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Establishment of immune tolerance to exogenous antigens
Ustavení imunitní tolerance k exogenním antigenům

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.

V Praze, 30.4.2025 Matúš Puskeiler

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Abstract
This bachelor's thesis investigates the mechanisms of immune tolerance to exogenous antigens, focusing on regulatory T cells in the intestinal mucosa. Although the discovery of regulatory T cells marked a paradigm shift in our understanding of dominant tolerance, the differentiation and functional specialization of two principal subsets, microbiota-specific Rorγ^{+} pTregs and food antigen-specific pTregs, remain partly unresolved. The thesis provides a systematic account of thymic T cell development, including positive and negative selection, examines the roles of key cytokines IL-2, TGF-β and the transcription factor FOXP3 in peripheral Treg induction, and identifies the antigen-presenting cell populations (ILC3s, Thetis cells) that mediate their differentiation. Particular emphasis is placed on maintaining mucosal homeostasis and on the therapeutic potential of modulating these cells in autoimmune disease. The aims of this thesis are to deliver a comprehensive overview of gut Treg development, differentiation, and function, and to evaluate their prospects for clinical application.

Key words: immune tolerance, regulatory T cells, exogenous antigens, intestinal mucosa, pTreg

Abstrakt
Táto bakalárska práca skúma mechanizmy imunologickej tolerancie voči exogénnym antigénom so zameraním na regulačné T bunky v črevnej sliznici. Hoci objav regulačných T buniek predstavoval významný posun v porozumení dominantnej tolerancie, diferenciácia a funkčná špecializácia dvoch hlavných populácií, mikrobiota-špecifických Rorγ^{+} pTreg a potravinovo-špecifických pTreg, zostáva čiastočne nejasná. Práca systematicky popisuje vývoj T buniek v thymuse vrátane pozitívnej a negatívnej selekcie, analyzuje úlohu kľúčových cytokínov IL-2, TGF-β a transkripčného faktora FOXP3 pri periférnej indukcii Treg buniek a identifikuje antigén-prezentujúce bunky (ILC3, Thetis cells), ktoré sprostredkúvajú ich diferenciáciu. Osobitný dôraz je kladený na udržiavanie slizničnej homeostázy a perspektívy modulácie týchto buniek v terapii autoimunitných ochorení. Cieľom práce je poskytnúť ucelený prehľad vývoja, diferenciácie a funkcie črevných Treg buniek a zhodnotiť ich potenciál v klinických aplikáciách.

Kľúčové slová: imunologická tolerancia, regulačné T bunky, exogénne antigény, črevná sliznica, pTreg

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Introduction

One of the most important cells of the mammalian immune system are T cells or T lymphocytes. These cells are essential for the maintenance of immune homeostasis and pathogen-free environment inside the organism. T cells contribute significantly to the adaptive immune responses that are initiated upon the recognition of foreign antigens. In recent decades, various distinct subsets of T cells were discovered and annotated, which differ in function(s) as well as the combination of specific cell surface markers. All T cell subsets originate from bone marrow and migrate to the thymus, where they undergo their development. Here, the highly complex process of generating immunocompetent T cells is facilitated by a plethora of antigen-presenting cells (APCs) which reside in thymic cortex or thymic medulla. These

thymic APCs express and/or present nearly an entire set of endogenously encoded self-antigens and are critical to limit the self-reactive potential of T cells. This is achieved via mechanisms referred to as positive and negative selection, which occur in the thymic cortex and thymic medulla, respectively, and collectively are known as the mechanism of "central tolerance".

Despite the process being highly regulated, some autoreactive T cells can escape central tolerance and can thus represent a serious threat to the host. In addition, some non-self-reactive T cells which egress from the thymus could be specific for the host's commensal and symbiotic microbiota, or food antigens and the activation of these T cells could result in uncontrolled immune reactions in immune periphery. Thus, to maintain the immune homeostasis, their activation must be kept at bay.

In recent decades, immunology experts have focused on a specific population of T cells, referred to as regulatory T cells (Tregs). These cells have immunosuppressive capabilities, are crucial for the maintenance of tolerance to self-antigens and can prevent the onset of autoimmune diseases. Tregs have also a critical role in the intestine, where they suppress immune responses to commensal microbiota and food antigens. In this thesis, I will focus on the development and function of intestinal Tregs which protect beneficial commensal intestinal microbiota. However, to better understand the emergence and role of intestinal Tregs in homeostasis of the gut, I will first briefly describe the development of T cells and selection processes to which they are subjected during their development in the thymus.

Early development of T cells

T cells originate from hematopoietic stem cells in bone marrow. After completing differentiation into multipotent progenitor and subsequently, into common lymphoid progenitor (CLPs), these cells transfer to the thymus via the bloodstream (Serwold et al., 2009). Upon arrival to the thymus, CLPs, from now on referred to as thymocytes, undergo the first developmental step, which is the T cell receptor (TCR) rearrangement. During this stage, thymocytes are denoted as double-negative (DN), because of the absence of both characteristic coreceptors, CD4 and CD8. TCR is the hallmark receptor of the whole T cell population. Each mature T cell has its own unique TCR, which binds to an antigen presented in the context of the major histocompatibility complex (MHC) on the surface of APC. This allows the immune system to recognize any potential antigen, that T cell can encounter. TCR consists of α and β chains, each containing the *variable (V)* and *joining (J)* gene segments, plus *diversity (D)* segments in β chains. Rearrangement of the V, D, and J gene segments is called V(D)J

recombination and it represents a fundamental principle of generating an enormous diversity of TCRs in the context of entire T cell population present in an organism (Tonegawa, 1988). β chain is the first to undergo this recombination process and α chain follows shortly after. This process is coupled with the upregulation of both CD4 and CD8, turning DN thymocytes into the double positive (DP) state. Due to the stochastic process of TCR generation, there is a risk of producing receptors with suboptimal affinity for the antigen-MHC complex, either too low, leading to ineffective immune responses, or too high, potentially causing autoimmunity.

