORγt$^+$ Treg development on the microbiota. We have established that NKp46$^-$ RORγt$^+$ type 3 innate immune lymphoid cells (ILC3) which can also induce Tregs, express the costimulatory molecules, but not PD-L1. Interestingly, our data showed that the development of peripheral Tregs expressing the transcription factor Helios occurs only in the pre-weaning period. Although this work did not directly show the peripheral induction of Tregs by RORγt$^+$ ILC3, it confirmed many findings about the development and physiology of these cell populations, and at the same time provided fresh inside into the influence of the microbiota on the development of Helios$^+$ Tregs and their ability to be selected on food antigens, which may open new avenues in research for treatment of food intolerance.

Key words:

peripheral tolerance, Tregs, microbiota, ILC3, colon, thymus, RORγt, Helios

Abstrakt

Vzájemná tolerance mezi organismy je klíčovým evolučním mechanismem vzniku komensálních a mutualistických vztahů. V případě imunitního systému je vytvoření tolerogenních mechanismů vůči vlastnímu tělu, ale také komensálně-mutualistickým organismům a potravním antigenům spolu s obrannou složkou imunity a jejich vyváženému působení nezbytnou podmínkou našeho přežití. Pro lepší obranu před patogeny se vyvinula adaptivní imunita s náhodnně sestavenými TCR a BCR receptory lymfocytů. T lymfocyty mající předpoklady rozpoznávat tělu vlastní antigeny jsou odstraněny nebo konvertovány na T regulační lymfocyty v dření brzlíku při ustanovení centrální tolerance. Oproti vzniku centrální tolerance ke stálým antigenům je ustanovení periferní tolerance náročnější proces z hlediska obrovské proměnlivosti na nás žijící mikroorganismů a přijmu široké palety potravních antigenů během ontogeneze. Bylo ukázáno, že lymfoidní buňky vrozené imunity exprimující RORγt transkripční faktor mají schopnost prezentovat antigeny odvozené od střevních mikroorganismů a provádět tak deleci T lymfocytů rozpoznávajících antigeny momentálně přítomné mikrobioty podobnou logikou jako epiteliální buňky v dřeni brzlíku a tedy i zda kormě delece dochází k indukci T regulačních lymfocytů. S využitím tkáňově specifických KO myších modelů a pokročilé průtokové cytometrie jsme identifikovali buňky provádějící deleci T lymfocytů v rámci prevence rozvoje kolitídy jako „LTi like cells“ a pozorovali přítomnost molekuly PD-L1 na těchto buňkách. Pozorovali jsme periferně indukované T regulační lymfocyty a jejich reakce na rozvoj kolitídy při narušení tolerance vůči střevní mikrobiotě. Díky depleci střevní mikrobioty pomocí koktejlu antibiotik jsme ověřili i závislost vývoje RORγt exprimujících T regulačních lymfocytů na mikrobiotě. Stejná závislost byla poprvé pozorována u periferně indukovaných T regulačních lymfocytů exprimujících transkripční faktor Helios, přičemž jsme díky adoptivnímu přenosu OT-II TCR transgenním T lyfocytů pozorovali, že tato populace se vyvíjí pouze v období před odstavem a, že tyto Tregulační buňky mohou být indukovány vůči potravním antigenům (OVA). Z možné zprostředkovatele periferní indukce T regulačních lymfocytů navrženy NKp46– lymfoidní buňky vrozené imunity 3. typu (ILC3) mající transkripční faktor RORγt. U těchto ILC3 jsme pozorovali přítomnost kostimulačních molekul a oproti LTi like cells také absenci PD-L1. Ačkoliv tato práce přímo neukázala periferní indukci T regulačních buněk pomocí ILC3, tak potvrdila mnoho poznatků o vývoji a fyziologii těchto buněk a zároveň přinesla nové poznatky o vlivu mikrobioty na vývoj Helios+ T regulačních buněk a schopnosti jejich selekce na potravních antigenech, což může otevřít nové cesty ve výzkumu a léčbě potravinových a jiných intolernací

Klíčová slova:

periferní tolerance, Treg, mikrobiota, střevo, brzlík, RORγt, Helios

Contents

Abbreviations

AIRE	Autoimmune regulator
APC(s)	Antigen presenting cell(s)
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
ATB	Antibiotics
BIM	Bcl-2-like protein
CAT	Cooperative antigen transfer
CD	Cluster of differentiation
cDC	Conventional dendritic cell
c-Kit	Tyrosine-protein kinase KIT
CMJ	Cortico-medullary junction
CNS	Conserved non-coding DNA sequence
cTEC(s)	Cortical TEC(s)
Ctrl	Control
DC(s)	Dendritic cell(s)
DN	Double negative
DP	Double positive
EOMES	Eomesodermin
FACS	Fluorescent Activated Cell Sorting
FoxN1	Forkhead box protein N1
FoxP3	Forkhead box protein P3
GFP	Green fluorescent protein
IL	Interleukin
ILC(s)	Innate lymphoid cell(s)
ILC1(s)	Type 1 innate lymphoid cell(s)
ILC2(s)	Type 2 innate lymphoid cell(s)
ILC3(s)	Type 3 innate lymphoid cell(s)
IMG	Institute of Molecular Genetics of the ASCR, v.v.i.
KO	Knock out
mDC(s)	Migratory dendritic cell(s)
MHC	Major histocompatibility complex
mTEC(s)	Medullary TEC(s)
OT-II	OVA-restricted CD4+ T cells
OVA	Ovalbumin

pDC(s)	Plasmacytoid dendritic cell(s)
pMHC(s)	MHC-peptide complex(es)
pTreg(s)	Peripheraly-derived Treg(s)
Sca1	Stem cells antigen-1
SP	Single positive
SPF	Specific-pathogen-free
TCR	T cell receptor
tDC(s)	Thymic-derived DC(s)
TEC(s)	Thymic epithelial cell(s)
TF	Transcription factor
Thymocytes	Developing T cells
TLR	Toll-like receptor
TNC(s)	Thymic nurse cell (s)
TRA(s)	Tissue-restricted antigen(s)
Treg(s)	T regulatory cell(s)
TSP	Thymic seeding progenitor
tTreg(s)	Thymic-derived Treg(s)
WT	Wild type

A. INTRODUCTION

The discrimination in the recognition between self and non-self entities forms an indispensable part of principal processes of life such as nutrition intake, self-defense as well as competition ability among species (Buchmann 2014). Jawed vertebrates are endowed with ~~dispose of~~ adaptive immunity which encompasses a diverse repertoire of T cells with a unique antigen specificity of its T cell receptor (TCR)(Ward and Rosenthal 2014). Given that TCR repertoires are generated via stochastic VDJ recombination, T cell repertoire includes clones reactive to non-self-antigens and unfortunately also to self-antigens. Thus, the elimination of self-reactive T cell clones in the specialized niche of the thymus is crucial to avoid the development of autoimmune diseases (Klein et al. 2014). The processes which dramatically limit the selfreactive repertoire of T cells in the thymus are collectively referred to as central tolerance.

In respect to the host, central tolerance covers the mechanisms of selfantigens production and presentation to developing T cells, the recognition of which results in either deletion of self-reactive T cells or their deviation into T regulatory cells (Treg). These processes are critically dependent on antigen-presenting cells (APCs) of the thymus whose major cell subsets are thymic epithelial cells (TECs) and dendritic cells (DCs). In case of depletion of these cells or loss of their self-antigen presentation capacity, the mice and humans develop various levels of autoimmunity which is caused by the escape of self-reactive T cells to the immune periphery (Malchow et al. 2016).

In respect to the surrounding, the main mission of immune system is here to protects us from environmental threats, chiefly pathogenic microbes. However, a vast majority of microorganisms which the host come into contact with are harmless, or even beneficial. Notably, our bodies are colonized by approximately 100 trillion microorganisms (bacteria, archaea, fungi and protozoans) just in the intestines. All together, it represents about 3 million structural genes encoded by gut microbiome, compared with approximately 23 000 genes of the host**.** Many of these microorganisms are commensal and mutualistic symbionts important for the effective acquisition of nutrients and essential amino acids or vitamins production (Valdes et al. 2018).

Since gut microbiota and food are inseparable and indispensable part of our life, self-tolerance to food antigens and gut microbiota with constantly changing diversity needs also to be established and enforced. In general, all mechanisms which impose immune tolerance outside of the thymus, are referred to as peripheral tolerance. In this respect, peripheral tolerance to gut commensal microbiota is mediated by mechanisms that parallel and complement central tolerance. Microbiota-specific T cells are silenced by deletion, anergy induction, or Tregs

conversion (ElTanbouly and Noelle 2021; Malchow et al. 2016). Similar to mechanisms of central tolerance, gut APCs, including DCs and innate lymphoid cells (ILCs), present microbiota-derived antigens to T lymphocytes, modulate the immune responses to the gut microbiota, and help to establish mucosal homeostasis (Hepworth et al. 2013). However, what remains still unclear, is the fate of microbiota-reactive T cells and what type of APCs in the gut are involved in the mechanisms controlling for their proliferation, deletion, or conversion to Tregs. Characterization of these processes is the main topic of my diploma thesis.

B. CURENT STATE OF KNOWLEDGE

1. CENTRAL TOLERANCE

Central tolerance is a system of interdependent and thymus localized mechanisms developed to eliminate self-reactive T cells (Klein et al. 2014). The thymus of mice and humans is a two-lobe organ situated in the chest cavity which consists of three main histological parts, with each playing an essential role in T cell maturation. Namely, it consists of the outer *cortex*, inner *medulla,* and *cortico-medullary junctions* (CMJ). CMJ is a highly vascularized tissue structure where cells can enter and leave the thymus. Cortex is a tissue where TCRs are tested to recognize the major histocompatibility complex molecules (MHC) during the positive selection of developing T cells. Finally, T cells are negatively selected or deviated into Tregs by the display of self-derived antigens in the thymic medulla (Rodewald 2008). From the evolutionary point of view, the mechanisms of central tolerance in jawed vertebrates developed simultaneously with the processes surrounding VDJ recombination (Hirano et al. 2011)

1.1. Cell composition of the thymus

Thymic cell populations could be divided into two main populations: (i) stromal cells lacking CD45 marker, and (ii) hematopoietic cells which consist of cells of lymphoid or myeloid origin. From the point of view of the cellularity, the absolute majority of the thymic cells are T cells present at various stages of their development. On the other hand, stromal cells, represented by Foxn1-expressing thymic epithelial cells (TECs) which are ontogenetically derived from the third pharyngeal pouch, form only a minor population. The crucial importance of TECs in T cells development is demonstrated by the deletion in human chromosome 22q11.2 that leads to Di George syndrome characterized by embryonal aberrances in pharyngeal pouches formation and thymus hypo/aplasia (McDonald-McGinn et al. 2015) and manifested by immunodeficiency caused by a significant reduction in T cells (Rota and Dhalla 2017).

Similarly, Foxn1 mutations in mice and humans result in the "nude" phenotype and Guarino-Pignata syndrome, respectively, and is usually manifested as an alopecia and/or thymic aplasia.

1.1.1. Developing of αβ T cells

In general, developing T cells in the thymus are called thymocytes. There are multiple developmental stages of thymocytes along with distinct selective pressures in both cortex and medulla which result in the formation of self-tolerant TCR repertoire of T cells which then can enter the immune periphery. T cell precursors originate in the bone marrow and enter the thymus via bloodstream in a stage called thymus seeding progenitors (TSP) (Lind et al. 2001) Upon entering the thymus, TSP first migrate to the cortex and become double negative (DN) thymocytes which do not express CD4 and CD8 co-receptors. There are four main developmental stages of double negative thymocytes, referred to as DN1-DN4. Once DN thymocytes successfully undergo VDJ recombination and start to express a complete TCR on their surface, they upregulate the expression of CD4 and CD8 co-receptor and become double positive (DP) T cells. They interact with specialized stromal cells, the cortical thymic epithelial cells (cTECs) which provide a survival signal allowing DP T cells to undergo the process known as positive selection (1.2.1). However, in most of cases, the TCR formation is unsuccessful, thymocytes do not acquire positive signals and they undergo apoptotic death by neglect. On the other hand, if their TCR is capable to engage peptide-MHC complexes (pMHC) presented by cTECs, they undergo positive selection during which they loose the expression of one of the co-receptors, either CD8 or CD4, according to their restriction to MHCI or MHCII, respectively, and become single positive thymocytes. Also, positive selection triggers the expression of chemokine receptors such as CCR7 which drives the migration of single positive thymocytes into the medulla region according to the gradient of CCL19 and CCL21 chemokines, which are highly expressed by medullary thymic epithelial cells (mTECs). In the medulla, TCRs are tested for their reactivity to self-antigens expressed on medullary thymic epithelial cells (mTECs). If TCR of a thymocyte recognizes presented selfantigens with a relatively high affinity, it is eliminated (negative selection, chapter 1.2.2) or converted into Treg, in the process of agonist Treg selection.

1.1.2. Thymic epithelial cells

TECs start to develop in the mouse embryo at embryonic day (E)10.5 of the gestation period. It was originally suggested, that the mutual approachment of the endoderm and ectoderm in the third pharyngeal lobe causes the invagination of the endoderm epithelia into the ectoderm layer,

creating a typical division of the thymus into the cortex and medulla, with cTECs) and mTECs, respectively (Rodewald et al. 2001). However, it seems that there is no predetermined germ layer origin of cTECs or mTECs and both types of TECs develop from a common bipotent progenitor from endodermal epithelial cells (Bleul et al. 2006).

Forkhead box protein N1 (Foxn1), transcription factor mentioned above, encoded by so called a "nude gene" is a specific marker for both cTECs and mTECs, and is necessary not only for their development but also for thymus regeneration and protection against its involution (Blackburn et al. 1996). Foxn1 is also irreplaceable in driving the expression of genes important for positive and negative selection of T cells, such as chemokines or components of antigen presentation machinery. There is one minor population of TECs that does not express Foxn1 and depends on another transcription factor: Pou2f3 which is morphologically and transcriptionally similar to tuft cells (chapter 1.1.1.1) present in mucosal tissues, such as gut or trachea (Kadouri et al. 2020).

