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The role of Rorγt^{+} antigen presenting cells in peripheral tolerance to microbiota

Úloha Rorγt^{+} antigen-prezentujících buněk v ustanovení tolerance k střevní mikrobiotě

Bachelor´s thesis

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

The intestinal immune system needs mechanisms that provide a balance between suppression and tolerance to microbiota, regulating beneficial and inhibiting pathogenic ones. One such mechanism is based on microbiota-specific Rorγt+ T regulatory cells (Tregs), which can regulate immunity upon recognition of a specific antigen. Rorγt+ antigen-presenting cells (APCs) have been hypothesized to promote microbiota-specific Tregs development, but the specific mechanism was poorly understood until recently. In my bachelor thesis I provide an introduction to Tregs biology, compare and review in detail studies indicating a possible role of lymphoid tissue inducers like group 3 innate lymphoid cells, Janus cells and newly discovered Thetis cells in the generation of Rorγt+ Tregs as well as a possible molecular mechanism based on the integrins Itgav and Itgb8.

Key words:

Rorγt+ antigen-presenting cells, peripheral tolerance, gut microbiota, regulatory T cells (Tregs), immune homeostasis, autoimmune diseases

Abstrakt

Střevní imunitní systém vyžaduje mechanismy, které zajistí rovnováhu mezi potlačením a tolerancí mikrobioty, regulací prospěšných a potlačením patogenních mikrobů. Jeden z takových mechanismů je založen na mikrobiota specifických Rorγt+ T regulačních buňkách (Tregs), které mohou regulovat imunitu po rozpoznání specifického antigenu. Předpokládá se, že Rorγt+ antigen prezentující buňky (APC) podporují rozvoj mikrobiota specifických Tregs, ale konkrétní mechanismus byl donedávna nedostatečně popsán. Ve své bakalářské práci uvádím úvod do biologie Tregs, porovnávám a podrobně rozebírám studie naznačující možnou roli LTi like ILC3, Janusových buněk a nově objevených Thetisových buněk v generaci Rorγt+ Tregs a také možný molekulární mechanismus založený na integrinech Itgav a Itgb8.

Klíčová slova:

Rorγt+ antigen-prezentující buňky, periferní tolerance, střevní mikrobiota, regulační T buňky (Tregs), imunitní homeostáza, autoimunitní onemocnění

Contents

Abbreviations

Aire	Autoimmune regulator
Akt	Protein kinase B
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AP-1	Activator protein 1
APC	Antigen presenting cells
CNS	Conserved noncoding regulatory site
Cxcr6	C-X-C chemokine receptor 6
CXCL1	C-X-C chemokine ligand 1
Ccr7	C-C chemokine receptor 7
CCL20	C-C chemokine ligand 20
CTLA-4	Cytotoxic T lymphocyte antigen 4
CIITA	Class II major histocompatibility complex transactivator
Clec9a	C-type lectin domain family 9 member A
DC	Dendritic cells
DDIT4	DNA-damage-inducible transcript 4
Dpp4	Dipeptidyl peptidase-4
Ebi3	Epstein-Barr virus-induced gene 3
EGFR	Epidermal growth factor receptor
Epcam	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
eTACs	Extrathymic Aire-expressing cells
eGFP	Enhanced green fluorescent protein
Foxp3	Forkhead transcription factor 3
GALT	Gut-associated lymphoid tissue
IBD	Inflammatory bowel disease

IL-12R	Interleukin 12 receptor
ILC	Innate lymphoid cell
ILC3	Group 3 innate lymphoid cell
ILFs	Isolated lymphoid follicles
IFNγ	Interferon gamma
ITAM	Immunoreceptor tyrosine-based activation motif
IgA	Immunoglobulin A
Ikzf2	Zinc finger protein Helios
Itg	Integrin
JC	Janus cells
JNK	Jun amino-terminal kinases
LAG-3	Lymphocyte-activation gene 3
Lck	Lymphocyte-specific protein tyrosine kinase
LP	Lamina propria
LI	Large intestine
LTi-like ILC3s	Lymphoid tissue inducer like ILC3s
Ly75	Lymphocyte antigen 75
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Mgat5	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A
mTECs	Medullary thymic epithelial cells
mTOR	Mammalian target of rapamycin
mLN	Mesenteric lymph nodes
Ncam1	Neural cell adhesion molecule 1
NK	Natural killer cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

PP	Peyer's patches
pTreg	Peripheral Treg
RAG	Recombination-activating gene
RegIII	Regenerating islet-derived protein 3
RORγt	RAR-related orphan receptor gamma t
SAPK	Stress-activated protein kinases
SCFAs	Short-chain fatty acids
Sca-1	Stem cell antigen-1
scRNA-seq	Single-cell RNA-sequencing
Siglec-G	Sialic acid binding Ig-like lectin G
Smad3	Mothers against decapentaplegic homolog 3
SFB	Segmented filamentous bacteria
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAT	Signal transducer and activator of transcription
T-bet	T-box transcription factor Tbx21
TC	Thetis cells
TCR	T cell receptor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TGF	Transforming growth factor
Treg	T regulatory cells
TF	Transcription factor
tTreg	Thymic Treg
TNF	Tumor necrosis factor
TNFRSF18	Tumor necrosis factor receptor superfamily member 18
Th	T helper cells
UTR	Untranslated region
Zbtb46	Zinc finger and BTB domain-containing protein 46

Introduction

The immune system is responsible for maintaining the homeostasis of the organism, promoting its survival and defense against external pathogens. A disorder in central tolerance can lead to the development of an immune response against the body's own tissues that can progress to T cell- or B cell-mediated autoimmune diseases (Rose & Bona, 1993) (Bieber et al., 2023). T cells undergo negative selection and self-reactive cells are eliminated during their development, which is one of the main mechanisms responsible for central tolerance (Kyewski & Klein, 2006). However, because some self-reactive T cells avoid negative selection in the thymus (Harrington et al., 1998) (Bouneaud et al., 2000), there must be another mechanism that suppresses immunity to harmless or self-antigens. One such mechanism is mediated by T regulatory cells (Treg) (Asano et al., 1996) (Hori et al., 2003). Initial evidence that T cells can actively prevent autoimmune diseases comes from thymectomy experiments, where the removal of the thymus from a mouse between the second and fourth day of life resulted in autoimmune inflammation that could be prevented by the transfer of T cells from the thymus or spleen of a not thymectomized mouse (Sakaguchi et al., 1982) (Nishizuka & Sakakura, 1969) (Asano et al., 1996). Later, T regulatory cells were associated with CD4+ T cells population expressing high levels of interleukin (IL)-2 receptor α-chain (Sakaguchi et al., 1995). IL-2 receptor consists of three chains: α (*CD25*), β (*CD122*) and γ (*CD132*) where CD25 ensures the high affinity of the receptor to IL-2, but the receptor is functional even without it (Ross & Cantrell, 2018). IL-2 receptor activation via signal transducer and activator of transcription 5 (STAT5) signaling cascade promotes expression of the transcription factor *forkhead transcription factor 3* (*Foxp3*) (Burchill, Yang, Vogtenhuber, et al., 2007). *Foxp3* gene is located on X-chromosome and it is the major transcription factor (TF) of Tregs required for their lineage identity (Fontenot et al., 2003) (Khattri et al., 2003) (Hori et al., 2003). Foxp3 is essential for the maintenance of suppressive functions of mature Tregs (Williams & Rudensky, 2007), and strikingly it is not only the presence of Foxp3, but also the level of its expression that plays a key role in the maintenance of Tregs suppressive functions. Low expression levels of Foxp3 may lead to the reconversion of Tregs back into effector cells (Y. Y. Wan & Flavell, 2007). The broadly defined group of CD25+ Foxp3+ Tregs can be divided into several subsets. Based on Tregs development in either the thymus or the immune periphery we can distinguish thymic Tregs or natural Tregs (tTreg) and the peripheral Tregs or induced ones (pTreg) (Josefowicz et al., 2012).