Positive selection

After the generation of TCR, DP thymocytes are navigated via CCR9 towards a special thymic cell population, expressing a CCR9 ligand, CCL25 (Uehara et al., 2002). These cells are a vital cellular component of positive selection machinery, referred to as the cortical thymic epithelial cells (cTECs) (see also Fig.1). cTECs present self-antigens to DP thymocytes via MHC molecules. This presentation allows for the selection of thymocytes capable of recognizing MHCs via TCR-MHC engagement. Thymocytes that receive a TCR signal pass the positive selection and downregulate either CD4 or CD8 coreceptor. In this process, the intracellular signaling protein, ZAP-70, is phosphorylated, triggering T-cell survival via anti-apoptotic signaling pathways (X. Liu et al., 2003). All thymocyte precursors are predetermined to develop CD8 expression thanks to Runx3, however, if TCR recognizes the peptide in the context of MHCII complex, another transcription factor ThPOK is activated, overriding the Runx3 program and committing the thymocyte to the CD4 lineage (Serroukh et al., 2018). This primary mechanism, referred to as “MHC restriction” is commonly accompanied by complementary mechanisms that help to regulate the choice between CD4+ and CD8+ lineage (Wang & Bosselut, 2009). Good example is a well-known process which relies on the strength of the TCR signal. A stronger TCR signal signifying the interaction with MHCII has the propensity to result in CD4+ lineage, while a weaker signal intensity resulting from the interaction with MHCI leads to CD8+ lineage commitment. However, approximately 90% of thymocytes due to inability of TCR-MHC interaction fail to receive this survival signal and undergo apoptosis, the process referred to as “death by neglect”. While positive selection ensures that thymocytes have sufficient affinity for MHC molecules, negative selection in the thymic medulla prevents autoimmunity by eliminating thymocytes whose TCRs bind too strongly to pMHC complexes.

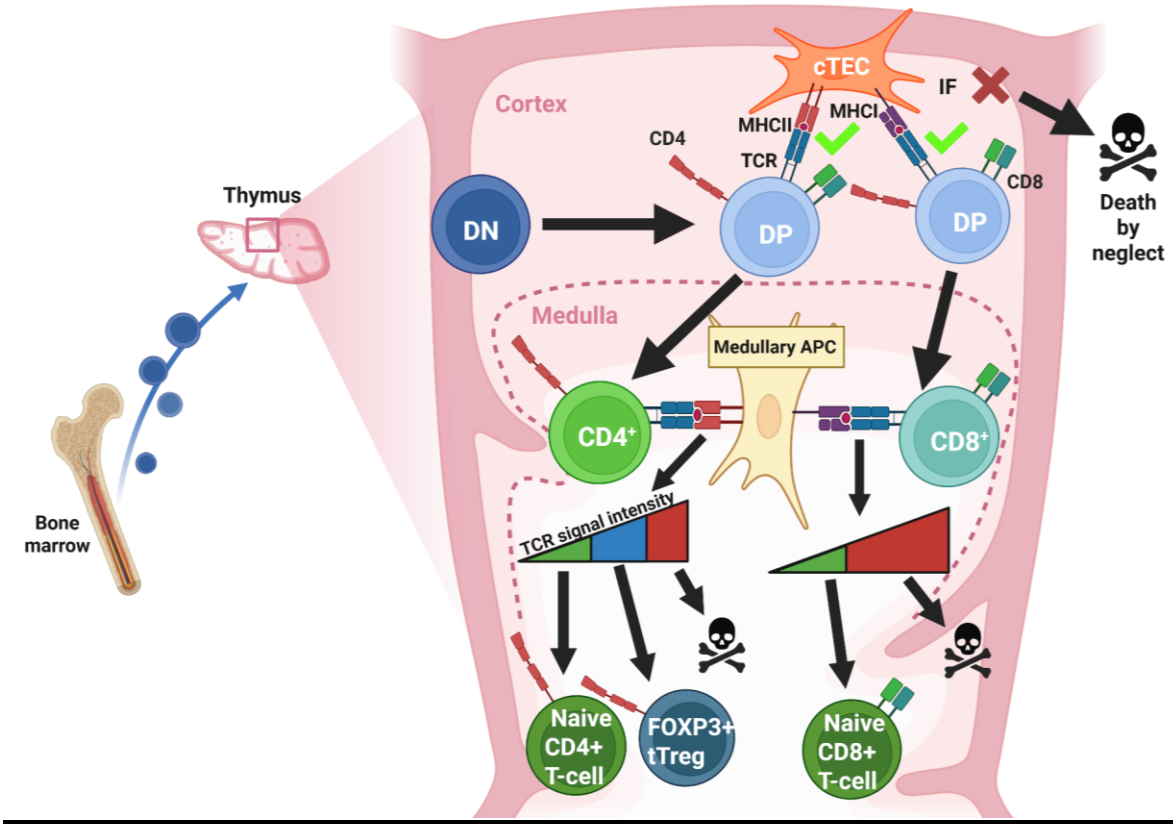

Fig.1: Thymic T cell development

Thymocyte precursors enter the thymus and undergo V(D)J recombination and enter the double-positive state. Depending on the TCR-MHC interaction during positive selection in cortex, they commit to either CD4+ or CD8+ lineage and enter the thymic medulla to undergo negative selection. Some CD4+ thymocytes can upregulate FOXP3 upon contact with medullary APC, therefore becoming tTregs. Majority of thymocytes undergo apoptosis either in positive or negative selection. Abbreviations: cTEC- cortical thymic epithelial cell, DN- double negative thymocyte, DP- double positive thymocyte, TCR- T cell receptor.