1.1.2.1. mTECs

mTECs are important for the antigen presentation to developing thymocytes during negative selection and agonist selection of Tregs. They present not only ubiquitously-expressed self-antigens but also antigens which are in the immune periphery expressed by single or very limited number of tissues referred to as tissue restricted antigens (TRAs). Thousands of TRAs from most of the peripheral tissues are uniquely expressed by mTECs. Major driver of the TRA expression in mTECs is a protein named autoimmune regulator (AIRE). AIRE is not acting as a traditional DNA sequence-specific TF. It rather navigaters transcription machinery to the silenced parts of chromatin (Koh et al. 2008) where double-strand breaks are made to stimulate RNA polymerase activity (Giraud et al. 2012; Guha et al. 2017).

mTECs could be divided at least into four cell subsets (Bornstein et al. 2018). *Immature mTECs^{low}* (*mTEC I*) with low expression of MHC II and CD80 are presumably containing a population of mTEC progenitors. They also comprise the population of CCL21-expressing mature cells, which guide single positive thymocytes from the cortex into the medulla (Lkhagvasuren et al. 2013). *mTECs II*, historically also called *mTECs^{high}* with the high expression of MHC II and CD80, are effector mTEC population participating in the processes of central tolerance (Kadouri et al. 2020). Expression of AIRE is highest in mTECs II. AIRE can initiate the expression of approx. 4000 genes most of which are TRAs. Interestingly, just half of mTECs^{high} produces AIRE, and not every mTEC^{high} expresses all TRAs under AIRE control (Derbinski et al. 2005). Since Aire operates stochastically, each TRA is expressed just

by 0,4-3 % of mTECs^{hi} (Derbinski et al. 2008). AIRE deficiency in humans causes autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome with loss of central tolerance and manifested as multiorgan autoimmunity (Kahaly and Frommer 2019; Kisand and Peterson 2015). Aire knockout (KO) mice also develop multiorgan autoimmunity which mimics the syndromes of APECED (Anderson et al. 2002; Chang et al. 2005).The third mTEC subset referred to as *Post-AIRE mTECs,* also referred to as *mTECs III.* Since these cells are formed by terminally differentiated corneocyte-like cells which are absent in AIRE KO mice (Metzger et al. 2013), they develop from Aire-expressing mTECs. Similar to skin-derived corneocytes, mTECs III upregulate involucrin, keratin 10 , desmoglein, or serin protease SPINK5 (X. Wang et al. 2012) and loose their nuclei (Kadouri et al. 2020). These terminally differentiated mTECs are localized in thymic Hassal´s corpuscules (J. Wang et al. 2019; Yano et al. 2008). It is not clear if Hassal´s corpuscules play any role in central tolerance in mice. But, in the human thymus, thymic stromal lymphopoietin (TSLP) produced in Hassal´s corpuscles was shown to contribute to the conversion of self-reactive thymocytes into Tregs (Watanabe et al. 2005). As mentioned previously, thymic tuft cells represent so far quite enigmatic subset of TECs. Similar to intestinal tuft cells, their thymic counterparts develop under the control of POU2F3 TF and they also express genes important for signaling pathways in taste reception. It is suggested that approx. 50% of thymic tuft cells originates from mTECs II (Bornstein et al. 2018; Jenkinson, Jenkinson, and Anderson 2011). The function of thymic tuft cells is unclear. Their importance for processes of central tolerance is discounted by their lower expression of MHC II compared with that of mTECs^{high} (Cowan et al. 2013). Intestinal tuft cells are presumed to be important in the upregulation of Th2 immune responses by the production of IL-25 which supports the development of type 2 innate lymphoid cells (ILC2) (Bornstein et al. 2018). Depletion of tuft cells in the thymus is marked by the reduction of type 2 natural killer T lymphocytes (NKT2) and CD8^{+} EOMES^{+} T lymphocytes critical for thymic Tregs development (Lucas et al. 2020). Recently published work showing the presence of small subsets of mTECs with distinct expression profile of specialized cells from the periphery may shed entirely new light on the evolution of the processes associated with central tolerance (Michelson et al. 2022). Since the expression of antigens by mTECs mimics the environment that T cells encounter in the periphery, it is possible that initially, various types of epithelial cells which formed the thymus presented a saving net mechanism to prevent autoimmunity against the host' related tissues.

1.1.3. Dendritic cells

Dendritic cells in the thymus play indispensable role in the processes of negative and Treg selection of developing thymocytes (Perry et al. 2014). Because of the very low number of mTECs compared to thymocytes, the cooperation between mTECs and DCs in the processes of central tolerance is vital. Importantly, DCs present not only ubiquitous and endogenous antigens, but are also capable to acquire TRAs from mTECs in a process called cooperative antigen transfer (CAT) (Perry et al. 2014; Vobořil et al. 2022). CAT represents a mechanism by which TRA are taken up by DCs, presumably by trogocytosis from living mTECs or by phagocytosis from apoptotic mTEC (Koble and Kyewski 2009; Perry et al. 2014). Subsequently, these antigens are presented by DCs. There are several phenotypically distinct populations of DCs in the thymus, but they can simply be divided into two main groups: plasmacytoid DCs (pDCs) and classical DCs (cDCs). cDCs can be further divided into cDC1 and cDC2 (Vobořil et al. 2022). During experiments with Aire-GFP mice, the uptake of GFP antigens by CAT was observed preferentially by the cDC1 group. On the other hand, cDC2s dominated in the acquisition of OVA antigen in the RIP-mOVA mouse model (Lancaster et al. 2019), where OVA is expressed mostly by mTEC I subset. Based on this data, it was assumed that DC subsets show distinct preferences for mTEC subsets regarding CAT. Indeed, it was established by our research group that cDC1 and activated forms of cDC1 and cDC2 preferentially mediate CAT in cooperation with those mTECs which express high levels of TRAs, implicating these DC subsets to the establishment of tolerance to TRAs.

Another subset of thymic DCs that was recognized recently are monocyte-derived DCs (moDCs). It was found that activation of mTECs via their Toll-like receptor 9 results in increased cellularity of moDCs in the thymus and enhanced agonist selection of Tregs (Vobořil et al. 2020). moDCs are very important for CAT. Notably, while they do not display preference for any TEC subsets, they excel in the repetitive acquisition of antigens from two or more mTECs and are the most potent in the acquisiton of antigens from other thymic DCs (Vobořil et al. 2022).

Interestingly, plasmacytoid DCs (pDCs), cDC2 and moDCs were shown to acquire antigens in the immune periphery, migrate into the thymus and present these peripheral antigens to developing T cells (Bonasio et al. 2006; Husein et al. 2013; Vollmann et al. 2021; Zegarra-Ruiz et al. 2021). This led to the assumption that the food and microbiota antigens are tolerized in the thymus. Indeed, moDC were recently found to present and tolerize to microbial (Zegarra-Ruiz et al. 2021) as well as blood-borne antigens in the thymus (Vollmann et al. 2021). Thus, thymic central tolerance imposes tolerance not only to selfantigens but also to microbial non-

self antigens to maintain immune homeostasis. Currently, a single-cell RNA sequencing revealed previously unappreciated heterogeneity of DCs populations in mice and humans (Park et al. 2021; Vobořil et al. 2020), since it can classified distinct activation/maturation states of cDCs as separate clusters. These analyses showed that in prder to tolerize T cells, cDCs are fully equipped with the checkpoint molecules such as PD-L1, OX40 or CD40 (Park et al. 2021). Importantly, several studies showed that activated cDCs are the most potent in CAT, however, their exact potential to tolerize autoreactive T cell remains to be determined (Ardouin et al. 2016; Vobořil et al. 2022).

1.2. Thymic T cells development

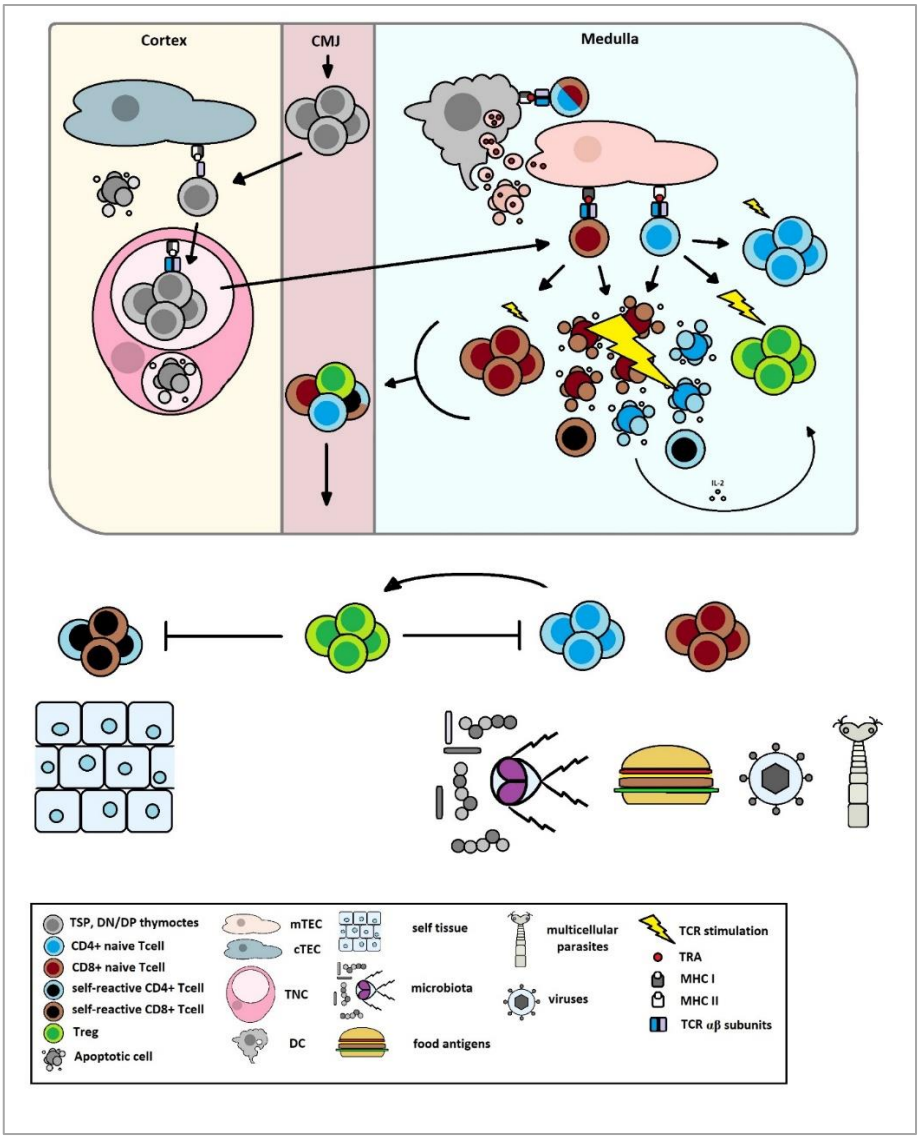
1.2.1. Positive selection.

Positive selection is a process taking place in the thymic cortex which leads to survival of those thymocytes that are capable to recognize pMHC molecules. In the beginning of T cell development, TSPs enter the thymus in CMJ by adhesion to P-selectin on capillar endotel (Zlotoff et al. 2010). $CD44^+$, c-KIT^+, $CD25^-$ DN1 thymocytes also express CXCR4 and CCR9 which mediate their chemoattraction to CXCL12 and CCL25 gradient made by cTECs and promotes their homing in the thymic cortex (Zlotoff et al. 2010). Development of thymocytes is then guided by Notch signaling. Notch ligands especially Notch ligand δ-like ligand 4 (DLL4) promotes thymocytes development to the DN3 stage at which point they start to express signature genes such as TCR and CD25, and gradually suppress their potential to develop into the natural killer (NK) cell, B lymphocyte, or myeloid cells (Koch et al. 2008). After $CD44^+$c-KIT^+CD25^+ DN2 stage, $CD44^-$c-KIT^-CD25^+ DN3 thymocytes undergo VDJ recombination. DN3 thymocytes start with recombination of the TCRβ chain, the first step of a unique αβTCR creation. TCRβ chain is displayed on the surface with the pre-TCRα chain (pTCRα) without variable domains. cTECs are APCs with the unusual self-antigen processing. The presence of β5t proteasome subunit, cathepsin L1, and TSSP proteases are under the transcription control of *Foxn1*, TEC-specific TF mentioned before (Calderón and Boehm 2012). The recognition of peptide pMHC complex by pre-TCR DN3 thymocytes enables a successful β chain recombination and development to DP thymocyte. If this process is unsuccessful, the particular thymocyte undergoes apoptosis. DP thymocytes with CD4, CD8, and pre-TCR initiate VDJ recombination of the TCRα chain. Their development is further mediated by a close interaction with the population of cTECs called thymic nurse cells (TNCs), whicg are capable to enclose up to 200 DP thymocytes. In these clusters, the TCRα chain is rearranged and associated with the TCRβ chain to form αβTCR. DP thymocytes are again positively selected for recognition

of MHC (Guyden and Pezzano 2002). TNCs express both MHCI and MHCII and display self-derived antigens. The MHCI binds peptides produced by the thymoproteasome with unique β5t subunit, while the MHCII engages peptides processed by TSSP and cathepsin L1 which are loaded into MHCII pathway via autophagy (Takahama et al. 2012). A thymocyte which is capable to recognize MHCI or MHCII upregulates CD69 and is released from TNC to become $CD4^+$ or $CD8^+$ SP thymocyte, depending on whether it recognizes MHCI or MHCII, respectively, during the positive selection (Siggs et al. 2014). DP thymocytes which fail to undergo positive selection dye and are scavenged by TNC. cTECs and TNCs are able, after mutual stimulation by the CD83 molecule, to upregulate the expression of MHCII and thus favor the development of $CD4^+$ lymphocytes over $CD8^+$ T cells (von Rohrscheidt et al. 2016).

1.2.2. Negative selection of T cells and their deviation into Tregs

Positively selected SP thymocytes are attracted to the medulla by their upregulation of CCR7 and mTEC-produced chemokines CCL19 and CCL21 (Lkhagvasuren et al. 2013). Chemoattraction of developing T cells into medulla is further enhanced by activated forms of classical DCs due to their high production of CCL17 and CCL22 which bind CCR4 on SP $CD4^+$ T cells (Hu et al. 2015; Park et al. 2021). In the medulla, repertoire of developing T cells is subjected to negative/Treg selection to reduce their autoreactive potential. In addition to mTECs, negative selection is effectively mediated also by DCs and other thymic APCs such as B lymphocytes and macrophages (Klein et al. 2014). Macrophages play also important role in scavenging apoptotic T cells (Kurd et al. 2019). Significant proportion of negative selection takes place also in the cortex, where DCs delete SP thymocytes even before their entry into the medulla (McCaughtry et al. 2008). In the cortex, the affinity of developing T cell to MHC with self-derived peptides may imprint for quicker activation ability to this T cells. These T cells could be defined by CD5 expression which positively correlates with strong TCR stimulation (Klein et al. 2014). While differences in the reactivity were not observed during pathogenic immune responses, $CD5^{high}$ cells are more efficient in IL2 production and their rapid activation could be caused by higher TCR tonic stimulation trough „little bit“ self-reactive TCR (Persaud et al. 2014). In the medulla, it was suggested, that number of mTECs using direct antigen presentation is insufficient to promote successful and effective negative selection (Lancaster et al. 2019). In this context, CAT with indirect presentation of TRAs on thymic DCs is of critical importance. Peripheral endogenous as well as exogenous antigens are distributed into the thymus by mDCs and pDCs. It is of note that pDCs and cDC2 which can carry intestine and



Scheme 1. Thymic development of T cells: *TSPs enter the thymus through the CMJ and are guided to the cortex where they undergo VDJ recombination and assembly of TCR αβ subunits. TCR functionality is tested by MHC expression on cTECs and TNCs. If the TCR is non-functional, the thymocyte dies in the process of positive selection. CD4/CD8 SP thymocytes migrate to the medulla, where they undergo negative selection against self-antigens. Self-antigens are presented by mTECs and DCs. TRAs under the control of AIRE are also presented by DCs via the mechanisms of CAT from TECs. The strength of TCR stimulation leads to either deletion with strong affinity for self-antigens or survival with low or no TCR affinity for self-antigens. Thymocytes that recognize the self-antigen not too strongly are converted to Tregs, the development of which is supported by the production of IL-2 by self-reactive T cells. Naïve CD4/CD8 SP T cells specific mainly for exogenous antigens such as microbial, parasitic or food antigens leave the thymus along with self-reactive T cells and Tregs. As part of their specificity, naïve T cells can be peripherally converted to Tregs promoting tolerance to exogenous antigens.*

microbiota-derived antigens express the CCR9 receptor which can navigate them into the thymus (Pathak and Lal 2020).. High-affinity recognition of pMHC during negative selection of thymocytes leads to Ca^{2+} influx, protein kinase C (PKC) activation, and upregulation of proapoptotic Bcl-2-like protein11 (BIM) ultimately resulting in the apoptosis of negatively selected thymocytes (Canté-Barrett et al. 2006). Part of self-reactive thymocytes with intermediate affinity is converted into the Treg population. This mechanism is discussed in chapter 1.3.1. There are other parameters which affect the selection of $CD4^+$ and $CD8^+$ SP thymocytes. For example, the average speed of thymocytes in the medulla is approx. 10 µm/min. $CD8^+$ T cells are moving slower than $CD4^+$ T cells. This mechanism probably evolved for a more effective deletion of $CD8^+$ T cells due to their cytotoxic activity and hence their potentially higher risk of damage to the host' tissues (Le Borgne et al. 2009). In general, selection-escaping autoreactive $CD4^+$ T cells could not be that much dangerous because they need a complex costimulation for their activation (2.1.).