1. Development of Tregs

1.1. Development of thymic Tregs

tTregs undergo a classical T-cell developmental pathway starting with the migration of lymphoid precursor from the bone marrow to the thymus where it undergoes V(D)J recombination, during which lymphoid-specific recombinase (RAG), an ubiquitously expressed DNA repair proteins mediate the somatic assembly of T cell receptor (TCR) genes (Bassing et al., 2002). This is followed by the positive selection in the cortex of the thymus, where developing T-cells test their reactivity to host-encoded MHC molecules (Klein et al., 2014). Depending on whether the T cell receptor (TCR) can interact with MHCII or MHC I, T cells become CD4+ or CD8+ single-positive cells, respectively, proliferate and undergo negative selection in the medulla of the thymus (Klein et al., 2014). During the negative selection, cells whose TCRs bind too strongly to the MHC self-antigen complex receive a

programmed cell death signal from the antigen-presenting cell (APC) or they are converted to tTregs. Based on the affinity model, the tTregs bear TCR with high affinity to self-antigen presented on MHC class II, but not high enough to be self-reactive. Cells that have low affinity to self-antigens become conventional T cells (Klein et al., 2014). Medullary thymic epithelial cells (mTECs) are essential for the negative selection as they can present tissue-restricted antigens because of the expression of a transcription factor called autoimmune regulator (Aire) which allows them to promiscuously express a wide spectrum of tissue-restricted antigens (Derbinski et al., 2001)(Aschenbrenner et al., 2007). Also, dendritic cells (DCs) can contribute to this process by self-antigen presentation either by bringing self-antigens from the periphery or by acquiring them from mTECs (Joffre et al., 2012). The development of Foxp3+ Tregs requires a costimulatory signal via CD28 molecule, which interacts with its ligands CD80 and CD86 on the surface of the APC (Salomon et al., 2000). Part of the reason for this is that stimulation of CD28 involving lymphocyte-specific protein tyrosine kinase (Lck) leads to activation of *IL-2* enhancer, which leads to increased *IL-2* expression, that in turn promotes the development of Foxp3+ Tregs by stimulating *Foxp3* expression and activating STAT5 signaling pathway (Burchill, Yang, Vogtenhuber, et al., 2007) (Fontenot et al., 2005) (Tai et al., 2005). Furthermore, it is suggested that CD28 via tyrosine kinase Lck directly rather than via IL-2 contributes to the expression of *Foxp3* (Tai et al., 2005).

1.2. Development of peripheral T regulatory cells

One of the earliest studies suggesting the existence of a T regulatory cell subtype, whose origin is independent of the thymus, was the study by Sakaguchi et al. Thymus-less Nude mice develop multiorgan autoimmune diseases. However, as the study showed, transfer of CD4+ CD25- T cells into this mice resulted in the restoration of the level of CD4+CD25+ cells and protected them from the development of autoimmune diseases (Sakaguchi et al., 1995). pTregs, in contrast to tTregs, arise upon TCR-mediated recognition of APC-presented antigen in the periphery of the body. Whereas tTreg reactivity repertoire is mainly restricted to self-antigens, pTregs are responsible for tolerance to a relatively broader scope of antigens including foreign ones like microbial or food antigens (Lathrop et al., 2011). Since pTreg are often induced by foreign antigens in the periphery, they are mainly studied in the gastrointestinal system (Wolff et al., 2012) and the skin (Scharschmidt et al., 2015). Mucosal sites are in close contact with the environment due to their large surface area and Treg are present there in large numbers.

In my bachelor thesis, I will mainly focus on the pTregs specific to microbial antigens and localized mainly in the lymphatic system associated with the intestine. Microbiota-specific Tregs can be broadly distinguished from other Tregs by the presence of *RAR-related orphan receptor gamma t* (*RORγt*) expression (Lochner et al., 2008), which is also a marker for T helper 17 cells (Th17) and group 3 innate lymphoid cell (ILC3) (Ivanov et al., 2006) (Eberl et al., 2004). RORγt is a ligand-dependent TF which is a shorter isoform of the related RORγ protein encoded by the *RORc* gene (Hirose et al., 1994) (He et al., 1998). Since RORγt+ Tregs are microbiota-specific, it is not surprising that microbiota-derived metabolic products such as short-chain fatty acids and retinoic acid induce the emergence of RORγt+ Tregs (Ohnmacht et al., 2015) (Nurieva et al., 2007) (Furusawa et al., 2013). This is consistent with the fact that in the mouse during weaning (between the 2nd and 3rd week of life) RORγt+ Tregs increase significantly. This can probably be explained by an increase in microbiota diversity (Al Nabhani et al., 2019).

Commonly, pTreg require for their development TGF-beta signaling, which via its receptor and through the Smad3 and NFAT signaling pathway leads to the activation of conserved noncoding regulatory sites 1 (CNS1) in the *Foxp3* locus that activates a *Foxp3* enhancer that increases its expression (Tone et al., 2007). It has been suggested that low TGF-beta concentrations, together with the pro-inflammatory cytokines like IL-6 and IL-21, promote IL-23 receptor expression and together leads to Th17 development, whereas high concentrations of TGF-beta suppresses IL-23 receptor expression and promotes pTreg development. (Zhou et al., 2008) (Bettelli et al., 2006). Thus, the main cytokines for pTreg generation are TGF-beta and IL-2, whereas Th17 polarization requires IL-6, IL-21 and IL-23 for its development (Burchill, Yang, Vang, et al., 2007) (Zhou et al., 2008) (Bettelli et al., 2006) (Nurieva et al., 2007) (Zhou et al., 2007) (Korn et al., 2007) (Mangan et al., 2006).
Besides the already mentioned differences between pTreg and tTreg there is evidence that CNS1 in the *Foxp3* locus, unlike CNS2 and CNS3, is required for pTreg development and does not affect tTreg development, thus distinguishing these two populations on the molecular level (Zheng et al., 2010). It is assumed that zinc finger transcription factor Helios (also called Ikzf2) and Neuropilin 1 which is a type 1 transmembrane receptor involved in semaphorin signaling and vascular endothelial growth factor signaling can be used as the markers of tTregs (Guo & Vander Kooi, 2015) (Thornton et al., 2010) (Thornton et al., 2019). However, there are studies demonstrating that these markers can be also expressed by a fraction of pTreg (Gottschalk et al., 2012) (Akimova et al., 2011) (Szurek et al., 2015).

2. Molecular mechanism of T regulatory cells functioning

Irrespective of their type, Tregs use several mechanisms to perform their suppressive function. They can be divided into molecules located on the plasma membrane of Tregs and performing suppressive functions by enzymatic or signaling activity and into molecules that exert their suppressive function after being secreted (Tab.1.).

Name of molecule	*Function description*	*Reference*
Membrane molecules		
CD25	Limits the availability of IL-2 to other effector T cells	(Pandiyan et al., 2007)
CD39	Creates a suppressive environment for the immune system by converting extracellular ATP to AMP	(Borsellino et al., 2007)
CD73	Conversion of AMP into adenosine whose signaling via A2A inhibits lymphocyte proliferation and cytokine expression	(Thiel et al., 2003) (Bopp et al., 2007) (Kobie et al., 2006) (S. Huang et al., 1997)
CTLA-4	Down-regulates *CD80* and *CD86* expression in dendritic cells	(Friedline et al., 2009) (Wing et al., 2008)
LAG-3	Inhibits dendritic cells by interacting with MHCII and also inhibits proliferation of natural killer (NK) cells, activation of CD4+ T cells and CD8+ T cells and stimulates the suppressive function of Treg	(Liang et al., 2008) (C. T. Huang et al., 2004) (Durham et al., 2014) (F. J. Li et al., 2013) (Byun et al., 2007)
TIGIT	Down-regulates *CD80* and MHCII expression in dendritic cells, inhibits T cells through suppression of the TCR signaling pathway, inhibits NK cells, and stimulates IL-10 production through Fgl2 signaling	(Chan et al., 2003) (Joller et al., 2014) (Levin et al., 2011) (Joller et al., 2011) (M. Li et al., 2014) (Liu et al., 2012)
Secreted molecules		
IL-10	A major anti-inflammatory cytokine. A broad spectrum of effects on effector cells. Promotes *Foxp3* expression	(Fiorentino et al., 1991) (Coomes et al., 2017) (Huber et al., 2011) (Turovskaya et al., 2009) (Ip et al., 2017) (Sauer et al., 2008) (Delgoffe et al., 2009)
TGF-beta	Suppresses differentiation in Th1 and Th2 and promotes differentiation in Th17 and Treg direction	(Nandan & Reiner, 1997) (Gorelik et al., 2002) (Gorham et al., 1998) (Gorelik et al., 2000) (Mangan et al., 2006)
IL-35	Induction of Treg proliferation	(Collison et al., 2012) (R. X. Wang et al., 2014)
perforins and granzymes	Lysis of effector cells	(Grossman et al., 2004)

Tab. 1. Molecules responsible for the suppressive function of T regulatory cells

2.1. Membrane molecules

As mentioned above, Tregs are characterized by high expression of CD25, which is a high-affinity subunit of the IL-2 receptor. Because Treg must consume IL-2 for their survival (Burchill, Yang, Vang, et al., 2007), but they do not produce it, they limit other effector T cells' access to this cytokine (Pandiyan et al., 2007).