Negative selection

Thymocytes that pass positive selection migrate to the thymic medulla, where negative selection occurs. This migration is facilitated by positive selection-induced expression of CCR7, a receptor that leads immature T cells to medullary thymic epithelial cells (mTECs), which express CCR7 ligands (Hikosaka et al., 2008). Since mTECs are endowed with the set unique antigen-presenting attributes, they appear to be a key cell population for negative selection. Expressing a unique transcription factor, the autoimmune regulator (AIRE), mTECs display a remarkable capacity to present almost every antigen that is endogenously encoded in the genome (Anderson et al., 2002), via the process of promiscuous gene expression (Derbinski

et al., 2001). This is especially important in regards of tissue-restricted antigens (TRAs) which are expressed only in a limited number of peripheral tissues. Malfunction of either AIRE or mTECs themselves results in severe autoimmune conditions, further proving the importance of presenting TRAs in thymic medulla (Bonito et al., 2013; Finnish-German APECED Consortium, 1997). Apart from Aire-dependent antigen presentation, mTECs are also able to load MHCs with their own ubiquitinated intracellular antigens. The third mechanism for a non-negligible antigen expression in mTECs is largely AIRE-independent ectopic expression of TRAs via specialized mTEC-derived "mimetic cells", which allow antigen presentation beyond the traditional expression profile (Ushio et al., 2024).

Despite being indispensable, mTECs are not the only APCs in the thymic medulla, with another important population being dendritic cells (DCs). In general, we can divide thymic DC population into plasmacytoid (pDCs) and classical DCs (cDCs), which further divide into cDCs type 1 and cDC type 2 (J. Li et al., 2009). cDC2s, which are the signal-regulatory protein-α (SIRPα) positive, sometimes also referred to as migratory DCs, along with pDCs, play a role in bringing peripheral antigens to the medulla and presenting them via MHC complexes, contributing to negative selection (Hadeiba et al., 2012). Another major role of thymic DCs is to create a mosaic pattern of antigen distribution and to present the antigens acquired from mTECs via the cooperative antigen transfer (Vobořil et al., 2022), with the crucial component here being CD8+ SIRPα- cDC1s, denoted as the resident DCs (Wu & Shortman, 2005). Apart from mTECs and all DC subsets, there is a minor B cell population, colocalized with mTECs and DCs, which plays a role in the elimination of self-reactive T cells (Isaacson et al., 1987; Perera et al., 2013; Yamano et al., 2015). Macrophages are also an indispensable part of thymic machinery, with their main task being the phagocytosis of apoptotic T cells, which would otherwise flood the thymic environment (Surh & Sprent, 1994). Using experiments that abrogated the process of negative selection, it was shown that negative selection can remove approximately a half of the remaining thymocytes that successfully passed positive selection (van Meerwijk et al., 1997).

Dominant vs recessive tolerance

Upon recognizing the self-antigens presented by APCs on the MHC complex with high affinity interaction, the self-reactive T cell receives a signal to undergo apoptosis. This process referred to as "TCR-mediated apoptosis" involves a diverse array of signaling molecules like Fas, Bcl- 2 family, and DAP3, which contribute to the activation of caspases, resulting in protein

degradation and cell death. This mechanism was also denoted as recessive tolerance. Negative selection of autoreactive T cells has long been thought to be the sole important process happening in the thymus.

This dramatically changed with the seminal discovery of the existence regulatory T cells (Tregs) and description of their development in the thymic environment (Sakaguchi et al., 1995). These cells were first characterized as CD4 positive (CD4+) CD25+, and later specified as forkhead box protein P3 positive (FOXP3+). It is thought that most Tregs in the body originate from the thymus, thus being called thymus-derived Tregs (tTregs), with their specific marker originally thought to be the Helios transcription factor (Thornton et al., 2010). Evidence shows that upon the recognition of self-antigen presented by thymic APC, thymocytes with an intermediate affinity to self-antigens presented in the context of MHCII can differentiate into Tregs, meaning that the windows of negative selection and Treg differentiation can overlap to some extent (Kawahata et al., 2002). In contrast to recessive tolerance induced by the deletion of autoreactive T cells, dominant tolerance was introduced as a term to describe the induction of regulatory T cells. Despite the fact, that negative selection occurs in thymic cortex, Treg differentiation in this part of the thymus remains is still a controversial topic. It has been proven that CD4+ SP thymocytes can effectively differentiate into Tregs and that the propensity to do so is negatively correlated with the age of the cells (Wirnsberger et al., 2009).

Regarding the intensity of signal needed for Treg induction, there has been a mathematical model that proposes that upon a single interaction with APC which presents high levels of cognate peptide-MHC (pMHC) complexes, immature T cells are immediately arrested and led to clonal deletion (Khailaie et al., 2014). On the other hand, this model suggests that a continuous stimulation of TCR by interacting with APCs with low levels of pMHC could lead to Treg differentiation. Analysis of TCR repertoire in the thymus has proven that mTEC or DCs have a non-redundant role in Treg differentiation, which could mean that there is no single subset of APC subset specialized exclusively for Treg selection (Perry et al., 2014). Interestingly, a special population of monocyte-derived DCs that require the signaling from mTECs has been identified as a key player in the thymic Treg output under inflammatory conditions (Vobořil et al., 2020). What separates each cell population involved thymic selection is also their cytokine production.