1.3. T regulatory cells

Tregs are an indispensable cell type in the regulation of immune reactions. This lymphocyte population originaly described as $CD4^+CD25^+$ cells are marked by the expression of the transcription factor forkhead box P3 (Foxp3), the mutation of which results in phenotype of mice so called „scurfy" (Ramsdell and Ziegler 2014; Schubert et al. 2001). Scurfy mice were the first known mouse model with lethal X chromosome-linked disease. The phenotype of these mice showed scaly epidermis, skin lesions, eye reddening, splenomegaly, and premature death around the third week of life (By and Gower 1969). More recently, the connection between autoimmunity and T lymphocytes was shown in this mouse strain (Van Den Berg et al. 2001; Ferber et al. 2016). The same condition in humans causes the manifestation of immune dysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome. Equally as in scurfy mice, affected male patients without bone marrow transplantation or intensive immunosuppression die within the first two years of life due to the severe multiorgan autoimmunity (Tan, Louie, and Sleasman 1993). Tregs are T lymphocyte population with self-reactive TCRs which engages mTECs with intermediate to high affinity and receive pro-survival signals that lead to the upregulation of Foxp3 expression which maintains their immunosupressive phenotype. In addition to these thymus-generated Tregs (tTregs) they could be also derived from conventional T cells as peripheraly induced Tregs (pTreg), discussed in chapter (2.2.).

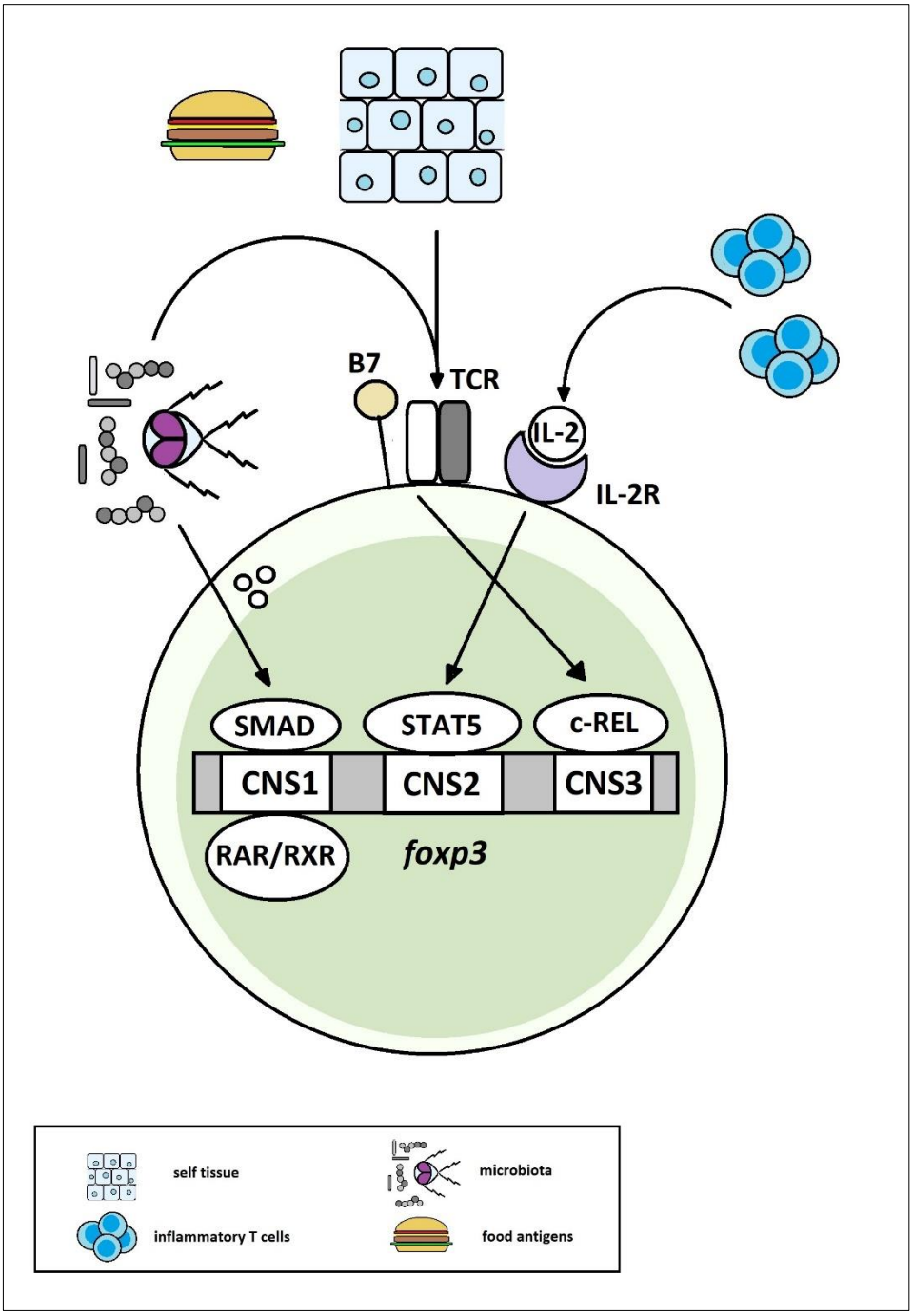
1.3.1. FoxP3 activation of expression

Expression of FoxP3 is regulated by 3 enhancers called conserved non-coding sequences (CNS1, CNS2, CNS3) which constitute a part of *FoxP3* intronic sequences (Tanoue, Atarashi, and Honda 2016). Every CNS binds different TFs under different stimulatory conditions and their deletion displays abnormality of Treg development both in the thymus and periphery. CNS1 is important for Tregs development on the periphery more than in the thymus as proved by its deletion showing a decreased population of pTregs, but no alterations in tTregs pool. CNS1 is under the control of SMAD and TGF-β signaling during inflammatory conditions (Ogawa et al. 2014). The importance of the TGF-β-SMAD pathway in regard to Tregs development is not clearly explained and will be discussed in the next chapter. CNS1 contains also binding sites for retinoic acid (RAR) and retinoid X receptor (RXR) as exogenous signal receptors which imply the importance microbiota for Tregs induction (Xu et al. 2010). CNS2 seems to be the most important for Tregs development and survival. It is regulated by IL2 cytokine important for all Tregs and conventional T lymphocyte proliferation. Demethylation of CNS2 by STAT5 activation as result of IL2-IL2R signaling allows binding of RUNX1 TF which results in the stable FoxP3 expression (Feng et al. 2014). CNS3 deficiency is manifested by the reduction of tTregs, with pTregs unaffected. Enhancing activity of FoxP3 gene is controled by c-Rel TF which can bind to CNS3 and upon TCRCD28 stimulation signals the recognition of pMHCII and costimulatory molecules on thymic APCs by Treg precursors during negative selection (Ye Zheng et al. 2010).

1.3.2. Thymic Tregs developement

Thymic-derived Tregs (tTregs) originate in the thymic medulla during the interactions of self-reactive T cells with pMHCs on mTECs. It was suggested, that tTregs are well marked by Helios TF from Ikaros TFs family (Thornton et al. 2010) but later observations showed, that there are also peripherally induced Tregs (pTregs) which form the Helios+ population (Pratama et al. 2020; Thornton et al. 2010)

In the thymus, Tregs are selected by the presentation of self-antigens in pMHC complexes. During their thymic generation, presence of AIRE in mTECs is crucial. Deletion of AIRE affects tTreg development likely by the absence of TRAs (Malchow et al. 2016; Perry et al. 2014). AIRE deficient mice similarly to APECED human patients show autoimmunity manifestation (Chapter 1.2.2.). The transfer of Tregs from wild-type mice to AIRE mice can cure autoimmunity (S. Yang et al. 2015). Similarly, it was shown using MHCII tetramers, that



Scheme 2. **FoxP3 activation of expression:** *The induction of Tregs is a very important step in the development of immune responses. CNS activation within the gene intronic sequences is important for FoxP3 activation and maintenance of its expression. CNS1 binds SMAD, RARs, and RXRs upon stimulation with microbial metabolites and is essential for the induction of pTregs. Demethylation of CNS2 is necessary for any induction of Tregs, and thus the stimulation with IL-2 and activation of STAT5 is necessary for the induction of Tregs both in the thymus and periphery. The development of Tregs is also under the control of antigen recognition and the presence of costimulatory molecules leading to the binding of TF c-Rel to CNS3. In the case of tTregs it is mainly the antigenic specificity to self-tissues and in case of pTregs, the microbiota and food antigens.*

the presentation of common and widespread tissue antigens leads to negative selection of reactive SP thymocytes contrasting with preservation of thymocytes recognizing rare TRAs and their conversion to FoxP3 expressing Tregs (Legoux et al. 2015). Together these observations suggest that tTregs are preferentially selected for rare TRAs under AIRE-controlled ectopic expression. There are differences between tTreg populations developed during lifespan. It was shown, that neonatal thymectomy before day 3 in mouse pups does not manifest in dec reasing Tregs, but in the development of multiorgan autoimmunity (Samy et al. 2008). These perinatal and neonatal Tregs constitute a different Treg population compared with adulthood-generated Tregs by much higher expressions of Treg effector genes as *Icos* or *St2* crucial for the regulation of immune reactions and more stable FoxP3 expression (S. Yang et al. 2015). Perinatal and neonatal Tregs exhibit TCR specificity to age and inflammation-dependent antigens, such as Padi4 which is connected with rheumatoid arthritis and promotes Treg conversion predominantly in the neonatal thymus in contrasts with a deletion in the adult thymus (Stadinski et al. 2019). Along with this, it was observed that the reduction in cDCs1 population in the perinatal thymus correlates with decreased Treg generation, suggesting importance of resident DCs for generation of this particular Treg population (S. Yang et al. 2015).

In the adult thymus, there are two distinct precursors of Tregs (TregsP) FoxP3^{low}TregsP and CD25^{+}TregsP which differ in FoxP3 expression induction, cytokine microenvironment dependence, TCR specificity and effectory function. CD25^{+} TregsP specific to myelin oligodendrocyte glycoprotein (MOG) tetrameres, increase their cellularity after blocking the apoptosis pathways. IL2 presence is crucial for Treg survival and maturation, but interestingly *CNS3* KO mouse does not show aberances in CD25^{+}TregsP population developement, which is in the contrast to FoxP3^{low} population that is unable to develop into mature Tregs in *CNS3* KO. Also, experiments with *Nfκb* KO showed importance of the activation of the NF kappa B signaling pathway during FoxP3^{low} TregsP selection. Unlike CD25^{+} TregsP, FoxP3^{low} TregsP are depend on IL4 and the reduction in their numbers was observed in *Pou2f3* KO mice which lacks thymic tuft cells that are important for NKT generation. These innate lymphoid cells produce IL4 as will be discussed in chapter 1.3.1. In case of Foxp3^{low}TregsP, their function in establishment of tolerance remains unclear. This is in marked contrast to the MOG-specific CD25^{+} TregsP which after their maturation show infiltration into sites of inflammation during experimental encephalomyelitits suggesting their importance in tissue tolerance homeostasis (Owen et al. 2019).

2. PERIPHERAL TOLERANCE

Although the complexity of selection processes in the thymus may seem as sufficient for maintaining immune tolerance, it has been shown that up to 25-40% lymphocytes that exit the thymus are self-reactive ones, capable to induce autoimmunity. Despite all this, no autoimmune phenotype was observed in WT mice (Bouneaud, Kourilsky, and Bousso 2000). It has been reported, that some mechanisms balance the ratios between the number of autoreactive lymphocyte and Tregs generated in the thymic medulla, by a virtue of utilizing IL-2 produced by stimulated autoreactive Tcells as one of the major FoxP3 activating factors (Hemmers et al. 2019). In addition to mechanisms selecting T cells based on their recognition of self-antigens, shaping of the population of T cells specific to exogenous, especialy microbial antigens, very likely occurrs already in the thymus (Zegarra-Ruiz et al. 2021). Despite this fact, both self-reactive and exogenous antigens specific T cells such as those recognizing a commensal microbiota or food antigens exit the thymus and seed the periphery. The mechanisms imposing the establishment of tolerance to these antigens are described in the following chapters

2.1. Mechanisms of T cell silencing

2.1.1. Quiescence

After leaving the thymus, naive T cells are exposed to several checkpoints which shape their future ability to perform effector functions or which lead to their death. Quiescence, the initial type of silencing mechanism as the protection against the expansion of self-reactive clones is mediated by locking of T-lymphocytes in the G0 phase of their cell cycle together with a low metabolic and translation activity and inhibition of IL-2 expression. It is mediated by the engagement of TGFβR and VISTA with their ligands TGFβ and TOB1 (ElTanbouly et al. 2020). Such engagement activates SMAD signaling pathway, leading to the activation of TF RUNX1 which is essential for the production of the quiescence-related TFs FOXO1, FOXP1, and KLF2 (Haaland, Yu, and Rice 2005). The importance of these TFs for quiescence is marked by the experiments with conditional deletion of *Foxo1* in T-lymphocytes which manifested in the form of severe pancreatitis and colitis (Wong et al. 2012), suggesting not just the disruption of tolerance to self-tissues, but also a failure to tolerate the microbiota.

2.1.2. Anergy

Anergy is yet another possibility of non-deletional tolerance, which reversibly silence T-lymphocytes, which is dependent on the signal(s) generated by TCR signaling. As opposed to the quiescence mechanism, anergy leads to a locked cell cycle during cell proliferation in the

G1 phase or during the transition from G1 to S phase (Li et al. 2006). T cell becomes anergic if it recognizes pMHC without receiving a costimulatory signal. Consequently, NFAT is induced without MAPK signaling which is extremely important for the nuclear translocation of FOS and JUN dimeric transcription factor AP-1. NFAT1 alone activates the TF ERG2 leading to the downregulation of IL-2 expression and to the suppression of autocrine positive feedback and proliferation. Furthermore, CBLB E3 ubiquitin ligase is activated (Yan Zheng et al. 2012). The deficiency of CBLB results in the development of type 1 diabetes (Hoyne et al. 2011), which demonstrates the importance of this enzyme and overall anergy in the establishment of peripheral tolerance. Induction of anergy may also be one of the escape strategies of bacteria and viruses, which can use superantigens to bridge MHC molecules with TCR and thus cause its excessive activation leading to the silencing events described above (Kurts et al. 1997).