A more direct anti-inflammatory effect is exerted by ectoenzyme CD39. It converts extracellular adenosine triphosphate (ATP), which appears in the intercellular space during tissue damage and contributes to the inflammatory response as the danger-associated molecular pattern (Vitiello et al., 2012) (P. Wan et al., 2016), into adenosine monophosphate (AMP), thus limiting the pro-inflammatory process (Borsellino et al., 2007). The effect of CD39 is synergic with another ectoenzyme expressed by Tregs, CD73. This enzyme converts AMP to adenosine, which in turn serves as a signaling molecule inhibiting the proliferation (S. Huang et al., 1997) and cytokine expression of effector Th1 and Th2 cells via A2A adenosine receptors (Kobie et al., 2006) (Bopp et al., 2007) (Thiel et al., 2003).

A fundamentally different way is used by Tregs to suppress antigen-presenting cells and their key activity of initiation of the immune response (Chen & Flies, 2013). One of the major molecules responsible for this function is cytotoxic T lymphocyte antigen 4 (CTLA-4) also known as CD152. The signal received by dendritic cells from CTLA-4 contributes to the downregulation of *CD80* and *CD86* expression. Thus, Tregs reduce the ability of dendritic cells to supply costimulatory signals to T lymphocytes, which is important for their activation, proliferation and overall this limits their effector function (Wing et al., 2008) (Friedline et al., 2009).

Another way how Tregs modulate DC activity is their ability to interact with MHCII directly by the transmembrane protein lymphocyte-activation gene 3 (LAG-3, CD233) (Huard et al., 1997). It has been demonstrated that expression of LAG-3 on CD4+ T cells contributes to the ability of Treg to regulate their proliferation capacity, presumably through effects on the IL-2 and STAT5 signaling pathway (Durham et al., 2014). In addition, LAG-3 interacts with MHCII and through the immunoreceptor tyrosine-based activation motif (ITAM) signaling pathway and activation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) suppresses the ability of DC to present antigens (Liang et al., 2008) (C. T. Huang et al., 2004) (Huard et al., 1994).

A molecule functionally comparable with LAG-3 and also expressed by Treg is the protein T cell immunoreceptor with Ig and ITIM domains (TIGIT) (Fuhrman et al., 2015). TIGIT can also directly interact with T cells by inhibiting their TCR signaling pathway and limiting their ability to be activated and proliferate (Levin et al., 2011) (Joller et al., 2011). TIGIT stimulation induces in Treg expression of Fgl2 in a CEBPa-dependent manner. Fgl2 is known for its ability to inhibit DC maturation through down-regulation of NF-κB, CD80 and MHCII expression and thus prevents polarization of effector T cells (Chan et al., 2003) (Joller et al., 2014). In addition, the same study indicates that Fgl2 regulates the production of suppressive cytokine interleukin 10 (IL-10) (Joller et al., 2014).

2.2. Secreted molecules

IL-10 also known as cytokine synthesis inhibitory factor is one of the major anti-inflammatory cytokines and one of the major effector molecules of Tregs (O'Garra et al., 2004). IL-10 which is known to also affect macrophages and dendritic cells in an antigen-

nonspecific manner, presumably by affecting their cytokine production (Fiorentino et al., 1991). IL-10 interacts with IL-10R on the surface of Th2 and induces the expression of granzyme B. Th2 start to express granzyme B, which causes restimulation of TCR and CD3 complex and their TCR-induced cell death (Coomes et al., 2017) (Oberg et al., 1997) (Devadas et al., 2006). IL-10 also directly inhibits the proliferation of Th17 cells through its receptor IL-10R-alfa. (Huber et al., 2011). Furthermore, IL-10 can directly affect CD8+ T cells by stimulating the expression of alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A (Mgat5), which through enhancing N-glycan branching on surface glycoproteins prevents TCR-mediated activation of CD8+ T cells (L. K. Smith et al., 2018). In addition, IL-10 promotes Treg development by enhancing *Foxp3* expression (Turovskaya et al., 2009). A possible mechanism for this is the ability of IL-10 to upregulate *DNA-damage-inducible transcript 4* (*DDIT4*) which product inhibits the mammalian target of rapamycin (mTOR) (Ip et al., 2017), which may lead to increased *Foxp3* expression levels (Sauer et al., 2008) (Delgoffe et al., 2009).
Together with IL-10, TGF-beta is one of the most important cytokines for Treg functionality and development. It has a wide range of suppressive functions, such as limiting the ability of APCs to present antigen by downregulating the MHC class ll expression via inhibition of class II trans-activator (CIITA) (Nandan & Reiner, 1997). It also inhibits polarization of naive T cells to Th1 by inhibiting *T-box transcription factor Tbx21* (*T-bet*) expression which is required for IL-12 receptor beta chain expression which in turn leads to decreased sensitivity to IL-12 and the ability to differentiate into Th1 cells (Gorelik et al., 2002) (Gorham et al., 1998). Similarly, TGF-beta inhibits differentiation in Th2 by down-regulating *Gata-3* expression, the major transcription factor of the Th2 lineage (Gorelik et al., 2000). In contrast, TGF-beta contributes to Th17 development by up-regulation of IL-23 receptor expression (Mangan et al., 2006). The combined effect of IL-10 and TGF-beta is also important for the inhibition of B cell activation (Komai et al., 2018).

IL-10 and TGF-beta are considered to be the main cytokines produced by Tregs. Among the less studied, but still important cytokines, belongs IL-35, which was initially thought to be Treg-specific but it was latter shown to have a regulatory function also when expressed by other cells, such as tolerogenic DCs or B cells (Shen et al., 2014) (Dixon et al., 2015) (Collison et al., 2007) (Niedbala et al., 2007). IL-35 is part of the IL-12 cytokine family, which includes IL-12 (Kobayashi et al., 1989), IL-23 (Oppmann et al., 2000), IL-27 (Pflanz et al., 2002), IL-35 (Collison et al., 2007) and probably IL-39 (Wang et al., 2016). IL-35 is a heterodimer consisting of the beta-chain Epstein-Barr virus-induced gene 3 (Ebi3) protein and the alpha-chain p35. This structure enables IL-35 binding to four different receptors: two homodimers IL-12Rβ2/IL-12Rβ2 and gp130/gp130, and two heterodimers IL-12Rβ2/gp130 and IL-12Rβ2/ IL-27Rα (Collison et al., 2012) (R. X. Wang et al., 2014). IL-35 via IL-12Rβ and IL-27Rβ receptor subunits activate STAT1 and STAT3 signaling pathways and induce differentiation of B cells into regulatory B cells which are capable of producing IL-35 and thereby promoting Treg proliferation (R. X. Wang et al., 2014). Mechanistically, the ability of IL-35 to convert naive T cells into IL-35 expressing regulatory T cells through activation of STAT1 and STAT4 signaling pathways has also been demonstrated (Collison et al., 2012).

Tregs can use the perforin/granzyme pathway (Grossman et al., 2004). It is a protein cascade that activates the programmed cell death in the target cell. Perforin creates pores in the membrane of the target cell, allowing granzymes to enter the cell and trigger cell death within its interior (Voskoboinik et al., 2015). Interestingly tTregs can be distinguished from pTregs

by granzyme A expression, while granzyme B is expressed in the later (Grossman et al., 2004).