Cytokines involved in Treg induction

The essential role of interleukin-2 (IL-2) as a limiting factor for thymic Treg differentiation has been recognized in the experiment in which the antibody-mediated blockage of IL-2 led to hindered tTreg production in thymus (Bayer et al., 2005). To put it into physiological context, a two-step process for Treg selection has been established. First, a strong TCR signal facilitates the expression of CD25, also known as IL-2Rα, which allows for IL-2 binding, resulting in FOXP3 upregulation (Lio & Hsieh, 2008). The main producers of IL-2 required for Treg cell differentiation seem to be neighboring T cells, while the concrete cell population remains unknown (D. L. Owen et al., 2018). This study also suggested that neighboring cDCs and mTECs may be able to sequester available IL-2, therefore creating a competitive environment among CD25+ FOXP3- immature Treg cells. This competition was later also demonstrated in recirculating Tregs, which home back to thymus from periphery and compete in this manner with thymic Treg precursors (Peligero-Cruz et al., 2020).

Another important Treg developmental factor is transforming growth factor-β (TGF-β). Scientists studying Tregs mainly associate this factor with peripheral induction of Tregs, which we will get into later. Still, it has been proven that mice deficient in TGF-β have a depleted pool of thymic Tregs in early developmental stages (Y. Liu et al., 2008). One study has proposed that TGF-β is not essential for tTreg induction but for the survival of T cells that receive agonist signal, including Tregs (Chen et al., 2001). A later study, however, has proven that there is no decrease in the tTreg population if the TGF-β receptor is switched off in later development, via the FOXP3- Cre system (Konkel et al., 2014). This study has also used TGF- β receptor-deficient mice to demonstrate higher intestinal inflammation because of absence of Tregs. While we know that TGF-β levels in the thymus increase steadily during the development of mice, TGF- β source cells and the exact mechanism regarding Treg development remain to be elucidated. Another intriguing molecule is the co-stimulatory receptor CD28, which was shown to be vital for CD4+ CD25+ Treg development (Salomon et al., 2000;Lio et al., 2010).

Role and induction of FOXP3

The pivotal role of the transcription factor FOXP3 in immunity regulation was discovered due to efforts of explaining the symptoms of patients suffering from Immune dysregulation, Polyendocrinopathy, and Enteropathy X-linked syndrome (IPEX). These patients were exclusively males, hemizygotes carrying a loss-of-function mutation in the FOXP3 gene. The

importance of FOXP3+ Tregs was highlighted by adoptive CD4+ CD25+ Treg transfer into neonatal FOXP3-deficient mice, which resulted in CD25+ Treg population expansion and rescue from the disease (Fontenot et al., 2003). Generally, the FOXP3 locus is known to include 3 conserved non-coding sequences called CNS1, CNS2, and CNS3 respectively, each of them being a transcription enhancer (Mantel et al., 2006) (see also Fig. 2).

CNS1 was shown to be a binding site for NFAT and SMAD3, which is a transcription factor activated by the aforementioned TGF-β (Tone et al., 2008). CNS2 contains a binding site for CREB, STAT5, and RUNX and is activated by TCR expression and IL2 (Y. Zheng et al., 2010). CNS2 is not considered to be essential for FOXP3 initial induction, rather it plays a role as a stabilizing module in proliferating Tregs. Authors also recognize the CNS3 region with its binding site for c-Rel, which is a crucial determinant of Treg abundance in both the thymus and the periphery. c-Rel is a subunit of NF-κB, and its binding allows chromatin to open and enable FOXP3 expression in response to TCR and CD28 signals. Hypoxia-inducible factor (HIP)-1α has also shown significance in FOXP3 induction, mainly as a part of local anti-inflammatory response (Clambey et al., 2012).

An unexpected discovery revealed a new CNS region, CNS0, which is crucial for FOXP3 induction in double positive thymocytes, referred to as the super-enhancer. CNS0 has a binding site for a special AT-rich sequence binding protein (Satb1), a chromatin-organizing transcription factor, which is expressed sooner than FOXP3, making it a “pioneer factor” (Kitagawa et al., 2017). Independently of acquiring FOXP3 expression, Treg cell precursors are required to carry a special epigenetic feature, a DNA hypomethylation pattern at Treg signature genes like Foxp3, Il2ra, Ctla4 and Ikzf2 (Ohkura et al., 2012). Another study has shown that during the development of Tregs, the levels of methylation on CNS2 are progressively decreasing, and thus correlate with the levels of surface CD24 which also diminishes during transition from immature to mature Tregs (Toker et al., 2013). Apart from demethylation, histone modifications were found to be vital for Treg differentiation, especially histone H3 or H4 acetylation or H3 polymethylation (Schmidl et al., 2009). It seems that the TCR signal induces some Treg-specific markers like CD25, CLA4, or GITR, but it is the FOXP3 that defines Treg identity afterward. As the master regulator, FOXP3 stabilizes the Treg cell program by suppressing inflammatory cytokine production while reinforcing its own transcription and stabilizing the anergic-like state of Tregs (Gavin et al., 2007). Notably, the most repressed FOXP3-dependent gene was cyclic nucleotide phosphodiesterase 3B (PDE3B), which when expressed can deregulate the anergic-like state of Tregs, presumably by inducing apoptosis caused by chronic IL-2-dependent proliferation.