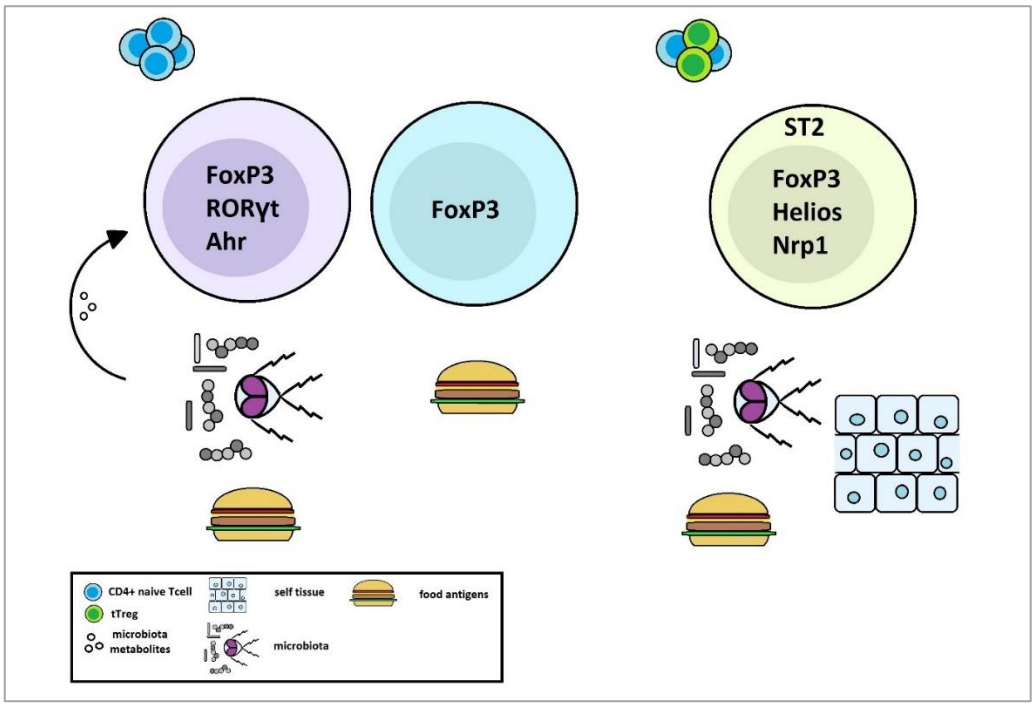
2.1.3. Deletional tolerance

Programmed cell death is a regulatory mechanism that T-lymphocytes encounter both in the thymus and immune periphery. In the periphery, T-lymphocytes upregulate the FAS death receptor in the terminal phase of immune responses and by the engagement of FAS ligand, unleash the caspases 8 and 3 cascade which triggers their apoptosis. This activation-induced cell death is very important for the reduction of amplified lymphocyte effector clones and the suppression of inflammatory reactions (Suzuki and Fink 2000). In the process of delaying the apoptosis of activated T-lymphocytes, the costimulation, and specifically the CD28 signaling plays a very important role, since it supports the upregulation of anti-apoptotic factors such as BCL-XL (Boise et al. 1995). The balance between the pro-apoptotic and anti-apoptotic factors also appears to be a major player in decision making process between the survival or death outcomes when T cells undergo the process of quiescence and anergy. Lymphocytes subjected to the quiescence and anergy mechanism show similar transcriptional profiles that are observable in cells entering apoptosis, such as the upregulation of Bim, or genes from the Nr4a family that negatively regulate the survival of T cells (Parish et al. 2009). Mice having a specific deletion of pro-apoptotic factor BIM in T-lymphocytes have been shown to have comparable levels of anergic T-lymphocytes to WT mice but exhibited a block in deletional mechanism. This suggests that naive T-lymphocytes are more sensitive to the signals that stimulate their transition to apoptosis and so quiescent T-lymphocytes are more prone to clonal deletion by the presentation of peripheral antigens which is by its mechanisms and induction very similar to the deletion of thymocytes observable in the thymic medulla (Chapter 1.2.2) as will be further discussed below (Hepworth 2013 a 2015).

2.2. Peripherally induced Tregs

As described above, under the appropriate influence of factors such as the production of the cytokines TGF-β and IL-2, TCR signaling, and costimulation, Tregs are induced from self-reactive T cells during agonist selection in the thymus (Klein et.al. 2019 Nature reviews Immunology). Alternatively, T cells restricted to self-antigens or exogenous antigens, including antigens derived from intestinal microbiota and food can be induced in the immune periphery. This chapter will focus on Tregs that are generated to establish tolerance in the intestinal system. In the gut, we can distinguish three major populations of Tregs, two of which are easily distinguishable by their transcription programs. The first group which was found relatively recently is represented by RORγt$^+$ Tregs occurring mainly in the lamina propria of the large intestine, where they form about 30-80% of all Tregs (B. H. Yang et al. 2016). Their development is strictly tied to the intestinal microbiota the complexity of which positively affects the formation of this population by increasing its frequencies (Al Nabhani et al. 2019; B. H. Yang et al. 2016). RORγt$^+$ Tregs are thus able to respond to specific species of microorganisms that support their development, such as *Clostridium ramosum* or *Bacillus thetaiotaomicron*, as shown by monocolonization of GF mice (Sefik et al. 2015). RORγt$^+$ Tregs arise predominantly after the weaning period (Pratama et al. 2020) from the naive CD4$^+$ T cells and to a little extent from tTregs, which respond to the Th17 polarizing signals by the IL-6, IL-21, IL-26 cytokines. These Tregs share RORγt TF and the presence of TGF-β appears to be the main criterion determining the activation of FoxP3 and conversion to Treg phenotype (Zhou et al. 2008). Although there is a stable Treg population in the gut, a small portion of these Tregs loose Foxp3 expression and switch to Th17 polarized T cells. Interestingly, the vast majority of RORγt$^+$ Tregs remain outside of the inflammatory environment (B. H. Yang et al. 2016). Another important population of Tregs in the gut and mLN are Helios$^+$ Tregs. In the case of these Tregs, it is a problem to distinguish their origin, as the Helios TF is a marker of thymic-derived Tregs but it is also present in some peripherally generated Tregs (Thornton et al. 2010). In part, Helios$^+$ Tregs are important population of Tregs in the gut due to their signature expression of ST2 which engages pro-inflammatory alarmin IL-33. Therefore, as shown by several independent studies, ST2$^+$ Tregs significantly contribute to the resolution of inflammation. Also, under inflammatory conditions in the intestine, this population expands considerably (Nascimento et al. 2017; Schiering et al. 2014). At the same time, Helios$^+$ Tregs express ST2-associated TF GATA3, indicating their Th2 polarization and thus healing functions after immunological and mechanical damage to mucosal and epithelial tissues. The association of Helios$^+$ Tregs population with the expression of ST2 predict that their

proliferation is physiologically more relevant and thus, functionally bound to the presence of IL-33 than antigen specificity (He et al. 2017). However, the peripheral induction of pTregs with Helios$^+$ phenotype is still dependent on TCR signaling. Specifically, the Helios$^+$ pTregs are dependent on intestinal epithelial cells (IECs), whose damage-promoted IL-33 production leads to a expansion of Helios$^+$ Tregs, the upregulation of MHCII on IECs through IL-33 production which depends on the degree of their stimulation via the aryl hydrocarbon receptor (Ahr) which recognizes the polyaromatic microbial metabolites with xenobiotic or host organism origin. By this mechanism, Helios$^+$ pTregs are induced directly in the intestinal mucosa (Yoshimatsu et al. 2022).



Scheme 3. Peripheraly induced Tregs: *RORγt$^+$ Tregs and unspecified pTregs originate from naive CD4$^+$ T cells. RORγt$^+$ Tregs are dependent on the presence of the microbiota and do not develop in its absence. Their specificity is mainly to microbiota and food antigens, as in the case of short-lived Tregs. Helios$^+$ Tregs may originate as already matured Tregs from the thymus or may be peripherally induced. Thus, this population can be specific to both self-antigens and exogenous antigens like microbiota and food antigens.*

The third significant population of intestinal pTregs which resides primarily in the small intestine but also in the large intestine is represented by Helios$^-$RORγt$^-$ double-negative, short-lived population of pTregs which dependent on antigens from complex, solid food. This population does not form in the case of antigen free diet applied to GF mice. Their short lifespan

and dependence on the availability of antigens predestine these pTregs to create a very flexible type of tolerance to variable food supplies (K. S. Kim et al. 2016).

2.3. Innate lymphoid cells

As their name suggests, innate lymphoid cells belong to the group of innate immune cells, yet they are of the lymphoid origin. In general, they serve as rapid polarizers of immune responses in both unperturbed and inflamed tissues due to their robust expression of cytokines. They also share various effector mechanisms and lineage-committed TFs with T cells. Accordingly, they are also traditionally divided into three groups (Vivier et al. 2018).

Type 1 innate lymphoid cells (ILC1) and NK cells share TF T-bet with Th1 polarized T-lymphocytes, all playing an irreplaceable role during the removal of viral infections or cancer.

Type 2 innate lymphoid cells (ILC2) expressing GATA3 similarly to Th2 polarized T-lymphocytes are important in defense against many multicellular parasites as well as providing aid in healing processes of damaged tissues.

RORγt transcription factor, which is typical for Th17 T-lymphocyte polarization is present in mutually unrelated type 3 innate lymphoid cells (ILC3) and lymphoid tissue inducers (LTi) (Vivier et al. 2018).

2.3.1. Type 3 innate lymphoid cells

ILC3 population, apart from the LTi subset whose distinction from other ILC3s will be discussed later, forms two fundamental subpopulations which can be distinguished by their expression of natural cytotoxicity triggering receptor 1 (NCR1), also known as NKp46 in mice and NKp44 in human (Vivier et al. 2018).

ILC3 doesn´t seem to be a stable ILC population. There is a transition between ILC1 and ILC3 which both share TF T-bet and only ILC3 initiates RORγt expression. If RORγt is downregulated, ILC3 are reciprocally converted back into ILC1. These rules are especially valid for NKp46^{+} ILC3 which share NKp46 expression with ILC1 (Klose et al. 2013). Under a specific cytokine environment (IL-2, IL-12), the conversion of ILC1 to NKp46^{-} ILC3 was observed *in vitro* (Bernink et al. 2015).

Except their function in rapid responses in the intestine and surrounding tissues by virtue of IL-17 and IL-22 cytokines production (Takatori et al. 2009), there is the question of their capacity to induce T lymphocytes proliferation. This takes into account the fact that ILC3 cells display the MHCII on their surface, but not strictly in all tissues. In mice, NKp46^{-} ILC3 are unable to

provide any T lymphocyte proliferation in small intestine lamina propria due to their MHCII downregulation under microbiota-induced IL-23 secretion (Lehmann et al. 2020). In the spleen, the expression of MHCII on NKp46$^-$ ILC3 is preserved along with the expression of costimulatory molecules such as CD80, CD86, and CD74. The expression of costimulatory molecules is reduced also in the small intestine lamina propria. This data suggest, that NKp46$^-$ ILC3 are potent APCs capable to support T lymphocyte proliferation but not in the intestines (Lehmann et al. 2020).

2.3.2. Lymphoid tissue inducers – a separate group of innate lymphoid cells

LTi play a crucial role in inducing the development of secondary lymphoid organs. The expression of TF RORγt by LTi is necessary for this event. If this TF is absent, LTi remain present, but secondary lymphoid organs such as lymph nodes and Peyer´s patches are not formed in Rorc KO mouse (Eberl et al. 2004). Unlike ILC3, the development of LTi is not dependent on the promyelocytic leukemia zinc finger TF (Plzf). If the gene for Plzf: *Zbtb16* is deleted, LTi are present, but not other innate lymphoid cell subsets. These observations contradict the existence of a common ILC progenitor and argue that LTi are not developmentally linked to ILC3 as previously proposed based on the RORγt expression in both cell types (Constantinides et al. 2014).

2.3.2.1. Origin, development and function of the lymphoid tissue inducers.

In general, there are two types of LTi with distinct functions which are divided according to their origin. Embryonicaly derived LTi in fetuses and newborns and LTi-like cells in adults of hematopoietic origin. The latter are unable to induce the secondary lymphoid tissues (M. Y. Kim et al. 2009). *Kim et.al. 2009* showed that adult LTi-like cells may be mistaken for ILC3. Embryonic hematopoietic cells capable to give rise to LTi are detectable using Cxcr4-cre reporter mouse model in which LTi derived from the hemogenic endothelial (HE) tissue can be distinguished from the cells of yolk sack origin (Simic et al. 2020). Subsequently, the fetal liver is colonized, when the maximum amount of hematopoietic precursors appears at E11.5 (Hoeffel et al. 2015). LTi precursors of HE origin were identified in the fetal liver along with other ILC precursors by the expression of integrin α4β7$^+$. However, LTi precursors also express RORγt TF (Possot et al. 2011). So far, the origin of LTi precursors derived from bone marrow in adults (LTi like cells) is unknown (Simic et al. 2020). LTi progenitors in the fetal liver need stimulation by the retinoic acid of maternal origin to induce RORγt expression, without which their conversion to LTi0 and afterwards into CD4$^+$ LTi4, is not possible (van de Pavert et al.

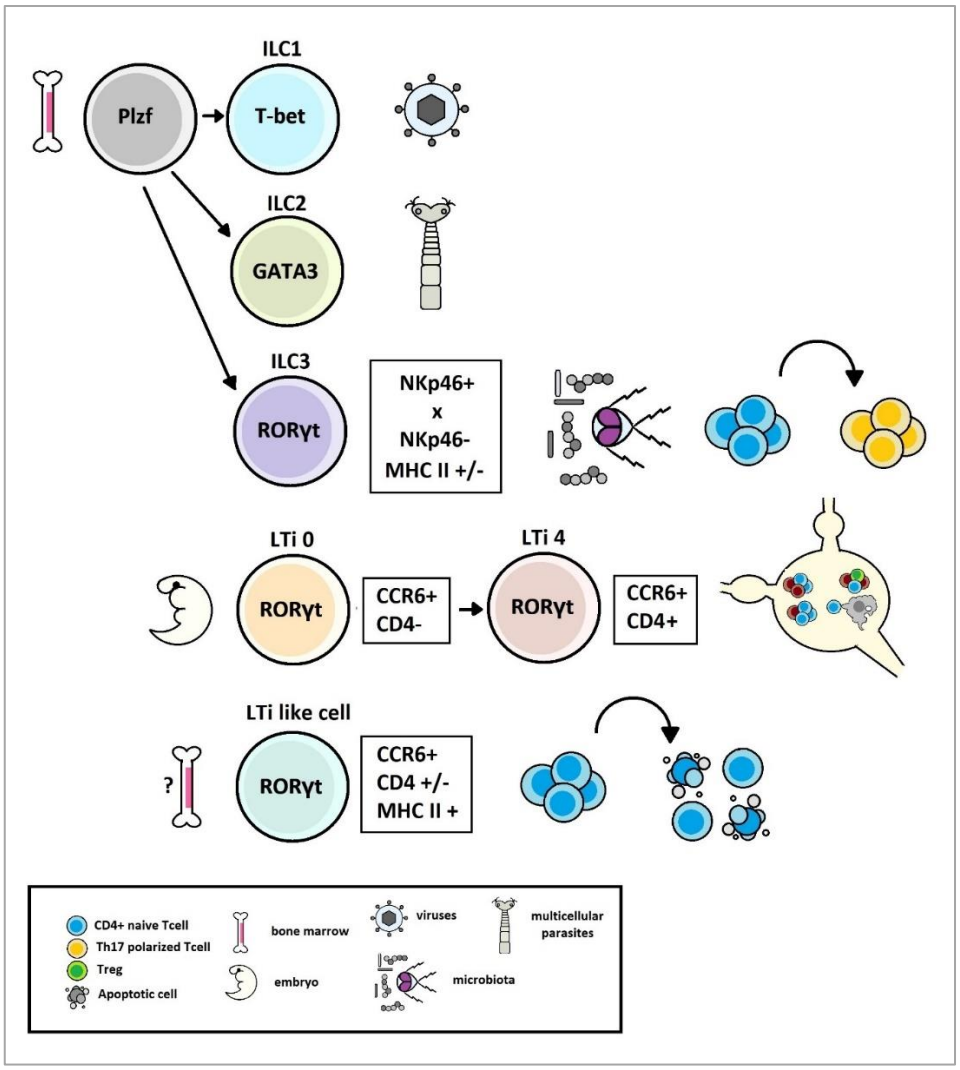
2014). Finally, LTi4 are capable of secondary lymphoid organ morphogenesis and their absence leads to a similar phenotype observed in Rorc KO mice (van de Pavert et al. 2014).