3. Intestinal immunity

3.1. Innate immunity

Before discussing the development of peripheral RORγt+ Tregs, I will briefly describe how the immunity in the intestine is organized. Between the intraepithelial environment of the intestine and its lumen is a layer of epithelial cells, interconnected by the apical junctional complex based on cadherins and occludins, which limit paracellular transport between the lumen and the internal environment of the body, thus contributing to the separation of these two environments (Farquhar & Palade, 1963) (Odenwald & Turner, 2017). Above the epithelium is a mucosal barrier created and maintained by epithelial cells, mainly due to mucin secretion by goblet cells (Pelaseyed et al., 2014). In addition to forming the physical barrier, intestinal epithelial cells produce antimicrobial peptides. An example in the mouse is the C-type lectin RegIII protein family (Vaishnava et al., 2011) that bind to bacteria via peptidoglycan carbohydrate and kill them by forming a hexameric membrane-permeabilizing oligomeric pore and also by preventing microbiota contact with the epithelium (Mukherjee et al., 2013) (Vaishnava et al., 2011). Other antimicrobicidals include Lysozyme C, which hydrolyzes proteoglycans in bacterial cell walls (Deckx et al., 1967) (Callewaert & Michiels, 2010) and phospholipase A2, which hydrolyzes cell membrane phospholipids, exerting a bactericidal effect on gram-positive but not gram-negative bacteria (Qu & Lehrer, 1998). In addition, Paneth cells also secrete alpha-defensins (Ayabe et al., 2000), such as cryptdins, which kills bacteria by permeabilization of bacterial membranes (Bevins, 2005) (Hadjicharalambous et al., 2008).

3.2. Gut-associated lymphoid tissue

The Gut-associated lymphoid tissue (GALT) includes the mesenteric lymph nodes (mLN) network near the intestine (Van den Broeck et al., 2006) (Brandtzaeg et al., 2008), Peyer's patches (PP) beneath the intestinal epithelium (Cornes, 1965), and isolated lymphoid follicles (ILF) mainly in the small intestine. Also, GALT includes the intraepithelial lymphocytes and the immune cells in the *lamina propria* (LP) (Hu & Edelblum, 2017). GALT is the major place for lymphocyte clustering, where they interact with each other and develop organized immune responses to external conditions.

3.3. Adaptive immunity

Adaptive immunity as well as innate immunity is able to control bacteria in the lumen of the intestine utilizing many effector molecules including immunoglobulin A (IgA). IgA is secreted by antigen-producing B cells and then binds through its J chain to the polymeric immunoglobulin receptor on epithelial cells. Then by so-called transcytosis it is transported to the lumen of the gut (Brandtzaeg & Prydz, 1984), where it is able to cause agglutination of dividing bacteria and contribute to their elimination (Moor et al., 2017). Among T cells, Th17 cells play a major role in the microbiota-specific inflammatory immune response coined by IL-17A and IL-22 production (Park et al., 2005) (Fujino et al., 2003). IL-17A together with

TNF-α activates epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways that positively regulates the production of the chemokines CXCL8 and CXCL1 responsible for recruitment of neutrophils (Das et al., 2010) (Sawant et al., 2016) and CCL20 recruiting Th17 cells (Hirota et al., 2007) (Zhang et al., 2015) (Lee et al., 2008). Also, through its receptor, IL-17A induces myeloid and endothelial cells to produce IL-6, which promotes mucin production and Th17 development, as well as TNF-alpha and IL-1-beta, which are pro-inflammatory cytokines (Kolls & Lindén, 2004) (Zhou et al., 2007). IL-22 is also known to promote goblet cell mucin production through STAT3 signaling pathway (Sugimoto et al., 2008) and through its receptor can also activate Akt, ERK1/2, and Stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) MAPK promoting intestinal epithelial cell proliferation and enhancing the expression of antimicrobial protein human beta-defensin-2 (Brand et al., 2006).

3.4. Beneficial effects of microbiota and its influence on Th17/Treg balance

Normally, the human bacterial microbiota is predominantly composed of phyla Bacteroidetes, Firmicutes and Actinobacteria, but if the mechanisms regulating the microbiota are disturbed, other commensal bacteria and opportunistic pathogens also may begin to overgrow, causing dysbiosis (Kumar et al., 2016) (Lupp et al., 2007) (Stecher et al., 2007) (Arumugam et al., 2011). The Th17 through IL-17A signaling pathway can inhibit the growth of microbiota and disruption of this function can lead to bacteria overgrowth and consequent dysbiosis (Kumar et al., 2016) (Ishigame et al., 2009). Studies suggest that the Th17-based microbiota regulation mechanism should also be regulated, presumably through Roryt+ Tregs, as their dysfunction leads to Th17-based inflammation such as inflammatory bowel disease (IBD) or colitis (B. H. Yang et al., 2016) (Kim et al., 2017) (Britton et al., 2019) (Chaudhry et al., 2009) (Leppkes et al., 2009) (Ogino et al., 2011).
Tregs expressing Roryt have been identified relatively recently and have been studied as a distinct cellular subtype of microbiota-specific Tregs with tolerogenic effects on immunity in the gut, such as suppressing the Th2 immune response (B. H. Yang et al., 2016) (Ohnmacht et al., 2015). Later, Suniti Bhaumik et al. studied the mechanism of Roryt action in more detail. They demonstrated that deletion of Roryt in Treg leads to decreased Foxp3 expression and loss of suppressive function, but simultaneous deletion of T-bet restored Foxp3 expression. This suggests that Roryt suppresses T-bet, which in turn is a negative regulator of Foxp3 which is the main TF responsible for the suppressive function of Treg (Bhaumik et al., 2021). The study by Esen Sefik et al. indicated Roryt+ Tregs and induced by specific members of the microbiota (Sefik et al., 2015). It is also known that colonization of the mouse intestine with specific bacterial species such as Helicobacter hepaticus or segmented filamentous bacteria (SFB) also can induce the emergence of RORγt+ Treg (M. Xu et al., 2018) or Th17 (Y. Yang et al., 2014), respectively. One factor influencing the balance between Roryt+ Treg and Th17 is that the cytokine environment for differentiation of these two cell types is very similar and they both require TGF-beta for their development and both express the transcription factor Roryt (Zhou et al., 2008). Given the proximity of these two cell types, it's not surprising that Treg can, in the presence of inflammatory cytokines such as IL-6 and in the absence of TGF-beta, re-convert to Th17 (L. Xu et al., 2007). Maintaining this balance is necessary because a normal microbiota also carries beneficial effects for the body, such as inhibiting the growth of pathogens by competing for limiting nutrients and ecological niche (Guiot, 1982) (Wilson & Perini, 1988). The microbiota, for example *Lactobacillus intestinalis,* is also actively involved in vitamin A metabolism, synthesizing retinoic acid (Bonakdar et al., 2022), as well as being known for their ability to produce short-chain fatty acids (SCFAs), such as acetate, propionate

and butyrate (Velázquez et al., 2000) (Bourquin et al., 1992), through which it can have a tolerogenic effect. For example, a study by Patrick M. Smith et al. shows that SCFAs stimulate Treg proliferation and affect the manifestation of colitis (P. M. Smith et al., 2013). As has been shown, the composition of the microbiota can also have a complex effect on the balance between Roryt+ Treg and Th17. Studies from Britton et al. demonstrate that transferring microbiota from IBD patients to germ-free mice leads to an increase in Th17 cells and a decrease in the RORγt+ Treg population, whereas microbiota from healthy patients did not induce such an effect (Britton et al., 2019).
In addition, there are studies showing an ambiguous role for RORγt+ Treg, for example some studies indicate that RORγt+ Treg may have inflammatory effects. In human pancreatic ductal adenocarcinoma, RORγt+ Tregs have been found to express *IL-17*, *IL-6* and other pro-inflammatory cytokines (Chellappa et al., 2016). Another example of this dual function of RORγt+ Treg is systemic lupus erythematosus where they secrete IL-17 and have a complex impact on disease progression (Kluger et al., 2016). Besides the anti-inflammatory effects in the gut, it has recently been demonstrated that RORγt+ Treg can promote regeneration of injury outside the gut by participating in the regulation of the balance of proliferation and differentiation of muscle stem cells (Hanna et al., 2023). This indicates that the function of RORγt+ Tregs as peripheral Tregs is not only focused on the microbiota but may have a broader spectrum of effects. However, the process of RORγt+ Treg generation has been poorly understood until recently.

4. Role of Rorγt+ APC in microbiota-specific-Treg cells differentiation

Over the past decade evidence has emerged that some cell types such as ILC3 (Hepworth et al., 2013) (Hepworth et al., 2015), DC (Brown et al., 2019) (Papaioannou et al., 2021) and extrathymic Aire-expressing cells (eTACs) (Yamano et al., 2019) (J. Wang et al., 2021) are capable of expressing *Roryt* and MHCII and can contribute to Roryt+ Treg generation.