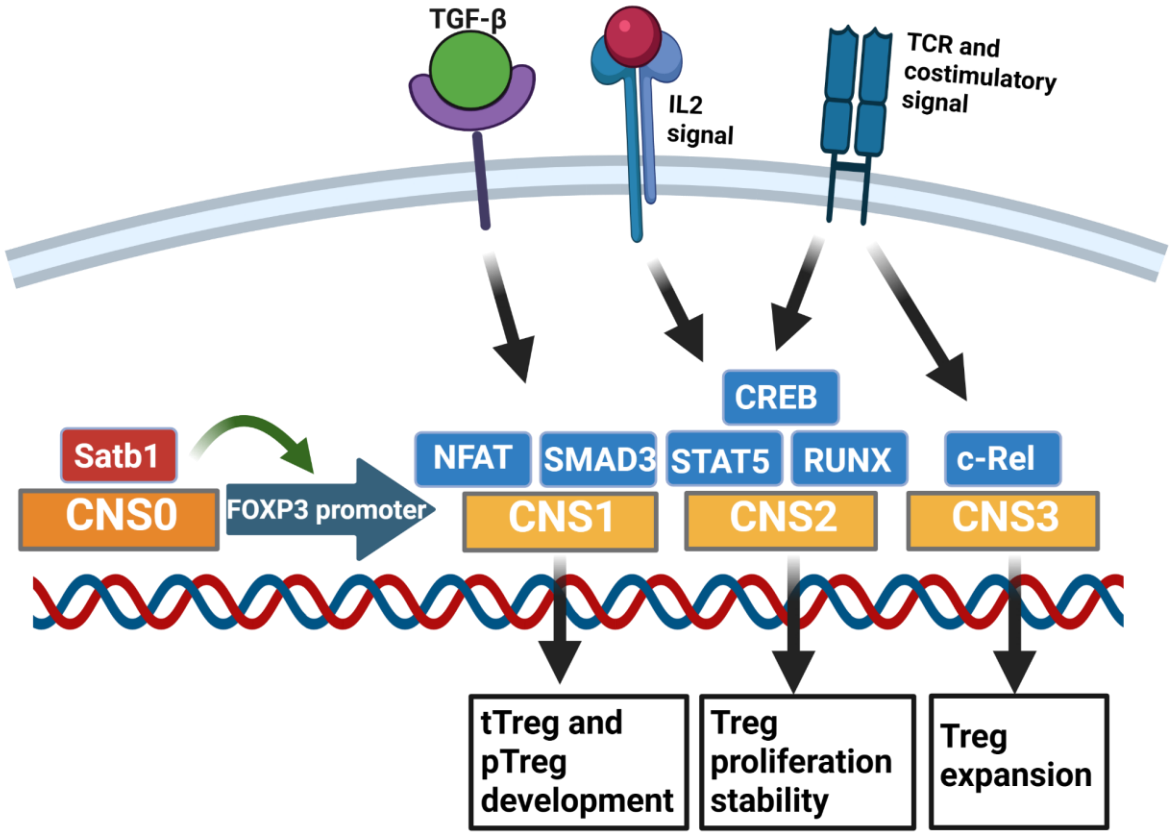

Fig.2: FOXP3 locus

The expression of a pioneer factor Satb1 precedes and regulates the expression of FOXP3 via super-enhancer CNS0. Downstream from FOXP3 promoter there are 3 sequences each of which can bind its specific transcription factors. CNS1, targeted by TGF-β signaling, regulates the development of Tregs. CNS2, targeted by IL2, TCR and costimulatory signaling, is crucial for Treg proliferation. CNS3 relies on TCR and costimulatory signals and keeps physiological numbers of Tregs in the organism. Abbreviations: CNS- conserved non-coding sequence.

Thymic escape and peripheral tolerance induction

Although the thymic machinery is very thorough in deleting autoreactive T cells, some regularly escape the thymic environment into the periphery and cause autoimmunity (Bouneaud et al., 2000). Playing a crucial role in dominant tolerance, tTregs control and suppress the autoreactive T cells on the periphery. When it comes to the localization of tTregs in the periphery, they are usually located in high numbers in draining lymph nodes, where self- antigens are commonly presented (Samy et al., 2005). It is also worth mentioning that they are relatively abundant in inflammation and infection sites and tumors (Belkaid et al., 2002). Depending on the target tissue, each FOXP3+ Treg possesses certain homing receptors,

which are key to assessing their tissue redistribution (Huehn & Hamann, 2005). The expression of homing receptors is similar among all T cells, as documented for CCR4+ Tregs and CCR4+ T effector cells (Iellem et al., 2003).

However, tTregs are not the only class of Tregs in the periphery because of peripherally-induced Tregs (pTregs in vivo, iTregs *in vitro*), which differentiate from naive T cells in the periphery. The existence of induced Tregs demonstrated *in vitro* by exposing naive T cells to TGF-β, which resulted in the FOXP3+ phenotype (Chen et al., 2003). Later, the possibility of experimental induction of Tregs *in vivo* was demonstrated by several research groups using various methods (Kretschmer et al., 2005); (Sun et al., 2007); (Verginis et al., 2008).

The importance of pTregs in establishing immune tolerance was highlighted by the neonatal transfer model, which treated FOXP3+ deficiency (Haribhai et al., 2011). This model has also proven that while being similar to tTregs, pTregs utilize a non-overlapping TCR repertoire and possibly perform different functions. After documenting the existence of pTregs and their importance, it was essential to characterize the exact mechanism of pTreg induction and its resemblance with tTregs. It is now a common consensus that upon stimulation with relevant antigen, CD4+ T cells can undergo differentiation into pTregs (Thornton et al., 2010) (see also Fig.3).