The basic developing structure of secondary lymphoid organs during embryonic development, called anlagen, is infiltrated by LTi which are guided by the CXCL13 gradient produced by a special type of stromal cells (Finke et al. 2002). CXCL13 acts on CXCR5 receptor on LTi, activates α1β4 integrin and thus mediates the attachment of LTi to these stromal cells. Then the Lymphotoxin α1β2 on LTi interacts with its LTβR on stromal cells and activates NF-κB signaling that leads to stromal cell activation and IL-7 and TNF-related activation-induced cytokine (TRANCE) expression (D. Kim et al. 2000). Both these cytokines support LTi in survival and enhance their lymphopoietic functions by upregulation of lymphotoxin α1β2 (Yoshida et al. 2002).

2.3.3. Role of ILC3 and LTi in T cell population development and shaping

LTi represent important players in the establishment of central as well as peripheral tolerance. They also play different roles as enhancers during central tolerance processing (Rossi et al. 2007) and mediators of similar processes like clonal deletion in the periphery (Hepworth et al. 2015). Notably, NFκB signaling which supports AIRE expression in mTECs and ultimately their development is maintained by the interaction of mTEC's receptor activator of NF-κB (RANK) with TRANCE on LTi that remain in the thymus for the whole lifespan from E14 during the fetal period in mice (Rossi et al. 2007).

At immune periphery, ILC3 play an important function as APCs by the expression of MHCII and costimulatory molecules CD80, CD86, ICOS-L, and CD40. In addition, AIRE expression is observed in these $CD4^{-}CD11c^{-}$ DN ILC3. Strikingly, the presence of Aire in ILC3 does not impact the expression of TRAs as in mTECs (Yamano et al. 2019). Thus, rather than selective deletion of peripheral T cells against TRAs, ILC3 might mediate the conversion of $CD4^{+}$ T cells to Tregs. A more specific question is whether ILC3 can induce microbiota-dependent and microbiota-specific Tregs. It is important to emphasize that during the course of experimental work on my thesis two publication presented data which indicated that this might be the case. First, it was reported that antigen presentation by $Aire^{+}ROR\gamma t^{+}$ ILC3 cells was both required and sufficient for induction of peripheral Tregs (Kedmi et al. 2022). And second, it has been shown that $AIRE^{+}$ ILC3s were capable of a direct presentation of Candida albicans antigens to induce Th17 polarized T cells (Dobeš et al. 2022). Although it is known that Lti-like cells I ILC3 can present microbiota-derived antigens, it has not yet been shown whether these antigens are obtained from the intestinal lumen directly or indirectly from other cells



Scheme 4. Types of ILCs and their functions: *Hematopoieticaly-derived ILCs mirror their functions according to the presence of specific polarizing TF common to T cells of adaptive immunity. ILC1 associated with Th1 polarization, ILC2 present in reactions against multicellular parasites or allergic reactions, and ILC3 associated with Th17 polarized T cells and capable of their induction due to antigen presentation capabilities. Mature, embryonic-derived LTi expressing CD4 induce the formation of secondary lymphoid organs. LTi like cells in adults are able to perform clonal deletion of T cells in the periphery due to the presentation of specific antigens.*

An obvious suspect forn this task could be ILC3s identified as $CCR6^{+}$ LTi /LTi-like cells that can perform a similar function as APCs in the thymic medulla. The difference is that LTi / LTi-like cells present via their MHCII the antigens of gut commensal organisms to naive or effector T lymphocytes. In addition, due to the absence of costimulatory molecules such as CD80, CD86, or CD40 on these cells, they are unable to activate T cells (Hepworth et al. 2013). Using OVA-specific OTII and flagellin-specific CBir TCR transgenic T cells it was suggested that

antigen presentation by LTi/LTi-like cells to T cells specific for intestinal commensal microorganisms leads into a state of anergy (Hepworth et al. 2013). Later, using the proapoptotic Bim KO model, it has been shown that there is not only anergy induction, but also the deletion of these commensal microbiota-specific T lymphocytes (Hepworth et al. 2015). At the same time, this system is also linked to the activity of microorganisms in the intestine, when the population of $MHCII^+$ ILC3 increases during infection due to signaling through the aryl hydrocarbon receptor (Ahr). Ahr KO mice show an increase in IFN-γ producing $CD4^+$ T cells in both bacterial colitis and Toxoplasma gondii infection (Wagage et al. 2015).

While the mechanism of ILC3 in the clonal deletion of microbiota-specific T cells is strikingly similar to that of mTECs during establishing of central tolerance via deletion of self-reactive T cells, the complementary mechanism which mediates the conversion of microbiota-specific T cells to Tregs based has never been convincingly documented. Thus, the main task of this work was to prove or dismiss the existence of such mechanism by MHCII+, RORγt + ILC3s.

Using the Rorc-cre x I-AB-flox mouse model in which MHCII is deleted on ILC3 and LTi/LTi like cells (Hepworth et al. 2013), we assessed the development of peripherally induced Tregs and analyzed other potential disturbances in ILC3 effector mechanisms.

C. MATERIAL AND METHODS

Mice:

Mice used for experiments were bred in the animal facility of the Institute of Molecular Genetics meeting the conditions for mouse breeding under SPF conditions under FELASA standards. Specific mouse strains breeding occurred on C57BL/6J mice background. All specific mice strains used in this thesis are described in **Tab. 1.** For organ dissection or cells isolation used mice were ranging in age from 3 to 12 weeks. They were humanly sacrificed by cervical dislocation.

Tab.1

Used mouse strains
Rorc-cre x I-AB-flox
Cd45.1. x (OT-II x Rag1 KO)
C57Bl6/J x Rorc(γt)-EGFP

Antibiotic treatment:

A cocktail of neomycin, ampicillin, vancomycin, and metronidazole in a concentration of 0.10 mg/ml was applied through drinking water in two different time windows split by weaning. Pups were treated during the preweaning period with the milk of females which took in ATB in drinking water. For successful milking, 5 times diluted solution of ATB cocktail were constantly present in drinking water in breeding cages due to the habit of tasting this solution to prevent its refuse during milking. Post-weaning treatment occurred from the 3rd week to the 6th week when the mice were analyzed.

Adoptive T cell transfer and OVA feeding.

For Adoptive Tcell transfer CD45.1 x (OT-II. x Rag1 KO) mice in age 3 and 6 weeks were used as a donor. T cells were isolated from mLN, pLN, and spleen using MACS column separation with anti-CD4-biotin coupled antibody and anti-biotin MACS beads for its enrichment. Cells were calculated in *Bürker chamber and diluted in PBS* to give $5x10^6$ cells per recipient mouse. Next, cells were intravenously transferred into recipient mice through the caudal vein in 100µl of PBS. After the transfer, OVA in concentration 10µg/ml was administred into drinking water of recipient mice for two weeks before analysis.

Cells isolations

Splenic and lymph node cells isolation

For soft isolation of lymphoid and other cells present in LNs and spleen, tissue was fragmented using scissors or needle and digested using collagenase D, RPMI solution in concentration 100 µl/ml and DNAse I in concentration 40 µl/ml at 37°C for 45 minutes. Digested tissue was squeezed through 40µm filter using a 5ml syringe plunger and washed with 10ml of 3%FCS 2mM EDTA PBS solution to stop the enzymatic reaction.

Tab. 2.

Targeted molecule	Conjugate	Manufactured	Dilution
CD3	PB	e-Bioscience	1:100
CD4	BV 605	BioLegend	1:400
CD45	APC/Cy7	BioLegend	1:200
CD80	PerCP/Cy5.5	BioLegend	1:200
CD86	APC/Cy7	BioLegend	1:200
RORγt	PE	BD Biosciences	1:300
Helios	APC	BioLegend	1:300
FoxP3	PerCP/Cy5.5	BioLegend	1:100
c-Kit	PE	BioLegend	1:200
Sca-1	PerCP/Cy5.5	e-Bioscience	1:200
MHC II.	FITC	BD Pharmigen	1:500
NKp46	PE/Cy7	BioLegend	1:200

Colon *lamina propria* cells isolation.

The cleaned colon was longitudinally opened and divided into 1cm long sections using scissors. To remove the epithelium, the intestinal sections were washed twice with 20 ml of 2 mM EDTA HBSS solution for 20 minutes at 37°C and vortexed twice, both for 10 seconds for maximum performance. Next, colon sections were fragmented to 1mm pieces using scissors and digested in collagenase D and DNAse I, RPMI solution in concentration 100µl/ml and 40 µl/ml at 37°C

for 60 minutes. After digestion, tissue was gently squeezed through a 40 µm filter using a 5 ml syringe plunger and washed with 10ml of 3%FCS 2mM EDTA PBS solution to stop the enzymatic reaction. After cell isolation, the lymphoid fraction was enriched in Percoll concentration gradient between 4ml 40% solution, where isolated cells were resuspended, and 2ml 80% solution on the bottom. Next, tubes with Percoll concentration gradient were centrifuged. The tubes were centrifuged at 800 rpm, 30 minutes, and 21 ° C with the brakes off

Flow cytometry and cell staining

All flow cytometric data published in this thesis were obtained from LSR II FACS analyzer using BDFACSDiva software and analyzed by FlowJo software. Used staining antibodies are shown in Tab.2.

Extracellular molecules staining

Cell suspension was stained by antibodies and viability dye eFluor 506 dissolved in 3%FCS 2mM EDTA PBS solution for 15 minutes on the ice.

Intracellular molecules staining

Intracellular staining was performed exclusively after extracellular staining when the cell suspension was fixed for 30 min at room temperature and then permeabilized using Foxp3 staining kit together with staining antibodies of intracellular molecules and also CD3 molecule. For intracellular staining, cells were incubated for 25 minutes on ice.

Statistics

For FACS data analysis, FlowJo software was used. Cell counts were calculated per 100,000 live cells and visualized by Prism-GraphPad where statistics were also calculated using an unpaired t-test

D. RESULTS

As indicated above, the main goal of this study was to:

(i) verify whether the mechanism of deletion of $CD4^+$ T cells in the periphery by ILC3 is operational;

(ii) determine whether $MHCII^+ROR\gamma t^+$ ILC3s are directly involved in the microbiota-specific induction of Tregs.

Rorc-cre x I-AB-flox mouse model and ILC3/LTi identification

To study the role of MHCII+; RORγt+ APCs in the induction of intestinal Tregs, we took advantage of the Rorc-cre x I-AB flox mouse model in which the expression of RORγt TF in ILC3 or LTi should result in the ablation of MHCII expression. Unfortunately, we found that MHCII is absent only in some RORγt+ ILCs. Nevertheless, the benefit of such defect is that the MHCII deletion is targeted into specific subpopulations of RORγt+ APCs as will be discussed further **(Fig.1b, c, d, e)**. To analyze the deletion of MHCII in specific sub sets of RORγt+ APCs we designed fa low cytometry gating strategy in which we first gated CD3-, B220- and CD11b- cells (Lin- cells) to get rid of T cells, B cells and myeloid cells, respectively **(Fig.1, a1).** Then, out of Lin- cells, we further analyzed those which were positive for RORγt, representing various subsets of ILCs **(Fig.1, a2)**. Within a population of RORγt+ ILCs, four main cell subsets were identified using MHCII and CD4 markers **(Fig. 1b).** RORγt+ ILCs include not only ILC3 but also cell types which share c-Kit and Sca1 surface markers with LTi cells (Moro et al. 2010). However, since LTi were originally described in prenatal and early postnatal mice (M. Y. Kim et al. 2009), we will refer to these cells as Lti-like cells. Interestingly, these LTi-like cells, similarly as LTi, form a CD4+ population of LTi4 and a CD4- population of LTi0. Using this gating strategy, we observed in Rorc-cre x I-AB-flox mice a depletion of CD4+ MHCII+ LTi-like cells **(Fig. 1. b1).** This is also obvious from the Sca1/c-Kit projection where double-positive population is reduced due to lack of MHCII in LTi-like cells **(Fig. 2b, c, d, e)**. To verify the identity of LTi like cells, the Sca1/c-Kit image is shown in projection onto the CD4/MHCII image **(Fig. 2f)**. We refered the other RORγt+ APCs as ILC3. We found that this MHCII ^{low} ILC3 population is primarily negative for NKp46 **(Fig. 1g)** and increases its cellularity in CD4-/$MHCII^{low}$ population in Rorc-cre x I-AB flox mice **(Fig. 1g, 2h, 7b)**. ILCs generally express fewer costimulatory molecules than other APCs. In this work, PDL1 was detected on LTi like cells **(Fig. 7c)** supporting the idea of their ability of negative regulation of T cells (Hepworth et al. 2013). In Rorc-cre x I-AB-flox mice, we see a total reduction of PD-L1+ LTi like cells compared to WT which is triggered by the lack of MHC II. **(Fig. 7c)** and,

conversely, we observed the expansion of the PD-L1- population represented by ILC3 **(Fig. 7c)**. In addition, a positive CD86 signal indicating the ability to induce lymphocyte proliferation was measured on this CD4-/MHCII+ ILC3 population **(Fig. 7d)**.