4.1. Rorγt+ APC types

4.1.1. Conventional dendritic cells as Rorγt+ APC

Conventional dendritic cells include Xcr1+ type 1 (cDC1) and Sirpa+ type 2 (cDC2) characterized by the expression of markers *CD1c*, *CD11c* and *CD11b* (Villani et al., 2017). In mouse spleen, a subtype of cDC2 called cDC2B was found to be able to express *Rorγt* reporter contributing to the complexity of Roryt+ APCs (Brown et al., 2019). However cDC2B were not identified among other Roryt+ cells in the paper from Mengze Lyu et al. (Lyu et al., 2022). That may be explained by the fact that Rorc/Rorγt+ cDC2 were originally found in the spleen and not in the mLN from where the cells were taken, or by the fact that cDC2B is a rather rare population, comprising about 0.2 percent of Rorc+ cells in mouse spleen (Brown et al., 2019), although Ranit Kedmi et al. indicate that using specific fate mapping for *Roryt* they found cDC of both types (cDC1 and cDC2) in mouse mLNs to have history of the *Roryt* locus activation. But according to their data, none of the cDC types they detected actively expressed Rorc in the time of analysis, so presumably they also did not observe cDC2B in mLN (Kedmi et al., 2022).

4.1.2. Innate lymphoid cells as Rorγt+ APC

ILCs are a cell type related to innate immunity, lacking recombination activating gene (*RAG*)-dependent rearranged of antigen receptors as well as phenotypic markers characteristic of myeloid cells and dendritic cells, and being morphologically similar to the lymphoid lineage (Spits et al., 2013). They are categorized into three groups based on their expression profile. ILC1s are characterized by *IFNγ* expression and dependence on T-bet in their development. ILC2s are defined by the ability to express Th2 cell-associated cytokines such as *IL-5*, *IL-6*, *IL-9*, *IL-13* and require IL-7 and Rora for their development (Ghaedi et al., 2020) (Spits et al., 2013). ILC3s are defined by their ability to produce IL-17A and IL-22 and also require Rorγt for their development (Spits et al., 2013). There is evidence that some ILC3s, in particular the lymphoid tissue inducer (LTi-like) ILC3s, can express MHCII (Hepworth et al., 2015) and that its deletion leads to intestinal inflammation driven by commensal bacteria-specific CD4+ T cells (Hepworth et al., 2013). The role of LTi-like ILC3s in Roryt+ Treg generation is discussed in more detail in a paper from Mengze Lyu et al. They used a genetically modified mouse whose cells expressed enhanced green fluorescent protein (*eGFP*) along with *Rorγt*, which allowed them to track Rorγt expression in cells. Single-cell RNA-sequencing (scRNA-seq) was used to analyze the transcripts of each individual Rorγt+ cell from the mLN of such a mouse. In addition to Treg, Th17 and γδ T cells were identified subtypes of ILCs, including LTi-like ILC3s, T-bet+ ILC3s and a small cluster of ILC2s. In addition, there were two clusters of eTACs with different expression of *Aire* (Lyu et al., 2022). In their article, they demonstrated differences in expression profiles between Aire+ Rorγt+ eTACs and LTi-like ILC3s. According to their data, eTACs have higher expression of sialic acid binding Ig-like lectin G (Siglec-G), dipeptidyl peptidáza-4 (Dpp4) and integrin alfa4 beta7 (Itga4b7), whereas LTi-like ILC3s have higher expression levels of Rorγt, CD25, C-X-C chemokine receptor typu 6 (Cxcr6) and stem cell antigen-1 (Sca-1) (Lyu et al., 2022).

4.1.3. Extrathymic Aire-expressing cells as Rorγt+ APC

eTACs are also capable of expressing MHCII and some of them co-express Aire and Roryt (Yamano et al., 2019). Such cells were termed Janus cells (JC) by others to highlight their unique properties (J. Wang et al., 2021), according to the recent report by Ranit Kedmi et al. they can be transcriptionally divided into three types (JC1- JC3), of which only JC1 and JC2 significantly express *Aire*, *Roryt, H2-Aa* and *H2-Ab1*, while the expression level of these markers in JC3 is much lower. However, JC2 and JC3 are characterized by high expression levels of *Itgav* and *Itgb8*, which will be important for us in the future discussion (Kedmi et al., 2022).

4.1.4. Thetis cells as Rorγt+ APC

Recently, in contrast to previous reports (Lyu et al., 2022) (Kedmi et al., 2022) (Hepworth et al., 2015) (Yamano et al., 2019), a study from Blossom Akagbosu et al. characterized a cell type that they called Thetis cells (TC) (Akagbosu et al., 2022). They identified them using scRNA-seq and single-cell assay for transposase-accessible chromatin of a genetically modified mouse they created. The mouse was engineered to express *Venus* fluorescent protein and a *creERT2* fusion protein, which activates Cre recombinase only when tamoxifen or its derivative is present. This construct was located downstream of exon 11 and upstream of the 3' untranslated region (UTR) end of the *Rorc* gene ($Rorc^{Venus\text{-}creERT2}$) (Akagbosu et al., 2022). Unfortunately, such a configuration is not *Rorγt* specific, as *Rorγt* and *Rorγ* differ in the first hundred nucleotides of the gene and share a common 3'UTR end (He et al., 1998) (Medvedev

et al., 1997). Because of this, the entire construct would be expressed with both *Rorγ* and *Rorγt* and such a model would not be specific for *Rorγt*. Further phenotyping of TCs allowed them to divide Rorc expressing cells to several TC groups (TC I - TC IV). TC I and TC III in contrast to TCII and TC IV have a significant level of Aire expression. However, TC IV and TC III have high expression of Cd11c and pseudo-bulk transcriptome analysis suggested that they belongs to cells previously identified by the same group as cDC2B (Brown et al., 2019). The intrinsically high level of Cd11c expression is characteristic of all TCs except TC I, and TC IV also expresses Cd11d. TC I and TC II are distinguished from other TCs by the highest level of MHCII expression and Ccr6 expression. However, TC I is distinguished from TC II by the expression of Ncam1 (CD56) and the absence of Epcam (CD326) expression. In addition, TC IV has the highest expression level of Itgav and Itgb8 compared to the other TCs, which will be important for us in the future discussion (Akagbosu et al., 2022).

4.2. Confirmation of impact Roryt+ MHCII+ cells on the generation of Roryt+ Treg

Two years ago, three new articles almost simultaneously published in Nature journal suggested that Roryt+ APCs are responsible for Roryt+ Treg generation (Akagbosu et al., 2022) (Kedmi et al., 2022) (Lyu et al., 2022). The articles by Ranit Kedmi et al., Mengze Lyu et al. and Blossom Akagbosu et al. studied the abundance of Rorγt+ Treg in a genetically modified mouse in which MHCII expression in *Rorγt*-expressing cells was turned off. This was achieved by Cre-mediated recombination guided by *Rorγt* (*Rorγt*^{Cre}) of floxed *H2-Ab1* MHCII subunit. Although the groups used mice of different ages to test the ability to generate Rorγt+ Treg, all three groups observed a significant drop in Rorγt+ Treg population compared to the control group in both LP and mLN (Akagbosu et al., 2022) (Kedmi et al., 2022) (Akagbosu et al., 2022). All three experiments prove that there are RORγt+ MHCII+ cells responsible for the emergence of Rorγt+ Treg (Akagbosu et al., 2022) (Kedmi et al., 2022) (Lyu et al., 2022).

In addition, another experiment described in an article from Ranit Kedmi et al. clearly demonstrates the need for MHCII specifically on Rorγt+ cells to generate pTreg. In the course of the experiment, mice had their bone marrow removed by irradiation and then replaced with genetically modified bone-marrow, thus producing chimeric mice. The mouse with bone marrow cells deprived of the ability to express MHCII on Cd11c+ cells (MHCII$^{\Delta Cd11c}$) was not able to generate pTreg. Whereas a mouse that in addition has half of the bone marrow consisting of cells with constant expression of MHCII on Rorγt+ cells was able to fully generate pTreg (Kedmi et al., 2022). Thus, this experiment shows that in addition to Roryt+ cells, the generation of Roryt+ Treg is promoted by Cd11c+ cells, as their deletion leads to a drop in Roryt+ Treg abundance (Kedmi et al., 2022). On the basis of this experiment it is impossible to judge whether they are the same cell type or different.