In an interesting discovery, it was shown that mice deficient in CNS1 in the FOXP3 region were capable of generating tTregs but not pTregs, which would suggest a different mechanism for peripheral induction of pTregs (Y. Zheng et al., 2010). This discovery led to the assumption that pTregs are responsible for preventing inappropriate allergic reactions at mucosal surfaces (Josefowicz et al., 2012). The essential cytokines required for FOXP3+ Treg conversion on the periphery are not the same as those in the thymus. As mentioned before, TGF-β plays a major role in peripheral induction, as proven by TGF-β neutralization, resulting in inhibited differentiation of FOXP3+ pTregs (Mucida et al., 2005). The exact mechanism was shown to be the induction of STAT3 and NFAT which bind to the CNS1 region. IL-2 neutralization also suggested that IL-2 is indispensable for pTreg induction, because of the highly reduced number of pTregs in the spleen (Setoguchi et al., 2005). Interestingly, the results of the experiments also showed that FOXP3 expression in the thymus remained almost unchanged. This was later proven to be true, because in the IL-2 absence, IL-15 takes its role and binds to IL-2Rβ, which is a receptor essential for thymic Treg development (Burchill et al., 2007). This is not true for the periphery, where the presence of IL-2 is essential for activating STAT5, which later binds to the FOXP3 gene. It is also important to mention CTLA-4, which is thought to be crucial for

TGF-β pTreg induction. Mice lacking this molecule were unable to express normal levels of TGF-β-induced FOXP3, which requires CTLA-4 ligation to CD80 (S. G. Zheng et al., 2006).

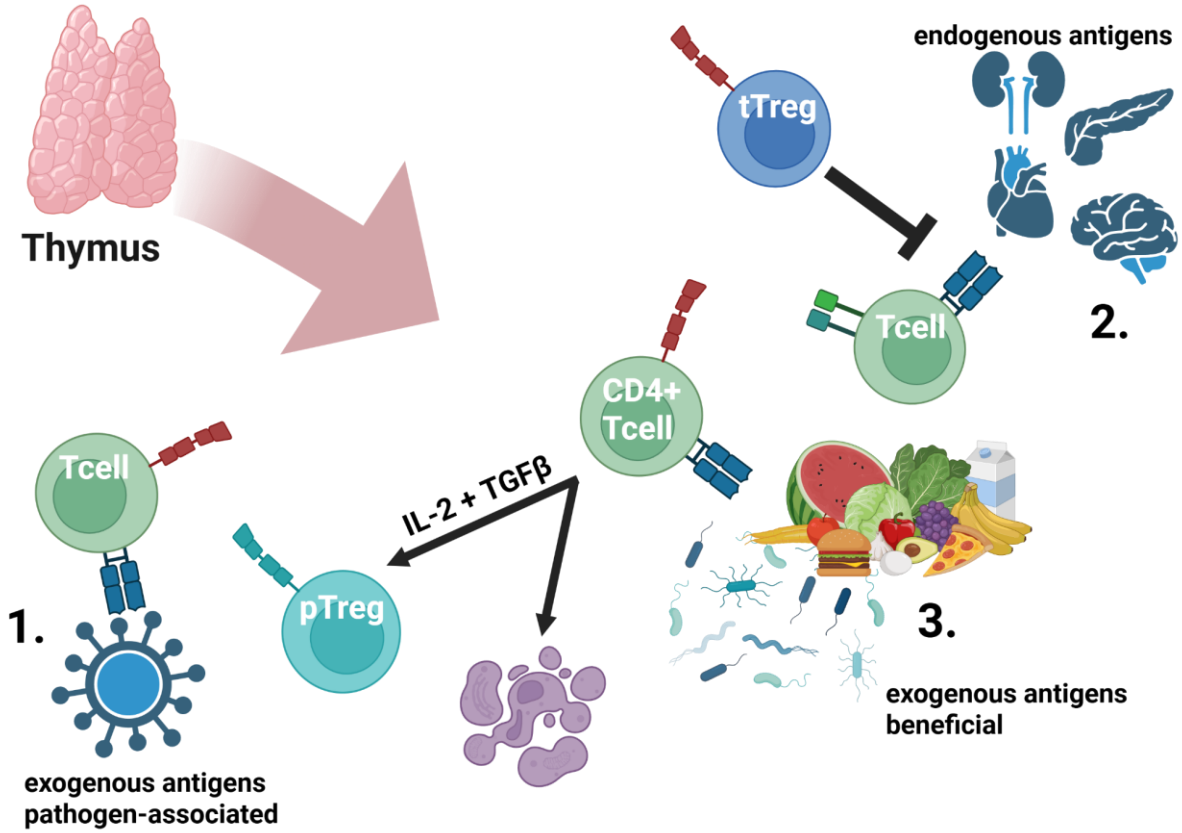

Fig.3: T cell fate in periphery

After leaving the thymus, T cells are allowed to interact with antigens of different origins. 1.The main purpose of T cell immunity is to interact with pathogen-associated antigens to invoke appropriate immune reactions. 2.However, some negative selection escapees may interact with endogenous self-antigens and these reactions are generally suppressed by tTregs. 3.Even some exogenous antigens like food and microbiota are beneficial for the organism and interacting with them leads a CD4+ T cell to pTreg phenotype or apoptosis. Abbreviations: pTreg- peripherally-induced Treg, tTreg- thymic-derived Treg.