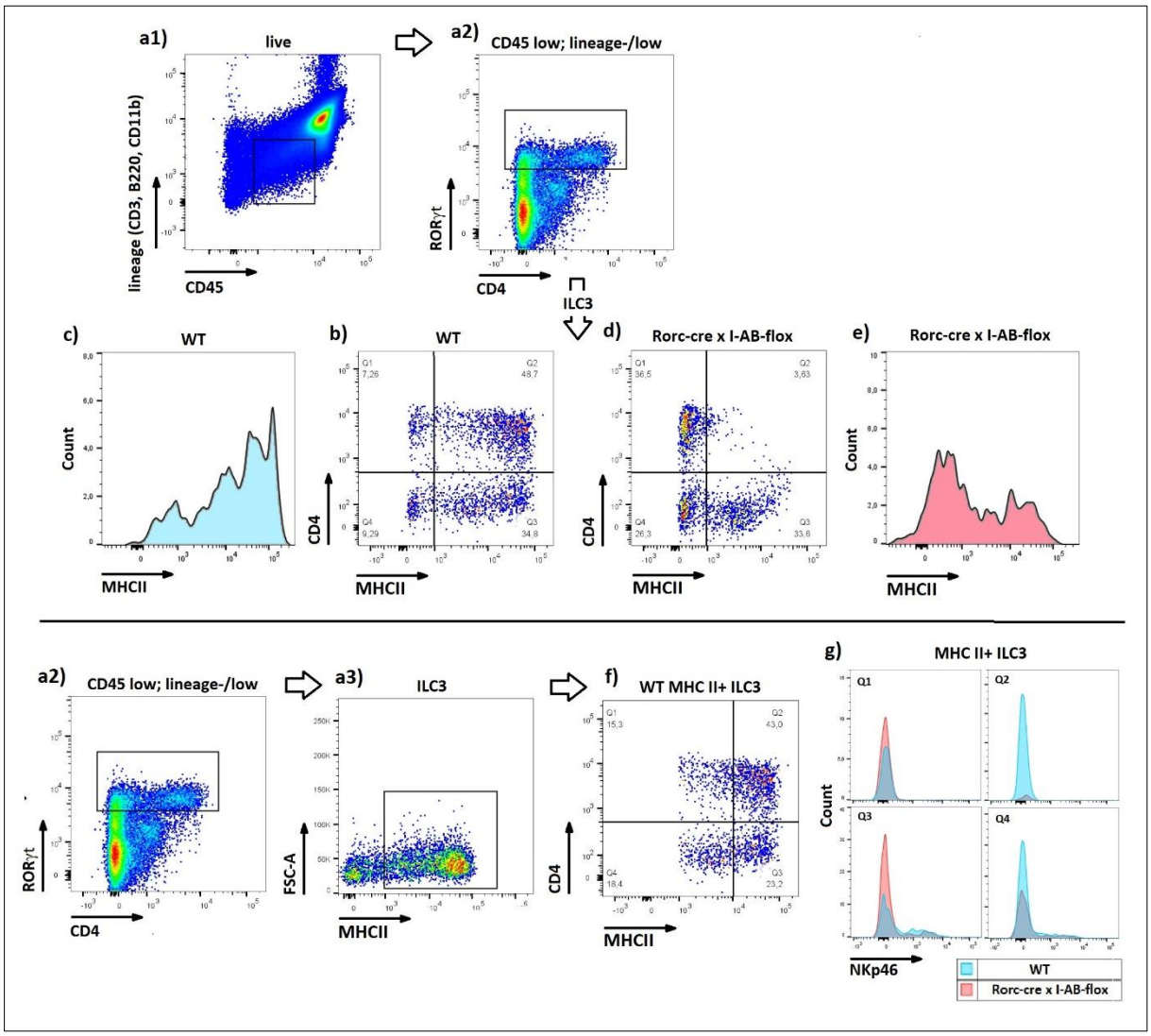


Figure 1.
a1) *Gating strategy of CD45^{lo} lineage (CD3, B220, CD11b) –/low.* **a2)** *Gating strategy of ILC3 projected as RORγt+CD4+/- cells.* **a3)** *Gating on MHCII+ILC3.* **b)** *Representative CD4/MHCII projection of ILC3 in WT.* **d)** *Representative CD4/MHCII projection of ILC3 in Rorc-cre x I-AB-flox. Representative visualization of MHCII expression on ILC3 in WT* ***c)*** *and Rorc-cre x I-AB-flox* **e)**. **f)** *CD4/MHCII projection of MHCII+ ILC3 in WT.* **g)** *Representative NKp46 expression in different populations of ILC3 in WT and Rorc-cre x I-AB-flox mice.*

Presence of MHCII on LTi like cells provides regulation of Th17 polarized T cells

The absence of MHCII on LTi like cells is manifested by an increase in the population of RORγt+ Th17 polarized T cells both in mLN and colon LP **(Fig. 3b, c)**. This observation is a reminiscence of the effect shown in the experiments with cBir T cells specific to clostridial flagellin, whose numbers in host Rorc-cre x I-AB-flox mice were dramatically increased

(Hepworth et al. 2015). When using ATB treatments, we again observed the negative regulation of Th17 T cell numbers by LTi-like cells. Notably, preweaning treatment with ATB causes a decrease in the number of LTi 4 like cells in the mLN of 6 weeks old mice (**Fig. 3f, g**). This diminuition is manifested by a significant increase in the number of Th17 polarized T cells **(Fig. 3i, j)**. The ATB postweaning treatment of mice showed no impact on Th17 T cells expansion due to the absence of microbiota due to which no inflammation is likely to develop **(Fig. 3i, j).** We decided for experimental ATB treatments before or after weaning due to the newly observed physiological phenomena related to weaning, as well as physical changes such as the transition to solid diet and the related development of the intestinal microbiota or the permeability of the intestinal epithelium (Al Nabhani et al. 2019). Unfortunately, preweaningly treated Rorc-cre x I-AB- flox mice results are not shown in any experimental setup since this cohort of mice suffered from a spontaneous whole-body MHCII KO (data not shown) manifested by the absence of MHCII on all APCs accompanied by a drastic reduction of CD4+ Tcells (data not shown).

Intestinal Tregs react to Th17 T cells expansion

Compared to the WT control, a significant increase in Helios+RORγt-Tregs in the colon lamina propria was observed in Rorc-cre x I-AB-flox mice **(Fig. 4b, d)**, which was also reflected by an overall increase in the number of Tregs in the lamina propria. No significant imbalance of this Helios+RORγt- Tregs population was not observed in the mLN **(Fig. 4b, c)**. Helios+ Tregs which express GATA3 TF and IL-33 receptor ST2, play a role in the regulation of colitis (Nascimento et al. 2017; Schiering et al. 2014) and could be of both tTregs and pTregs origin (Thornton et al. 2010). In contrast, there is a significant decrease of the RORγt+Helios- Tregs population in mLN **(Fig. 4c). S**imilar tendencies, although not significant, were also observed in the lamina propria of the colon **(Fig. 4d)**, which suggests the ability of some ILC3 or surprisingly, LTi like cells, to induce these RORγt+ Tregs (Kedmi et al. 2022). Other Treg populations were not significantly affected in any of both tissues.

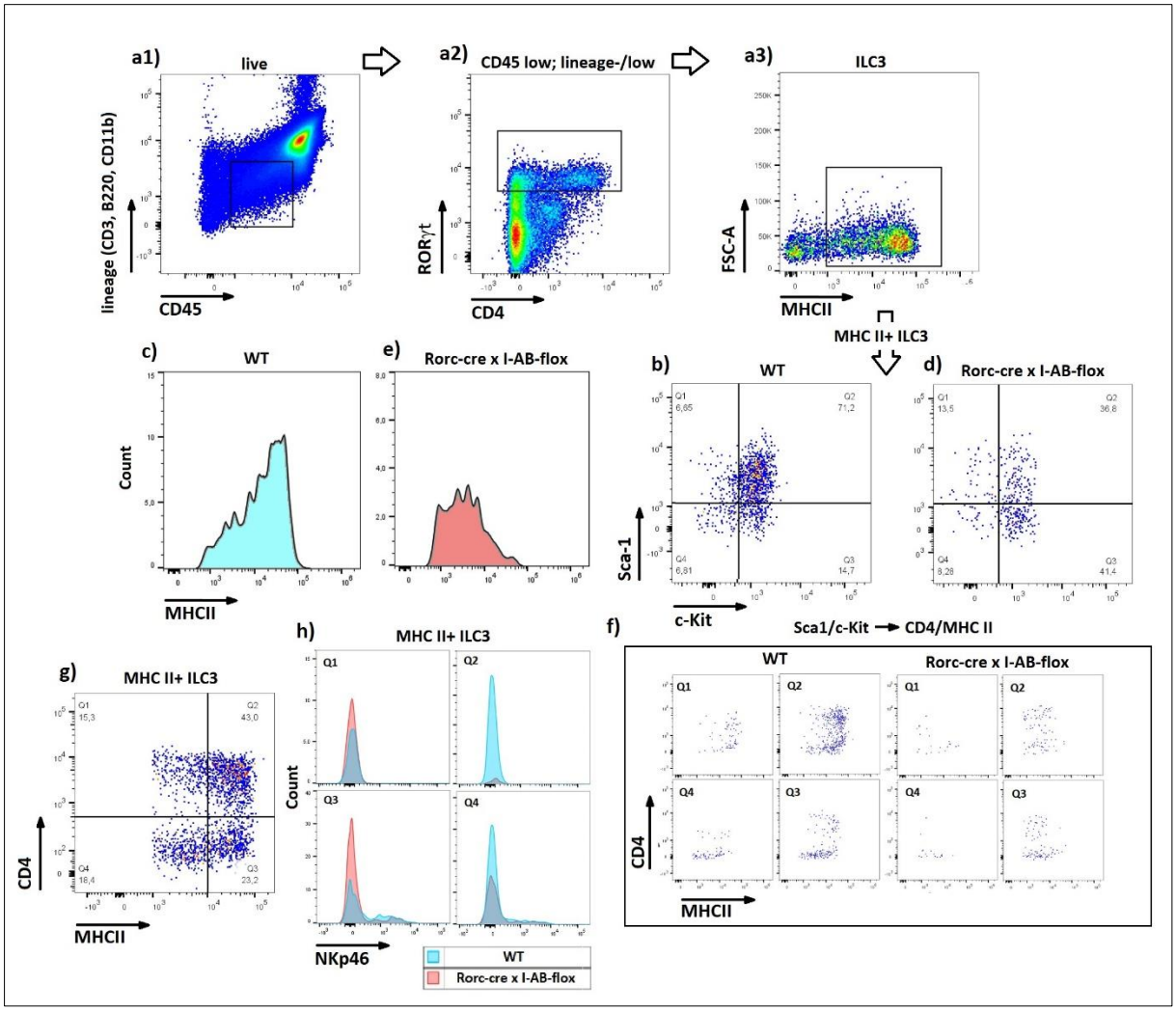


Figure 2.
a1) *Gating strategy of CD45^{low} lineage (CD3, B220, CD11b) –/low.* **a2)** *Gating strategy of ILC3 projected as RORγt+CD4+/- cells.* **a3)** *Gating on MHCII+ILC3.* **b)** *Representative Sca1/c-Kit projection of MHCII+ILC3 in WT.* **d)** *Representative Sca1/c-Kit projection of MHCII+ILC3 in Rorc-cre x I-AB-flox. Representative visualization of MHCII expression on ILC3 in WT* ***c)*** *and Rorc-cre x I-AB-flox* **e). f)** *Projection of individual quadrants form ILC3 Sca1/cKit visualization into CD4/MHCII visualization.* **g)** *Representative NKp46 expression in different populations of ILC3 in WT and Rorc-cre x I-AB flox mice.*

Some pTregs are dependent on the presence of microbita

Due to aberrations in Treg populations in mLN and in colon LP, especially in Helios+RORγt- Tregs, we were interested if the expanding cells are tissue-specific healing Tregs, or are somehow dependent on the microbiota or exogenous antigens. For this reason, experiments such as ATB treatments were designed. When the mice were treated either prior weaning or post weaning with ATB cocktail, the RORγt+ population of Tregs was almost absent in both MLN and colon. This scenario was expected since the presence of RORγt+ Tregs is known to depend on the microbiota and its metabolites (Sefik et al. 2015) **(Fig. 5d, e)**.

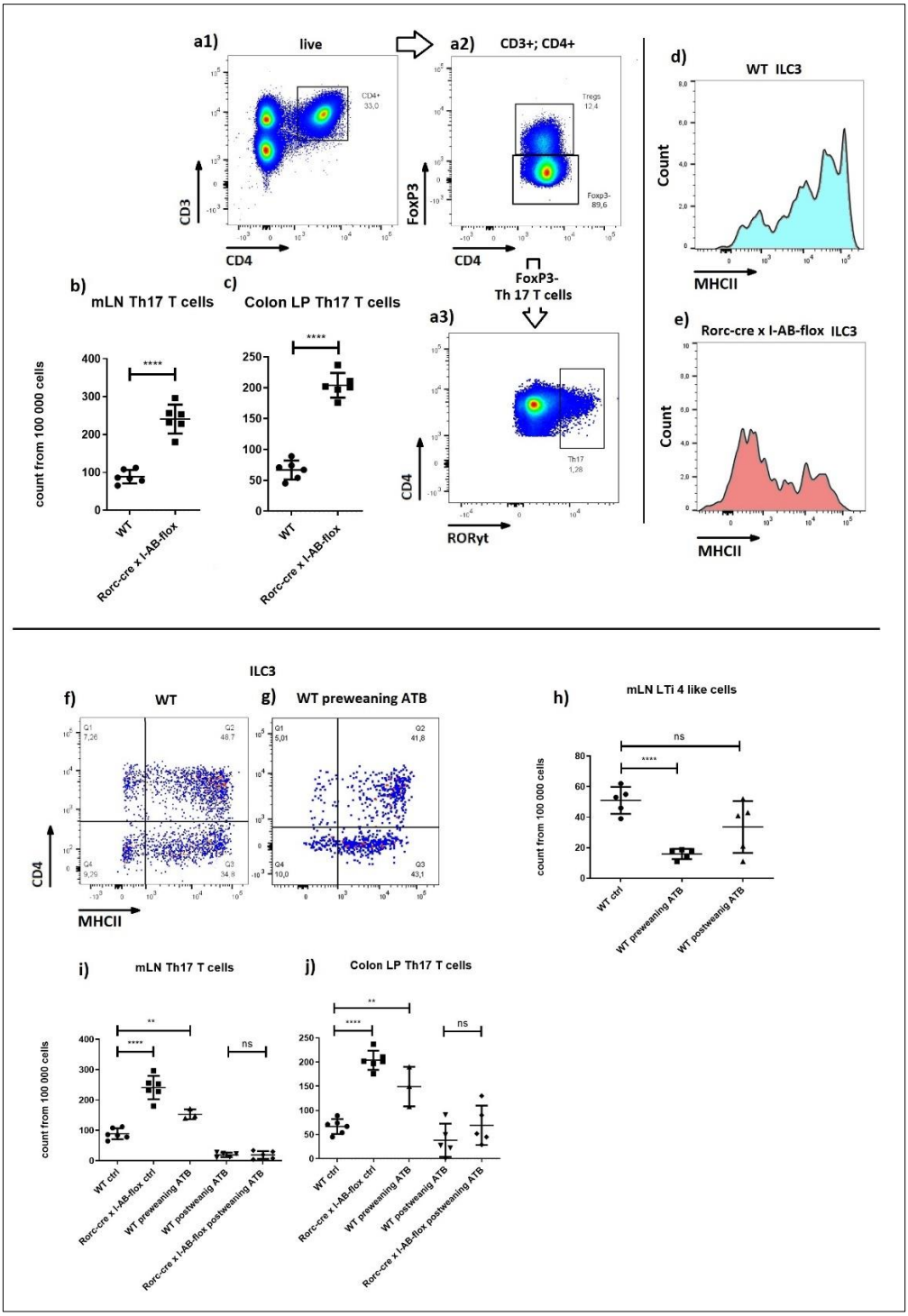


Figure 3:

a1) *Gating strategy of CD3–CD4+ cells.* **a2)** *Gating strategy of Tregs and non-Tregs CD4+ T cells by FoxP3/CD4 visualization.* **a3)** *Gating on RORγt+ Th17 polarized T cells. Counts of Th17 polarized T cells from WT and Rorc-cre x I-AB-flox in mLN* **(b)** *and colon LP* ***(c)****. MHCII expression on ILC3 in WT* **(d)** *and Rorc-cre x I-AB-flox* **(e)***. Representative CD4/MHCII projection of ILC3 in ctrl WT* **(f)** *and preweanig ATB treated WT* **(g). h)** *Counts of LTi4 like cells from ctrl WT, preweanig ATB treated WT and postweaning ATB treated WT in mLN. Counts of Th17 polarized T cells from the control (ctrl), preweaning ATB treated and postweaning ATB treated WT and Rorc-cre x I-AB-flox in mLN* **(i)** *and colon LP* **(j)**.