4.3. Role of integrins in the generation of Rorγt+ Treg

All three studies agree that a possible mechanism for Rorγt+ Treg generation is integrins. However, they disagree on which integrins are responsible for this (Kedmi et al., 2022) (Lyu et al., 2022) (Akagbosu et al., 2022). As mentioned earlier, pTreg differentiation is dependent

on TGF-beta signaling (Nutsch et al., 2016) (M. Xu et al., 2018) and for this to happen, TGF-beta must be activated from the latent complex by dimmer integrin av and integrin b6 (*Itgavb6*) or b8 (*Itgavb8*) (Qin et al., 2018) (R. Wang et al., 2012). There was also evidence that Itgav and Itgb8 heterodimer pair is involved in Treg differentiation and their deletion in myeloid cells can cause colitis (Travis et al., 2007) (Lacy-Hulbert et al., 2007).

4.3.1. Itgav

In this regard, an article from Mengze Lyu et al. demonstrates an experiment in which, Rorγt+ CD4+ T cells isolated from LI-LP and mLN were cultured for duration of 72-hour with LTi-like ILC3s in the presence of neutralizing antibodies against Itgav (Cd51), Itgb1 (Cd29), Itgb3 (Cd61), or Itgavb6. They found that in the absence of antibodies, the percentage of Rorγt+ Treg relative to Th17 almost doubled, while blocking Itgav or Itgb3 leads to a significant decrease in Rorγt+ Treg frequency and an increase in Th17 frequency (Lyu et al., 2022). There was a significant decrease in the Rorγt+ Treg population and an increase in the Th17 population in $Itgav^{\Delta Ror\gamma t}$ mice, with a less pronounced changes in mLN than in IL and LP (Lyu et al., 2022). Partially similarly, Ranit Kedmi et al. confirmed that deletion of *Itgav* on Rorγt+ cells ($Itgav^{\Delta Ror\gamma t}$) or on Cd11c+ cells ($Itgav^{\Delta Cd11c}$) leads to a significant decrease in the number of Rorγt+ Treg. In another experiment, a chimeric mouse with half of its bone marrow not expressing MHCII on Cd11c+ cells and the other half lacking *Itgav* expression on Rorγt+ cells showed almost complete inability to generate Rorγt+ Treg, in contrast to a mouse with a combined bone marrow from $MHCII^{\Delta Cd11c}$ and a healthy wild-type mouse, which retained a normal ability to generate Rorγt+ Treg (Kedmi et al., 2022). In addition, Mengze Lyu et al. performed an experiment in which they demonstrated that deletion of *Itgav* in mouse Cd4+ cells did not affect the abundance of Rorγt+ Treg even though Cd4+ cells express *Itgav*. Considering the above data, this suggests that *Itgav* expression is required at APCs for Rorγt+ Treg generation (Lyu et al., 2022). In summary, the data from Ranit Kedmi et al. and Mengze Lyu et al. agree that $Itgav^{\Delta Ror\gamma t}$ and $Itgav^{\Delta Cd11c}$ mice have suppressed generation of Rorγt+ Treg. However, these data do not exclude but also do not allow us to assert that Cd11c, Rorγt and Itgav are expressed by a single cell type contributing to the generation of Rorγt+ Treg. (Kedmi et al., 2022) (Lyu et al., 2022).

4.3.2. Itgb8

The role of *Itgb8* was studied in more detail in a study by Blossom Akagbosu et al. according to which deletion of *Itgb8* in Rorc+ cells ($Itgb8^{\Delta Rorc}$) leads to a significant decline in the Rorγt+ Treg population. However, a chimeric mouse in which half of the bone marrow was from the $Itgb8^{\Delta Rorc}$ mice and the other half from the $MHCII^{\Delta Rorc}$ mice had the same percentage of Rorγt+ Treg population as the mouse with bone marrow from $MHCII^{\Delta Rorc}$ mice, which casts doubt regarding the importance of *Itgb8* in the induction of Rorγt+ Treg differentiation or suggests that MHCII and Itgb8 do not have to be expressed on the same cell type (Akagbosu et al., 2022). By generating mice lacking *Itgb8* expression in *Cd4*-expressing cells ($Itgb8^{\Delta Cd4}$), Blossom Akagbosu et al. demonstrated that this does not lead to a decrease in the abundance of Rorγt+ Treg. This indicates that Itgb8 expression is not required on Rorγt+ Treg precursors for their maturation (Akagbosu et al., 2022). It should be noted that in addition to Cd4+ T cells, Ranit Kedmi et al. confirm that ILC3 and cDC also express Cd4, but since the deletion did not lead to changes in the Rorγt+ Treg population, it can be assumed that Itgb8 on the surface of these cells does not play a significant role in the generation of Rorγt+ Treg.

Also, according to Blossom Akagbosu et al. ILC3 do not express Itgb8 (Akagbosu et al., 2022) (Kedmi et al., 2022).

4.4. Role of Rorγt+ APC in the generation of Rorγt+ Treg

4.4.1. Thetis cells

Blossom Akagbosu et al. argue that TC IV participate in the generation of Rorγt+ Treg early in mouse life. According to their data TC IV are characterized by the highest level of Itgb8 expression among all TCs and also express Itgav (Akagbosu et al., 2022). In addition, they express Rorc, MHCII and Ccr7, which makes them potent Rorγt+ APCs but does not prove their involvement in the generation of Rorγt+Treg (Akagbosu et al., 2022). Thus Blossom Akagbosu et al. provide only indirect evidence that allows us to assume TC involvement in the process, but does not prove it.

4.4.2. LTi-like ILC3s

The article from Mengze Lyu et al. provides data suggesting a role for ILC3s in the generation of Rorγt+ Treg. In the experiment deletion of the *H2-Ab1* gene was performed in cells expressing *Il-22*, which is a good specific marker to distinguish ILC3s from other Rorγt+ APCs. They demonstrated that such deletion almost triples down the population of MHCII+ LTi-like ILC3s and leads to a significant decrease of Rorγt+ Treg population in LP and LI, but unfortunately Mengze Lyu et al. do not provide data on Rorγt+ Treg population in mLN, where the differentiation process presumably takes place. Their study using immunofluorescence and image analysis demonstrated that approximately 40% of Rorγt+ cells (CD3- IL-7Rα+ Rorγt+, presumably ILC3s) and Rorγt+ Treg (CD3+ IL-7Rα- Foxp3+ Rorγt+) are in mLN in close intercellular contact (Lyu et al., 2022). They also provide data indicating that almost all LTi-like ILC3s in mLNs express *Itgav*, and deletion of Itgav in *Rorγt*-expressing cells ($Itgav^{\Delta Ror\gamma t}$) leads to a twofold decrease in *Itgav* expression among LTi-like ILC3s, suggesting the existence of LTi-like ILC3s that express *Itgav* and *Rorγt* simultaneously (Lyu et al., 2022). However, Blossom Akagbosu et al. and Ranit Kedmi et al. in their studies indicate that ILC3 do not express *Itgb8* or express it only very weakly. Furthermore, the article by Blossom Akagbosu et al. presents data that was interpreted by them as disproving the role of ILC3s in the generation of Rorγt+ Treg. They used Rora Cre-driver for deletion of MHCII ($MHCII^{\Delta Rora}$), in their article this is interpreted as the ILC3s specific deletion. Experiments by Blossom Akagbosu et al. demonstrate that $MHCII^{\Delta Rora}$ and $MHCII^{\Delta IL\text{-}22}$ mice did not show a decrease in Rorγt+ Treg population in mLN and large intestine (LI), which is inconsistent with data from Mengze Lyu et al. (Akagbosu et al., 2022) (Lyu et al., 2022). This discrepancy in the data may be due to the fact that Mengze Lyu et al. and Blossom Akagbosu et al. used mice of different ages and different models (Akagbosu et al., 2022) (Lyu et al., 2022). In addition, ILC3s are known to differ from cDCs and JCs in its high level of *Rora* expression (Kedmi et al., 2022) (Messing et al., 2018) (Spits et al., 2013), but *Rora* is also expressed by ILC2s (Ghaedi et al., 2020). ILC3s also differ from cDCs, JCs and ILC2 in their ability to express *IL-22* (Kedmi et al., 2022) (Spits et al., 2013), but it can also be expressed by other NKp46+ CD56+ ILCs (Crellin et al., 2010). Thus, neither *IL-22* nor *Rora* are specific makers for ILC3s. More reliable data would be required including studies with a larger number of mice and the use of a more specific model, for example, in which cells have a deletion of MHCII only if they express *Rora* and *IL-22* simultaneously or use *Rorγt* expression as a marker. Thus, the role of LTi-like ILC3s is still questionable and

requires further investigation, but it is possible that they participate in the generation of Rorγt+ Treg by *Itgav*.