Functions of Tregs

Since the breakthrough discovery of Tregs, immunologists have unearthed many mechanisms of Treg-mediated immune suppression. Understandably, the Treg mediated T conventional cell (Tconv) suppression mechanism was the first to focus on. One direct mechanism mediated by Tregs is to kill the activated CD4+ and CD8+ T cells along with dendritic cells via a perforin-dependent pathway (Grossman et al., 2004). Later studies found a different, perforin-

independent suppression pathway involving a molecule called granzyme B (Gondek et al., 2005).

Another direct mechanism of Treg suppression is the expression of TNF-related apoptosis-inducing ligand (TRAIL), which binds to Tconv death receptor 5 (DR5), effectively inducing apoptosis via a cascade involving caspase-8 (Ren et al., 2007). It is also important to mention the molecule PD-1 and its ligand PD-L1, which are both expressed on Tregs and are able to induce Treg differentiation or Tconv anergic state upon Tconv PD-1 and Treg PD-L1 interaction (J. A. Brown et al., 2003) (Francisco et al., 2009).

Apart from direct mechanisms, Tregs can also influence the immune environment. As mentioned earlier, CD25 or IL-2R has an important role in the induction and stabilization of Tregs population, but it also serves as a receptor for depletion of IL-2 from the environment (Chinen et al., 2016). This depletion results in limited CD8+ Tconv proliferation, with lesser effect on CD4+ T cell population. Active Tregs also secrete TGF-β, IL-10, and IL-35, which have a wide variety of functions, ranging from T and B effector cell activation and proliferation suppression, pTreg cell induction to DC tolerogenic phenotype switch (X. Li et al., 2019) (Tsuchida et al., 2017) (Boks et al., 2012). Another interesting aspect is the expression of CD39 and CD73 on Tregs. While CD39 converts ATP and ADP to AMP, CD73 catalyzes the hydrolysis of AMP to adenosine (Deaglio et al., 2007). The decrease in ATP and elevated adenosine levels suppress T effector cell and DC functions. Tregs also suppress the activity of Tconvs by disrupting intracellular Ca2+ mobilization, resulting in decreased activation of nuclear factors NFAT and NF-κB, which are important factors for T cell activation (Schmidt et al., 2011). The authors used a method of coculturing Tregs with Tcons, a method that might not be the most authentic in imitating in vivo conditions.

Tconvs are not the only immune cell population that is targeted by Treg suppression mechanisms. Tregs have been shown to induce DC maturation, resulting in heightened production of anti-inflammatory IL-10 and lowered production of pro-inflammatory IL-12, using a molecule called TIGIT (Yu et al., 2009). TIGIT, along with another molecule, Th-1 polarizing transcription factor T-bet, were later shown to inhibit the proinflammatory activity of Th1 and Th17 cells (Joller et al., 2014). Tregs can also regulate antigen presentation in a process called antigen-specific suppression. One example of this type of suppression is the process of forming aggregates on the surface of DCs, while simultaneously downregulating CD80 and CD86 costimulatory receptors of these APCs (Onishi et al., 2008). In this process, Tregs can physically block binding sites on DCs while reducing the number of costimulatory receptors needed for T cell activation, with the molecule CTLA-4 being needed for this to

occur. Apart from CD80/86 downregulation, CTLA-4 binding also results in higher expression of the enzyme indoleamine 2,3-dioxygenase (IDO) by DCs (Fallarino et al., 2003). High levels of IDO hinder the tryptophan catabolism in DCs, which results in suppression of activated T cell proliferation. In another interesting type of antigen-specific suppression, Tregs have been observed physically removing pMHCII complexes from the surface of DCs in vitro, in a process of trans-endocytosis (Akkaya et al., 2019). In this process, Tregs remove the pMHCII complexes based on the antigen specificity, while leaving the other peptides untouched. While the above-described mechanisms may seem numerous, many of them can act in synergistic manner with others, while contributing to the signature function of Tregs.

Exogenous antigens and Tregs

Apart from protecting the organism from unwanted autoreactivity, the immune system faces another challenge in establishing tolerance to antigens that are foreign to the body, but their presence is inevitable. One of the sites of massive antigen turnover is the intestine, colonized with approximately 3.8·10^13 microbial cells (Sender et al., 2016). Apart from microbes, food antigens are present in high numbers and the immune system must not perceive them as danger. The interplay of the immune system, commensal microbiota, and food antigens is therefore very intriguing. The small intestine, consisting of duodenum, jejunum, and ileum, is adapted for maximal food absorption; consequently, we could assume that food antigens are the majority in this part of the gastrointestinal tract (GT).

On the other hand, the large intestine, comprising the caecum, colon and rectum, absorbs a majority of water and minerals, while providing a suitable habitat for the microbiota. The role of Tregs in oral tolerance was foreshadowed by using mice models with disrupted TGF-β signaling, which resulted in intestinal inflammation (Gorelik & Flavell, 2000). Even earlier studies have shown that mice deficient in IL10 were developing symptoms similar to irritable bowel disease (IBD) (Kühn et al., 1993). These results were later attributed to the inability of the intestinal Treg population of IL10-deficient mice to suppress inflammation. Germ-free (GF) mouse models have shown that portion of intestinal Tregs of GF mice fail to express sufficient levels of FOXP3 and are therefore less efficient suppressors (Ostman et al., 2006).