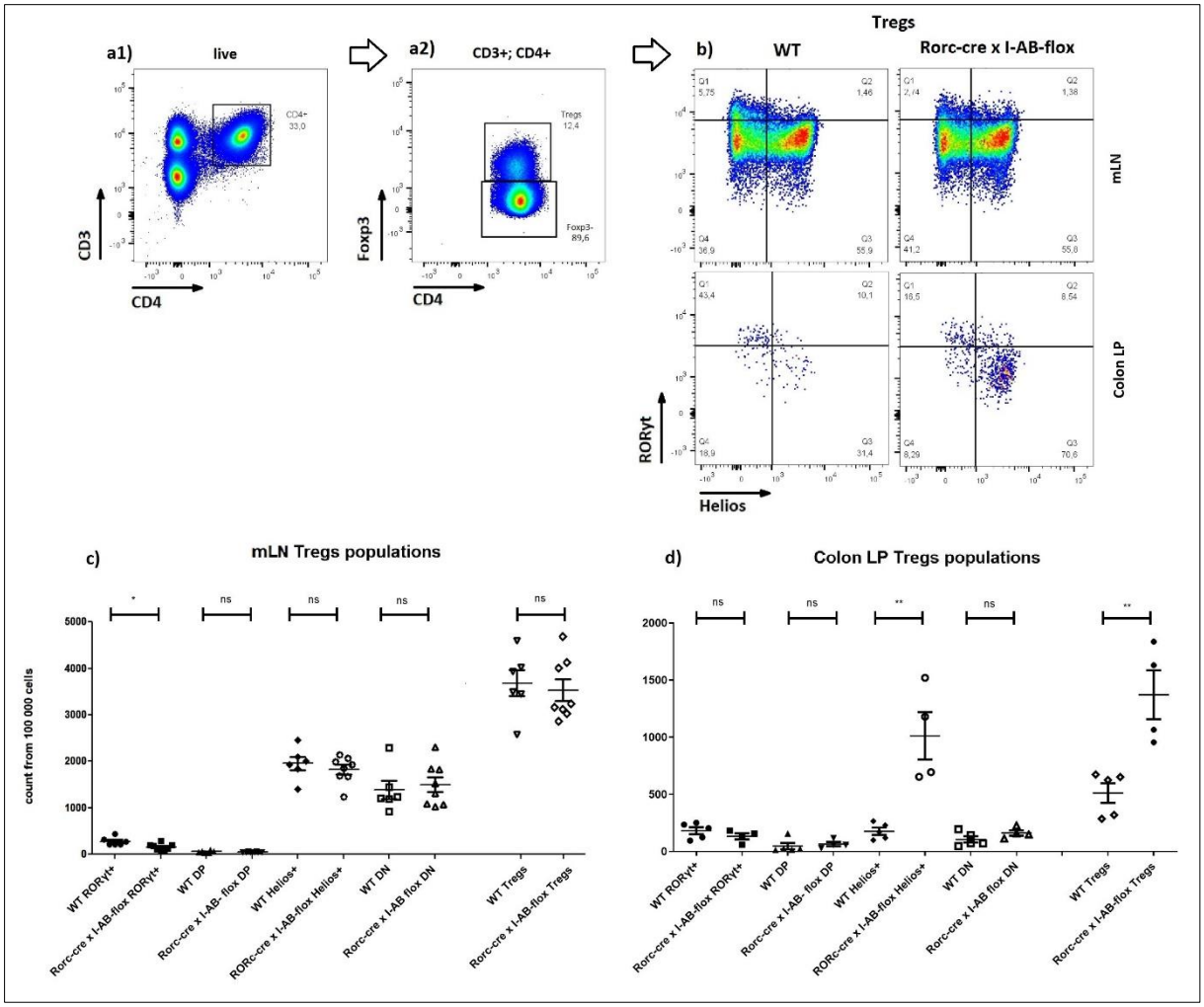


Figure 4:
a1) *Gating strategy of CD3–CD4+ cells.* **a2)** *Gating strategy of Tregs and other CD4+ T cells by FoxP3/ CD4 visualization.* **b)** *Representative RORγt/Helios projection of Tregs from WT and Rorc-cre x I-AB-flox in mLN and clon LP. Counts of RORγ+, Helios+, DN and DP Tregs populations in mLN* **(c)** *and colon LP* **(d)**.

However, this was not the case for Helios+RORγt- Treg population, where we observed the reduction in the number of Helios+RORγt- Tregs in MLN when ATB were administered before weaning. Strikingly, when the mice were treated with ATB after the weaning, Helios+RORγt- Tregs were unaffected in MLN **(Fig. 5f, g)**. Hence, it seems that before the weaning period, there is a time window in which the numbers of Helios+RORγt-Tregs are affected by the presence of microbiota. In the colon, ATB treatment of Rorc-cre x I-AB-flox dampens the increase in the number of Helios+ Tregs observed in untreated Rorc-cre x I-AB-flox mice to the WT control levels **(Fig. 5g)**. This implies that such increase is completely dependent on the presence of microbiota. This result together with the previously published data (Nascimento et al. 2017; Schiering et al. 2014) implies that Helios+ Tregs expand in the Rorc-cre x I-AB-flox to supress inflammation that is targeted against the microbiota.

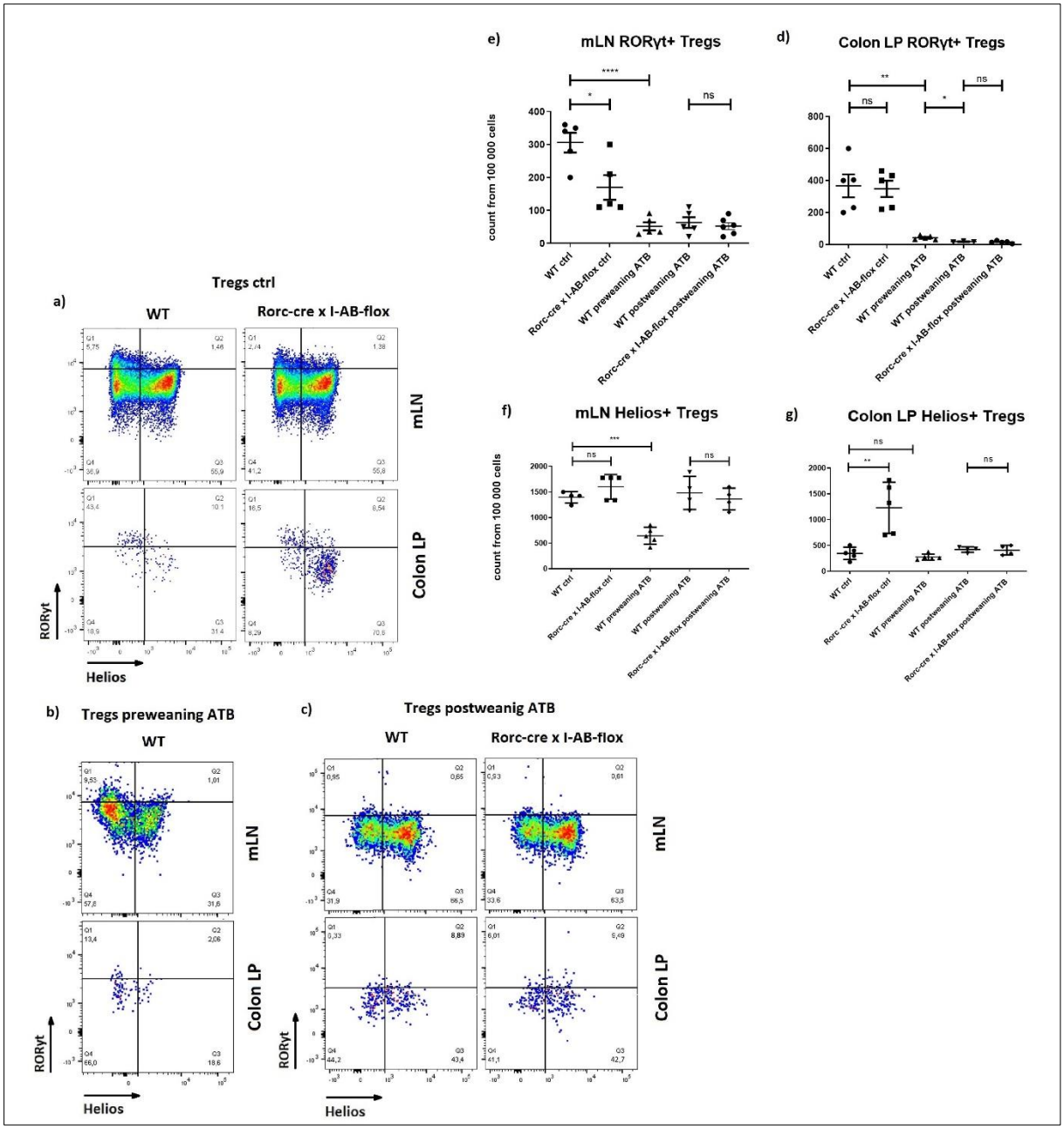


Figure 5:

Representative RORγt/Helios projection of Tregs from WT and Rorc-cre x I-AB-flox in mLN and colon LP in ctrl mice **(a),** *preweanig ATB treated mice* **(b),** *and postweaning ATB treated mice* **(c)**. *Counts of LTi4 like cells from ctrl WT, preweanig ATB treated WT and postweaning ATB treated WT in mLN. Counts of RORγt+ Tregs from ctrl, preweanig ATB treated and postweaning ATB treated WT and Rorc-cre x I-AB-flox mice in mLN* **(d)** *and colon* LP **(e)**. *Counts of RORγt+ Tregs from ctrl, preweanig ATB treated and postweaning ATB treated WT and Rorc-cre x I-AB-flox mice in mLN* **(f)** *and colon* LP **(g)**

Helios+ pTregs have a short induction time window and are generated through the presentation of exogenous antigens from the intestine.

It is the expansion of the Helios+RORγt- population of Tregs in the colon lamina propria which has not yet been observed in Rorc-cre x I-AB flox mouse model (Hepworth et al. 2013, 2015). This begs the question, if the expanding Helios+RORγt- Tregs are induced in the thymus or peripherally in the gut. Helios+ Tregs are generated during both establishment of central tolerance and by induction of peripheral tolerance (Thornton et al. 2010). Therefore, we have designed experiments which should demonstrate the peripheral induction of Helios+RORyt- Treg population. Moreover, our experimental setup should proof the antigen specificity of the Helios+ RORyt- Treg population to exogenous antigens. In other words, we wanted to see whether the Helios+RORyt- Treg population can be induced peripherally by the presentation of exogenous antigens and to determine the role of RORyt+ ILC3 in this process.

Intravenous transfer of CD4+ T cells specific to OVA antigen was performed from donor, congenic OT-II x Rag1 KO (CD45.1+CD45.2+) into Rorc-Cre x I-AB-flox and WT (CD45.2+) mice. Peripheral induction of Tregs in the mLN was observed after two weeks of feeding the recipient mice with OVA peptide in drinking water. Enumeration of OVA-specific Tregs by FACS analysis was performed in mLNs but not in the lamina propria of the colon where we failed to collect a sufficient number of cells for their analysis. It has been confirmed that the induction of a particular Tregs population is dependent on the age of the donor mouse (Pratama et al. 2020). Accordingly, CD4+ T cells from three weeks old OT-II x Rag1 KO mice gave rise to Helios+RORγt- Tregs population **(Fig. 6b)**. In marked contrast, using 6-week old adult donor, only RORγt+ Tregs were generated **(Fig. 6e).** Strikingly, the significant increase in the number of OVA specific Helios+RORγt- Tregs was observed in Rorc-cre x I-AB-flox when compared to WT recipients **(Fig. 6d)**. This apparently occurred as a count remeasure to the expansion of Th17 T cells in the host **(Fig. 6c)**, indicating that peripheral induction of these Tregs in the gut in this mouse model is appropriate under the perturbed/inflammatory conditions.

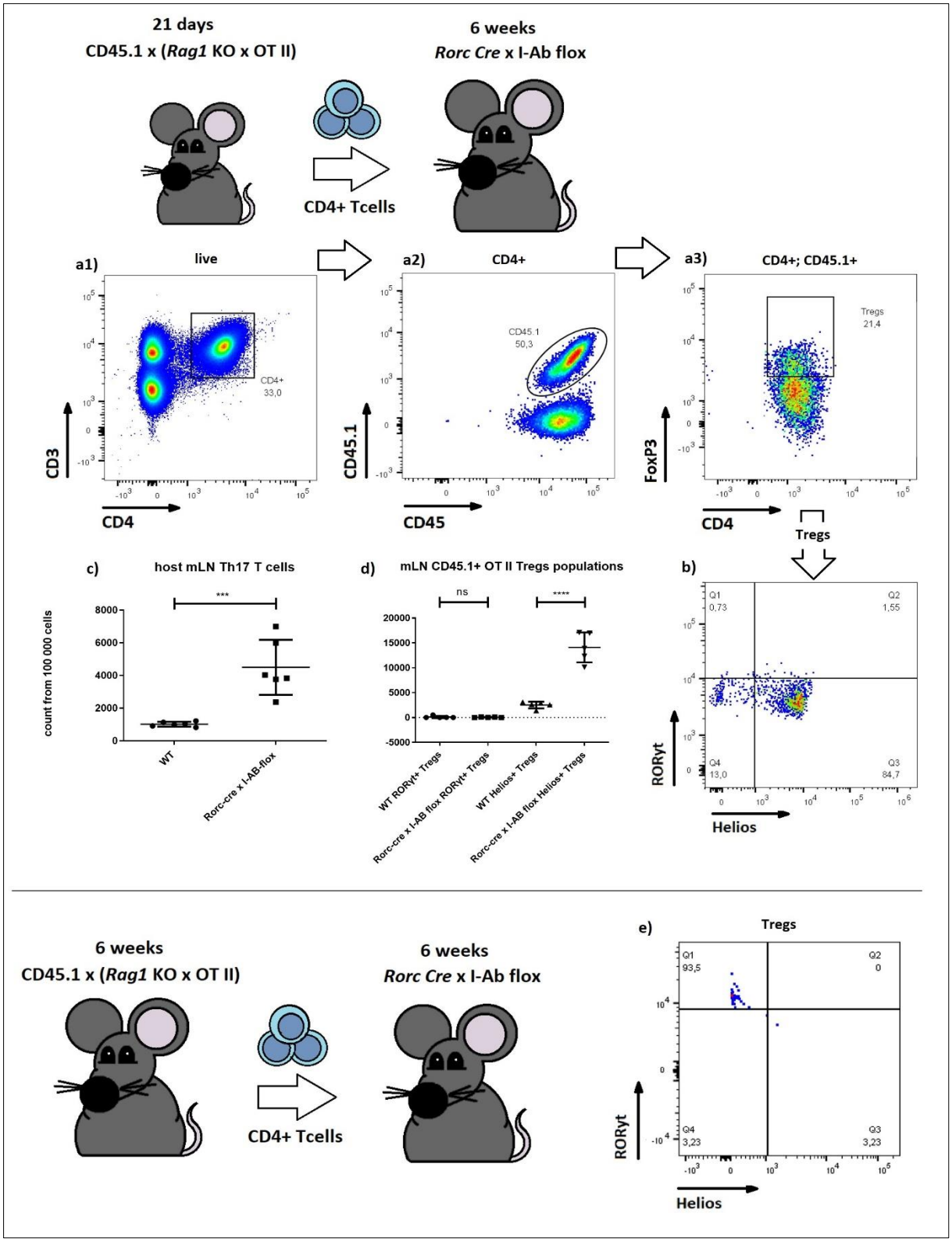


Figure 6:
a1) *Gating strategy of CD3–CD4+ cells.* **a2)** *Gating strategy of donor CD45.1; CD45 T cells.* **a3)** *Gating strategy of Tregs.* **b)** *Representative RORγt/Helios projection of induced pTregs which originate from 3 weeks old donors.* **c)** *Counts of Th17 polarized T cells in LNs from the host WT and Rorc-cre x I-AB-flox mice.* **d)** *Counts of 3 weeks old donors-derived RORγt+ and Helios+ Tregs in LNs from WT and Rorc-cre x I-AB-flox mice in mLN.* **e)** *Representative RORγt/Helios projection of induced pTregs originating from 6 weeks old donors.*