4.4.3. Janus cells

Data from the study by Mengze Lyu et al. indicate that MHCII$^{\Delta Aire}$, Aire$^{\Delta Rorc}$ and Rorc$^{\Delta Aire}$ mice retain the ability to generate Rorγt+ Treg at normal levels. Thus, these data suggest that Aire-expressing cells do not participate in the generation of Rorγt+ Treg. As previously described, JCs are related to Rorγt+ eTACs, but JC1 and JC3 have only weak Aire expression, which allows to assume that they can be involved in the process. In their study, Ranit Kedmi et al. indicated that *Itgb8* and *Itgav* are strongly expressed by JC2 and JC3 (Kedmi et al., 2022). However, experiment from study by Mengze Lyu et al. indicates that deletion of *Itgav* in *Rorγt*-expressing cells (*Itgav*$^{\Delta Rorγt}$) does not affect the level of *Itgav* expression in eTACs, suggesting that those *Rorγt*-expressing eTACs are a separate subtype from those that express *Itgav* (Lyu et al., 2022). Additionally, they conducted an experiment in which they demonstrated that deletion of Ccr7 in Cd11c+ cells (*Ccr7* $^{\Delta Cd11c}$) leading to Rorγt+ Treg proliferation, as does deletion of Ccr7 in Rorγt+ cells (*Ccr7* $^{\Delta Rorγt}$). Ccr7 promotes cell migration from tissue to draining lymph nodes, which is essential for the presentation of peripheral antigens thereby (Hauser et al., 2016) (Hirao et al., 2000). Given this, it is logical that cells expressing Ccr7 participate in the generation of Rorγt+ Treg. According to their own data, JCs outcompete ILC3s in Ccr7 and Cd11c expression (Kedmi et al., 2022). The low level of Ccr7 expression in ILC3s is also confirmed by Blossom Akagbosu et al (Akagbosu et al., 2022). However, there is no evidence that all these markers must be expressed by a single cell type. Furthermore, since the samples for both experiments were taken from mLN it can be assumed that the expression level of Ccr7 was down-regulated since the cells no longer needed to migrate. It should be noted that both ILC3s, JC and TC express Ccr7, even though at different levels, so one of the cell types should not be excluded on the basis of its expression (Akagbosu et al., 2022) (Kedmi et al., 2022) (Lyu et al., 2022). Thus, the role of JC in Rorγt+ Treg generation remains questionable, but whether for example JC3 contribute to this process through the expression of *Itgb8* and *Itgav* requires further investigation.

4.4.4. Rorγt+ cDC2s

Since Rorγt+ cDC2 was not detected among cells expressing Rorγt in the mLN by any of the three articles discussed (Akagbosu et al., 2022) (Kedmi et al., 2022) (Lyu et al., 2022), it can be assumed that this cell type is not involved in Rorγt+ Treg generation. Blossom Akagbosu et al. demonstrate an experiment in which using C-type lectin domain family 9 member A (*Clec9a*) as a specific marker for DC, they demonstrated that deletion of MHCII in Clec9a+ cells does not affect Roryt+ Treg abundance (Akagbosu et al., 2022). However, since Clec9a is not a specific marker for cDC2 this experiment cannot be considered as evidence.

4.5. Comparison of TC and JC

A newly discovered TC cell type was discovered by Blossom Akagbosu et al. using a model specific for Rorc rather than Roryt. Given this, it is interesting that some of the TC subtypes share many similarities with the JC subtypes. TC I and TC III are characterized by high levels of Aire expression, but the other TCs also express it, albeit at much lower levels. This and the expression of characteristic markers such as Epcam and Zbtb46 suggest that they may belong to the already known eTACs. TC I, judging by the expression of such markers as Aire, Rorc, Ccr6 and Ccr7 has similarities with JC1. Considerable overlap in the expressed profiles is observed in pairs JC2 and TC III, as well as JC3 and TC IV (Fig. 1.).

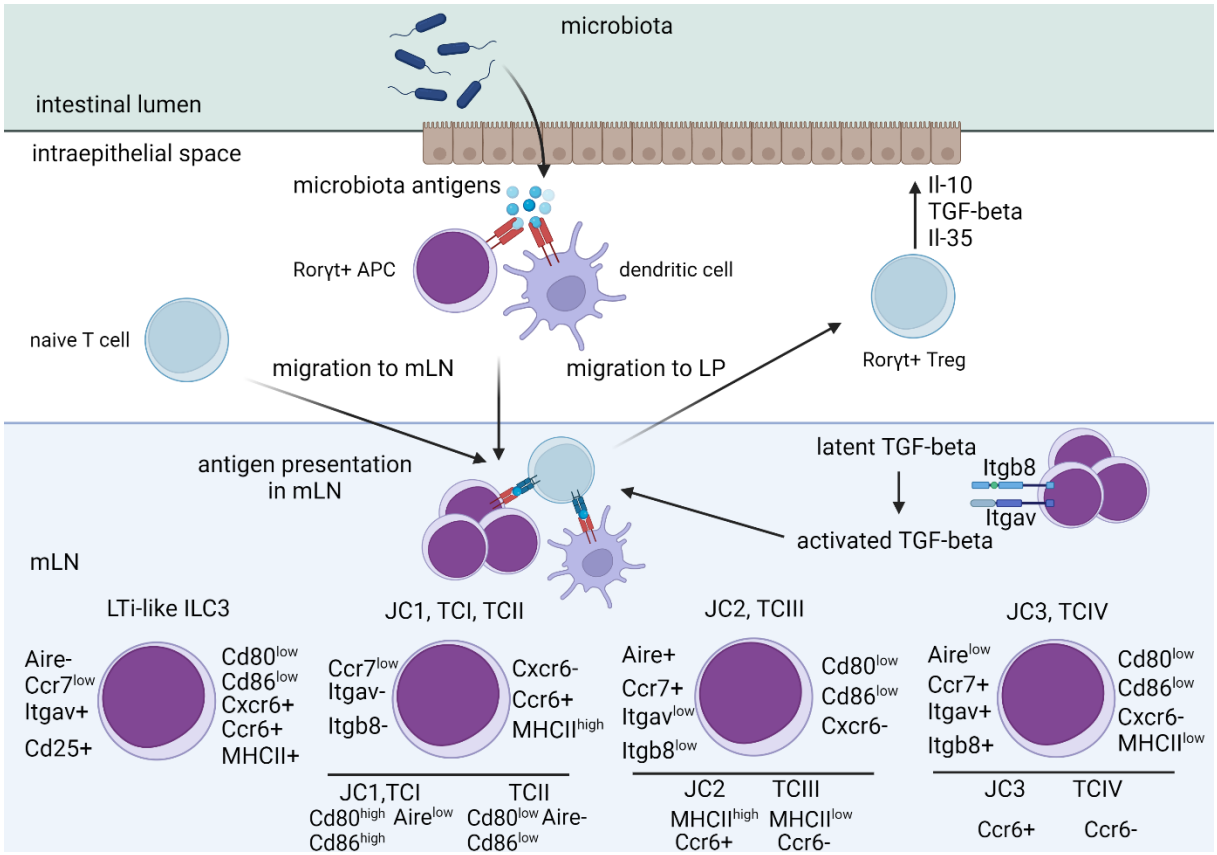

Fig. 1. Schematic representation of the generation of Roryt+ Tregs. (Antigen presenting cells (APC) in the *lamina propria* (LP) of the intestine capture extracellular antigen and migrate to the mesenteric lymph node (mLN) where they present antigen to naive T cells. Those that recognize the microbiota-specific antigen under the action of signals from TCRs, co-stimulatory molecules and cytokines, including TGF-beta, differentiate into Roryt+ Tregs and migrate to the LP where they produce anti-inflammatory cytokines and suppress the Th17 immune response.) Created with BioRender.com.

Discussion and conclusion

Three almost simultaneously published articles agree on the existence of Rorγt+ APCs that through integrins and antigen presentation are responsible for the generation of Rorγt+ Treg. The article by Ranit Kedmi et al. emphasizes the role of Ccr7 and Cd11c suggesting that the described Rorγt+ APC population expresses these markers (Kedmi et al., 2022). In addition,

they indicate that these Rorγt+ APCs must express Itgav to perform their function, and Mengze Lyu et al. confirm this with their data and complement this by suggesting a role for Itgb3 together with Itgav (Kedmi et al., 2022) (Lyu et al., 2022). As a possible cell type responsible for microbiota Tregs inducing Rorγt+ APCs, Mengze Lyu et al. suggest the LTi-like ILC3 (Lyu et al., 2022). ILC3 are able to express Itgav but do not express Itgb8 which makes them possible candidates for the role of APCs involved in the development of Rorγt+ Treg (Akagbosu et al., 2022) (Kedmi et al., 2022). Mengze Lyu et al. present data indicating that mouse models of MHCII$^{\Delta Aire}$, *Aire*$^{\Delta Roryt}$ and *Rorγt* $^{\Delta Aire}$ did not show changes in the level of population of microbiota-specific Tregs. This suggests that cells expressing *Aire* do not significantly participate in Rorγt+ Treg generation, which does not contradict the suggestion of Ranit Kedmi et al. that JCs may be responsible for this, because JC3 express both Itgav and Itgb8, while having a negligible Aire expression (Lyu et al., 2022) (Kedmi et al., 2022).

An outstanding opinion is offered by Blossom Akagbosu et al. who demonstrate that Itgb8 like Itgav takes part in the generation of Rorγt+ Treg, and TC IV have the highest expression of Itgb8 among all TCs, which suggests a role of TC IV in the generation of Rorγt+ Treg (Akagbosu et al., 2022). However, this is neither confirmed nor refuted by the data of Ranit Kedmi et al. and Mengze Lyu et al. as they did not observe cell-type matching TC IV at all, possibly caused by the use of a different mouse model (Kedmi et al., 2022) (Lyu et al., 2022). TC IV and TC II have weak expression of Aire, Cd80, Cd86 and H2-DMa, but TCIV also have high expression levels of Itgb8 and Itgav. This makes them very similar to JC3. TC IV express Epcam, which makes this cell type even more similar to eTAC or activated DCs (Gardner et al., 2013) (Akagbosu et al., 2022) (Kedmi et al., 2022). It is possible that to some degree both LTi-like ILC3 and cDCs contribute to this process, as there does not have to be one single cell type responsible. The experiment performed by Blossom Akagbosu et al. was specific for cDC1 because Clec9a is a specific marker for cDC1, and to make the model specific for cDC2 they should use mouse cre-drivers like Cd172a (SIRPα) or Clec10a (Wu & Shortman, 2005) (Akagbosu et al., 2022) (Huysamen et al., 2008) (Crozat et al., 2010) (Dzionek et al., 2000). Furthermore, Ranit Kedmi et al. and Mengze Lyu et al. did not observe this cell type in mLN, but Blossom Akagbosu et al. report the presence of TC IV, which out of all TCs has the closest expression profile to cDC2. Even so, the presence of Roryt+ cDC2 in mLN has not been directly proven by any of the three groups. Perhaps the difference is that Blossom Akagbosu et al. used younger, 2-week-old mice and a different, Rorγt non-specific, model whereas the others used older mice and Rorγt-specific (Akagbosu et al., 2022) (Kedmi et al., 2022) (Lyu et al., 2022). This indicates the need to explore the possible role of cDC2 in younger mice.

Moreover, other aspects of the process are still unclear. For example, it is necessary to study the relationship between JC and TC. Both of these cell types are quite similar in terms of expressed markers and probably some of them belong to eTAC. JC and TC may be just two ways to describe the same cell population. Speaking of this, it is important to remember that TC were discovered in a mouse model that was specific for Rorc and JC were studied in models specific for Rorγt. To clarify this issue, it would be necessary to try to discover the TC population in a Rorγt-specific mouse model and to perform a more detailed phenotyping of both cell types in order to have a more reliable and accurate picture of the markers they express. Nevertheless, in their study, Mengze Lyu et al. provide convincing evidence for the non-involvement of Aire-expressing cells in the process of Rorγt+ Treg generation. In future studies, this should be tested and the role of JC and TC should be judged with this in mind.

The articles discussed above clearly demonstrate the role of Itgb8 and Itgav in the generation of Rorγt+ Treg (Kedmi et al., 2022) (Lyu et al., 2022) (Akagbosu et al., 2022). But it remains

unclear whether the integrins must be expressed by APC together with MHCII or whether multiple cell types are involved in the process and the integrins are expressed by other cells separately from MHCII and antigen úresentation. In order to test this hypotheses it would be necessary to create a chimerical mouse, half of whose bone marrow would be from *Itgav*$^{\Delta MHCII}$ and the other half from MHCII $^{\Delta Itgav}$ mice. Thus, we would observe a drop in the Rorγt+ Treg population if both MHCII and Itgav on the same cell type are required for their generation. Another option would be to create a genetically modified *Itgav*$^{\Delta Ror\gamma t}$MHCII$^{\Delta Ror\gamma t}$ mouse model followed by a rescue experiment in which the mouse would be transferred with a suspensions of cells expressing *Itgav*, MHCII or both molecules at once. Otherwise, it is assumed that the mouse will not lose the ability to generate Rorγt+ Tregs because MHCII and Itgav will be present on different cell types

The role of integrins in the process of Rorγt+ Treg generation also remains unclear. Deletion of Itgb8 or Itgav separately led to a decrease in the number of Rorγt+ Treg but not to a complete loss of the ability to generate them. The fact that deletion of one of the integrins does not lead to complete loss of function suggests that their simultaneous expression is not a prerequisite for Rorγt+ Treg generation. However, it is not obvious whether integrins Itgb8 and Itgav function as a dimer and other mechanisms of Rorγt+ Treg generation are involved in the absence of one of them, or whether co-expression of integrins is not required and they function independently together with other integrin-family members. For a better understanding of this topic, it would be useful to establish which other integrins play a significant role, given that in addition to Itgav Mengze Lyu et al. data indicate a role of Itgb3 (Lyu et al., 2022). Experiments in which known integrins will be blocked with neutralizing antibodies and naive H. hepaticus-specific T cells will be cultured together with Rorγt+ APCs as it was done in the studies of Mengze Lyu et al. may help in understanding other mechanisms responsible for the generation of Rorγt+ Treg including those independent of above mentioned integrins (Lyu et al., 2022).

It is also important to note that only the study by Ranit Kedmi et al. used six or more mice in most of their experiments (Kedmi et al., 2022). In the studies by Blossom Akagbosu et al. and Mengze Lyu et al. the experimental groups consisted of 3-4 mice in most cases. In my opinion in order to confidently assess the statistical significance of the experiments, the experimental groups should have been larger and contain at least 6-8 mice in each experimental group (Lyu et al., 2022) (Akagbosu et al., 2022). However, the fact that the studies by Mengze Lyu et al. and Ranit Kedmi et al. largely confirm each other's data is in favor of their validity (Kedmi et al., 2022) (Lyu et al., 2022).

An important note to the article by Blossom Akagbosu et al. is their choice of age of mice for the experiments. In their paper, they indicate a peak in TC abundance between 1 and 3 weeks of mouse life. From about the second week onwards, TC abundance begins to decline and reaches a minimum in the 20 day old mouse. However, most of the Blossom Akagbosu et al. analyses were performed on mice at 18 or 21 days of age after birth, when TC abundance is already significantly reduced (Akagbosu et al., 2022). A more appropriate age to test the role of TC in Treg generation would be 14 to 15 days of age, because according to their own data, between 10 and 14 days of age, Roryt+ Foxp3+ pTregs first appear in the mLN and their abundance increases significantly after day 14, which coincides with a maximum in TC abundance. It was 2-week-old mice that they used for scRNA-seq analysis in which TCs were identified, but such an old mice were not used for the rest of the experiments (Akagbosu et al., 2022).

In conclusion, my thesis provides an introduction to Treg biology, differentiation and function, focusing on Rorγt+ Treg and the contribution of Rorγt+ APCs. I also review recent

more studies demonstrating the heterogeneity of Rorγt+ APC populations and point out the differences in their approaches and results, highlighting their peculiarities and possible future directions for research in this area. Personally, I consider the possibility that different types of Rorγt+ APCs including LTi-like ILC3s and Janus cells contribute to peripheral tolerance through the generation of Rorγt+ Treg as highly possible. And I find the evidence for the involvement of LTi-like ILC3s via Itgav provided by Mengze Lyu et al. and Ranit Kedmi et al. as compelling (Kedmi et al., 2022) (Lyu et al., 2022).

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(*secondary citations)

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