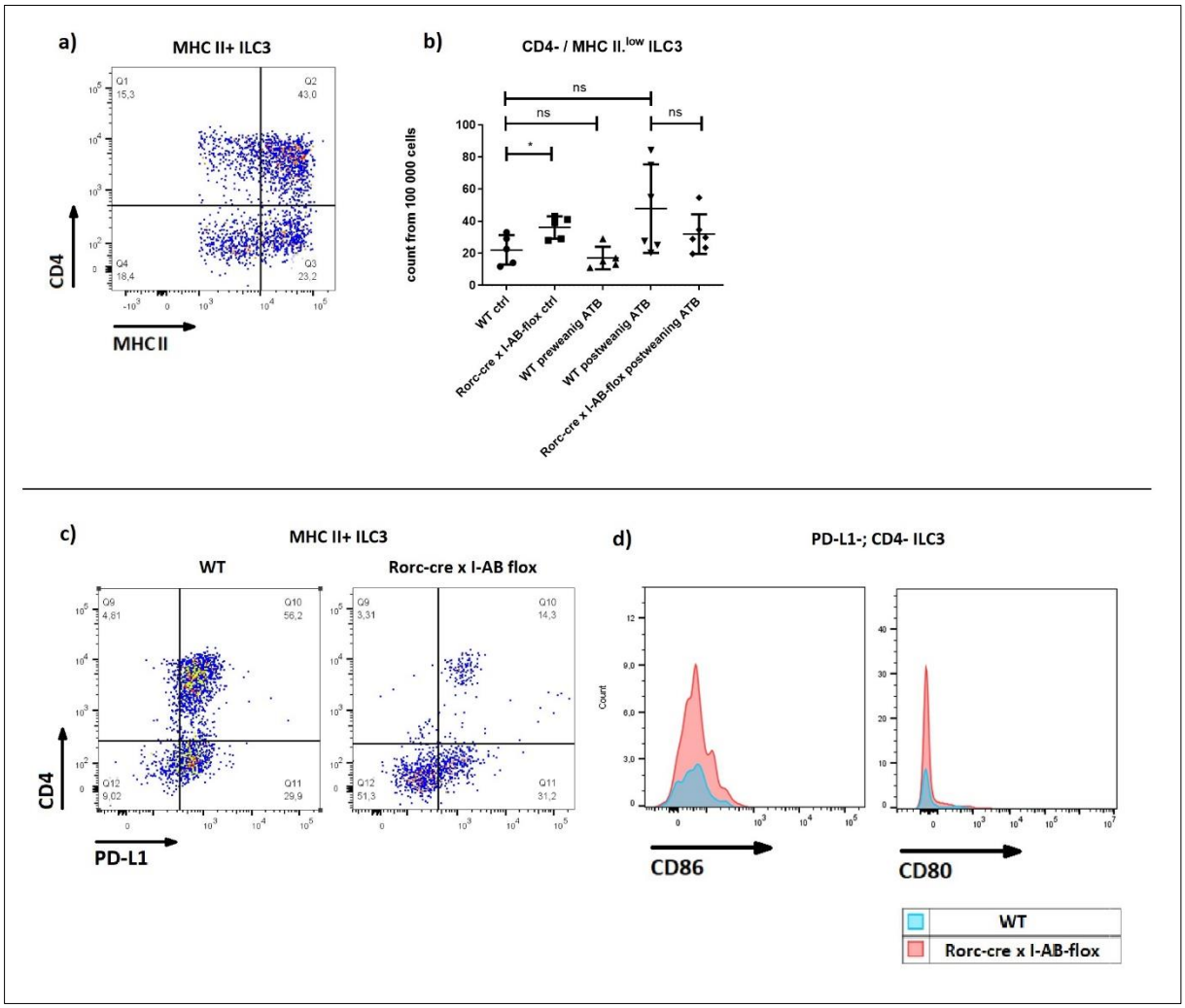


Figure 7:
Representative CD4/MHCII projection of MHCII+ ILC3 in ctrl WT **(a)**. *Counts of CD4-MHCII* ^{low} *ILC3 in LNs from ctrl, preweanig ATB treated and postweaning ATB treated WT and Rorc-cre x I-AB-flox mice* **(b)**. *Representative CD4/PD-L1 projection of MHCII+ILC3 in WT and Rorc-cre x I-AB-flox mice. CD80 and CD86 expression on CD4–PD-L1– ILC3 in WT and Rorc-cre x I-AB-flox* mice **(d).**

E. DISCUSSION

The main objectives of this work was to assess the importance of antigenic presentation by RORγt+ILCs in maintaining intestinal homeostasis by contributing to peripheral recessive and dominant tolerance. We observed novel biological properties of peripherally induced Tregs, and confirmed several previously published observations relevant to this topic. These observations and experiences, embedded into the context of the current understanding of mechanisms underpinning peripheral tolerance are discussed in this chapter. The main issues which emerged from the presented work are as follows:

1. Suitability of the Rorc-cre x I-AB-flox mouse model
2. Generation of Th17 T cells is under the control of LTi like cells
3. ILC3 as potent inducers of pTregs
4. Helios+ pTregs time window induction and antigenic specificites

1. Suitability of the Rorc-cre x I-AB-flox mouse model

Although Rorc-cre x I-AB-flox is an excellent model that deletes MHCII in a specific population of APCs (LTi like cells) **(Fig. 1b, c, d, e 2b, c, d, e)**, its functionality should be considered with caution. Disorders associated with such deletion have been observed previously (Hepworth et al. 2015), but the causality is still unclear. The most probable scenario is the presence of a non-coding regulatory sequences, which, for example, are insufficiently stimulated by TFs which are in turn activated by microbial metabolites. At the same time, however, it is interesting that frequent whole-body MHCII KO have been noted in some breeding cages in F1 generation, which in the course of time occurs with absolute certainty. Unfortunately, it is not possible to identify the founders of such whole-body MHCII KO by conventional genotyping. The best approach to determine the functionality of this model and to avoid this undesirable phenotype, it is necessary to characterized MHCII expression by FACS analysis. Specifically, to ensure the correct course of the experiment, I propose to perform preventive blood sampling of used mice and check their blood counts. The whole-body KO is manifested by the absence of CD4+ T cells, and this lack of lymphocytes in the blood is a quick and inexpensive indicator for the elimination of affected mice from the experiment to save time and resources. Despite the fact that in the Rorc-cre x I-AB-flox mice the deletion of MHCII on ILC3 is incomplete and subset specific **(Fig. 1b, c, d, e)** it turned out to be a very useful model. Paradoxically, among all ILC3, it genetically ablates MHCII only on LTi like cells. In preliminary experiments we used also Cd11c-cre x I-AB-flox model. Except classical CD11c+

APCs such as dendritic cells, deletion in MHCII locus affected also LTi-like cells but similarly to the Rorc-cre x I-AB-flox model, ILC3 were left unaltered. This observation resulted in use of a lineage staining mixture without an anti-CD11c antibody to successfully show ILC3 and LTi like cells during experiments on Rorc-cre x I-AB-flox mice **(Fig. 1a1, 2a1**).

2. Generation of Th17 Tcells population is under the control of LTi like cells

Th17 T cells are potentially pro-inflammatory cells that may be specific to the gut microbiota. Due to the high expression of MHCII and PD-L1, LTi like cells are involved in the deletion of T cells specific for exogenous antigens in the periphery (Hepworth et al. 2015). In the case of MHCII deletion in these cells, Th17 polarized T cells expand **(Fig. 3b, c)** as they loose the capacity to present antigens **(Fig. 3d, e)**. Th17 T cells also expand in the case of WT treated by ATB before weaning **(Fig. 3i, j)**. The population of LTi4-like cells is greatly reduced in these mice **(Fig. 3h)**, which indicates that the development of LTi-like cells occurs mainly in young mice and requires the presence of microbiota. In addition, bacterial metabolites (retinoic acid) present in milk are essential stimulants tor the development of RORγt+ ILCs (van de Pavert et al. 2014). We have shown that LTi like cells are responsible for the deletion of T cells specific to exogenous antigens, and thereby can potentially regulate immune reactions to intestinal microbiota. At the same time we observed that the presence of ATBs before weaning period is reflected in adulthood by insufficient deletion of microbiota specific T cells in the periphery.

3. ILC3 as potent inducers of pTregs

The main goal of the work was to find out whether ILC3 can induce Tregs. This question specifically relates to the mechanism of negative selection in the thymus by mTECs and DCs, in a sense to assess whether similar mechanisms, i.e. clonal deletion as well as the conversion to Tregs, can be mediated by ILC3 subset. As a consequence of MHCII deletion on RORγt+ ILCs, a significant reduction in RORγt+ Tregs was observed in the mLN **(Fig. 4c, 5d)**, but not in the colon LP **(Fig. 4d, 5e)**. This is likely caused by the higher concentration of Tregs in the effector tissue which in the case of colitis is colon LP. However, the observed reduction in RORγt Tregs in Rorc-cre x I-AB-flox mice strongly suggest that a proportion of these Tregs must be induced by MHCII+PD-L1+LTi like cells.

Two distinct populations of precursor Tregs are generated in the thymus. One of them, FoxP3^{low}, is specific mainly to exogenous antigens (Owen et al. 2019) and could represent a precursor with imprinted Treg phenotype. This feature might protect this precursor from peripheral deletion mediated by LTi like cells, as is the case for naive T cells. Helios+ pTregs

could also be of similar origin with imprinted Treg phenotype (Thornton et al. 2010). The ability to activate T cells was also shown for ILC3. In this work, we have shown that MHCII+CD4– PD-L1– ILC3 express the costimulatory molecule, CD86 **(Fig. 7d)**. At the same time, we showed that in the case of Rorc-cre x I-AB-flox mouse model expansion of ILC3 grows significantly **(Fig. 7b)**. The reason for the growth of this population may be an increase in the expression of MHCII, whereby they fall into the MHCII+ population as part of the gating strategy. A similar effect is observed in the epithelial cells of the colon, which are thus capable of peripherally inducing Helios+ pTregs (Yoshimatsu et al. 2022). In this case, the increase in MHCII is under the control of Ahr, which in colitis is stimulated by a greater amount of microbial metabolites due to increased intestinal permeability (Yoshimatsu et al. 2022). ILC3s also express Ahr (Wagage et al. 2015), which in the same situation also increases the antigen-presenting abilities of ILC3s and thus the induction of Tregs or Th17 T cells.

4. Helios+ pTregs time window induction and antigenic specificites

Helios+RORγt–Tregs excel in their ability to suppress inflammation in mucosal and epithelial tissues by producing soluble ST2 and thus neutralizing IL-33 (Schiering et al. 2014). In this work, we observed an increase in this population in Rorc-cre x I-AB-flox mice in colon LP **(Fig. 4d)**. Interestingly, using the same Rorc-cre x I-AB-flox mice, Hepworth and colleagues reported that when the population of CBir specific Tcells (i.e. microbial flagelin specific T cells) increased, they did not observe an increase in the population of all Tregs in LP of the gut (Hepworth et al. 2013). In contrast our study clearly demonstarted that the expansion of Th17 polarized T cells is accompanied by an increase in the number of Tregs, especially Helios+ Tregs in the colon LP. This difference may be due to a different composition of the microbiota in the animal facility. The microbiota plays a critical role in the induction of pTregs, and some specific microorganisms, especially the *Helicobacter* genus as pathobiontic bacteria, are very potent inducers of pTregs development (Chai et al. 2017). Helios+ Tregs are induced in both thymically and peripherally (Thornton et al. 2010). In this work, it was shown that Helios+ Tregs increase their cellularity in the inflammatory environment of the colon. In addition, we showed that these Tregs can be induced in the periphery by exogenous antigens **(Fig. 6)**. Our T cell adoptive transfer experiments with OVA-specific T cells showed that Helios+ pTregs can be effectively induced only in the first 3-4 weeks of life **(Fig. 6b)** and afterwards the induction of Tregs is restricted to the RORγt+Helios– phenotype **(Fig. 6e)** (Pratama et al. 2020). In this context it has been reported that the expansion of these Tregs depends on the presence of IL-33 (Schiering et al. 2014), the situation which in our experimental setting was mimicked by the

expansion of Th17 T cells in RORc-cre x I-AB-flox mice resembling the conditions of induced colitis **(Fig.6c)**. However, in the Rorc-cre x I-AB-flox model we see the expansion of Tregs in colon LP and not in mLN, but in case of donor OT-II we observe the expansion of OVA specific Tregs in mLN **(Fig. 6)** and perhaps also in the colon LP. However, due to the low number of CD45.1 cells in colon LP, it was not possible to generate the statistics. Thus, this experiment will be repeated in a near future. Importantly, our experiment suggests that Helios+ Tregs can also be specific to food antigens (in our case, the OVA peptide). It is therefore possible that the induction of pTregs specific to food antigens takes place in mLN and the migration to effector tissues like lamina propria occur after that. Helios+ pTregs are also dependent on the presence of microbiota before weaning, and with ATB treatment during childhood their resulting population in adults is in mLNs reduced. It is interesting that the population of Helios+ pTregs in the colon LP in the Rorc-cre x I-AB-flox mice is comparable to WT ctrl, which is most likely caused by a high concentration of Tregs in effector tissues. Thus, the most important novelty of this work is that Helios+ pTregs can be microbiota and food antigen specific and their induction is limited to the period before and around weaning.

F. CONCLUSONS AND FUTURE PERSPECTIVES

The main and very general aim of the work, to determine the role of ILC3 in the induction of pTregs was clearly not fulfilled completely and many questions remain. However, new properties of LTi like cells and their contribution to the generation of Helios+ pTregs with specificty for food and microbiota antigens, as well as and the utility of the Roc-cre x I-AB-flox model were investigated in this thesis.

- We observed an increase number of Tregs in the colon LP of the Rorc-cre x I-AB-flox mouse model in response to the ongoing colitis induced by a dysfunctional deletion of the microbiota specific CD4+ T cells.
- We confirmed the existence of a specific time windows in the induction of pTregs, namely, the preference in the induction of Helios+ pTregs before weaning period and RORγt+ pTregs after weaning period.
- We showed the dependence of Helios+ pTregs on the microbiota.
- We found that Helios+ Tregs can be specific to food antigens.

- We showed that exposure to ATB before the weaning leads to defects in the development of LTi like cells, which could be manifested in adulthood by breaking intestinal tolerance.
- According to the expression of costimulatory molecules, we indirectly showed the potential of ILC3 as professional APCs.

In the future, it would be appropriate to create in vitro assays for direct observation of the ability of individual subsets of ILC3 to induce Tregs. Furthermore, it is very tempting to examine whether there are predetermined precursors of pTregs in the thymus that could elegantly explain the relationships between the Helios+ pTregs and RORγt+ pTregs populations. In our work, we found that Helios+ pTregs can have a broad antigenic specificity. This population reacts to the presence of microbiota and food antigens. Due to their exceptional ability to regulate immune reactions and the narrow time window opportunity for their induction, they can be used as an easily controlled therapeutic target in the prevention of food intolerances. This is primarily related to the need to better understand their ontogeny and the stimuli behind their induction.

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