Historically, Peyer's patches (PP) were considered the exclusive mediators of oral tolerance. This consensus has shifted after the successful induction of oral tolerance in mice without PP, highlighting the function of mesenteric lymph nodes (MLN) (Spahn et al., 2002). Present in PP and other lymphoid aggregations, microfold cells (M cells) allow for antigen transport to

lamina propria (LP) (Mabbott et al., 2013). Antigen present in LP can then be uptaken by LP dendritic cells and presented either in situ or transported to lymph nodes and presented there (Chirdo et al., 2005). It is also worth mentioning the ability of LP DCs to open epithelial tight junctions, allowing them to sample antigens from the gut lumen directly (Rescigno et al., 2001). The importance of DCs and MLN was further highlighted by the deletion of CCR7, a receptor responsible for DC migration into MLN (Worbs et al., 2006). Mice deficient in CCR7 were unable to establish oral tolerance without DC-mediated antigen sampling and transport. These data allowed the intestinal immunology field to dive deeper into the exact mechanisms of oral tolerance to food and microbiota antigens.

Intestinal immune environment

Throughout its evolution, the intestine was continually exposed to a large number of antigens, and it adapted accordingly, becoming one of the most important immunological sites in the human body. The digestive tract is regionalized, with each part playing a distinct, often a non-overlapping role. Therefore, it is important to describe each region of the intestine and its immunological function to understand the interplay between digestion and oral tolerance. To maximize the absorbing surface, the cells of absortive epithelial enterocytes of small intestine forms of a finger-like lumen-protruding structures referred to as villi. Between these villi, a small invagination, denoted as the crypts of Lieberkühn, are readily observed, which serve as loci where the multipotent intestinal stem cells are localized. In addition, in the crypts there are also cells denoted as Paneth cells which play a critical role in regulating the microbiota by production of antimicrobial peptides (Ayabe et al., 2000). Goblet cells also play an important role in producing mucus, which covers the small intestine in one layer and the large intestine in two layers (Atuma et al., 2001).

The purpose of a mucus layer is to form a physical barrier between epithelial cells and microbiota, which is commonly found in the layer proximal to the lumen of the large intestine, in contrast to the inner layer, which is devoid of bacteria (Johansson et al., 2008). The layer which is in a direct contact with the lumen is called mucosa, which harbors most immune cells. In the outward direction, there is a connective tissue called submucosa, a muscle layer, and a fibrous layer called serosa.

As mentioned in the previous chapter, gut-associated lymphoid tissue in the small intestine is represented by PP. Analogous to PP in the small intestine, the large intestine contains caecal and colonic patches, which are an important site for T cell priming (R. L. Owen et al., 1991).

A simple experiment consisting of injecting a blue dye into a rat's intestine revealed the character of lymph drainage in the GT (Carter & Collins, 1974). While MLNs drain the major parts of the GT, mainly the jejunum, distal ileum, caecum, and ascending colon, they are not the only lymph nodes to play a part. The duodenum and transverse colon were shown to drain to the duodenopancreatic lymph nodes, while the caudal lymph node drains the descending colon and rectum.

It is important to emphasize that the heterogeneity of lymphocyte subsets present in GT goes beyond the scope of description in this thesis and varies greatly depending on the localization and particular region of interest. It is important to mention the inverse correlation between Th17 and Tregs, with the Treg population reaching the highest number in the colon (Denning et al., 2011). This study also provides an insight into functional specialization of DCs and macrophages in respect to the regulation of Th17 versus Tregs ratio in various intestinal regions. Lacking antigen receptors, innate lymphoid cells shape the intestinal immune environment via the production of cytokines and other diverse mechanisms (Spits & Di Santo, 2011). Due to the constantly changing antigenic environment of the intestine, a given analysis of immune cell composition thus can provides only a snapshot insight into the ever-changing attributes of intestinal immunity.

Intestinal Treg populations

Opposing the Th1, Th2, and Th17 cell proinflammatory function, intestinal Tregs are a vital part of the suppression machinery. Initial experiments using colonization of GF mice with B. fragilis have proved that Th17 and Treg levels react to the presence of microorganisms, with Treg numbers increasing 2-fold after the colonization of colon (Round & Mazmanian, 2010). Further, apart from the microbiota-specific population, another, microbiota-independent population of pTregs was found in the small intestine (Weiss et al., 2012), which was later found to be food-antigen specific. Using an interesting antigen-free mouse model, another study demonstrated that dietary antigens induce pTregs mainly in the small intestine lamina propria and that this process occurs upon introduction to solid food antigens (Kim et al., 2016). Together, the data mentioned earlier presume the existence of two distinct pTreg populations, one microbiota-specific, and one food antigen-specific, both indispensable for oral tolerance induction.

Previously thought to be an antagonist factor to FOXP3, Retinoid orphan receptor gamma (Rorγ) was shown to be the factor responsible for Treg response to symbiotic microbiota (Sefik

et al., 2015). Mutually exclusive to Rorγ, transcription factor GATA3 plays a role in Treg inflammation response, and GATA3+ Tregs were found to be a subset of Helios+ Tregs (Wohlfert et al., 2011). Interestingly, it is the Helios+ GATA3+ Treg population that responds to alarmin IL33, signaling tissue damage (Schiering et al., 2014). These results led to the speculations that GATA+ Tregs respond to dangerous gut pathogens, while Rorγ Tregs play a role in communication between the host and symbionts. Later identified transcription factor c-MAF was proven to be responsible for the differentiation and function of microbiota-specific Tregs, even in the presence of possibly pathogenic microbe as *Helicobacter hepaticus*, capable of causing IBD (Xu et al., 2018a).

While not always correct, for the sake of clarity it is possible to differentiate two major intestinal Treg populations, with one being Helios+ GATA3+ Tregs, not induced by gut microbiota. The second population of Helios- Rorγ+ c-MAF+ Tregs is induced by gut microbiota and was even found to be partially independent of FOXP3, with Rorγ partially compensating for the lack of FOXP3 (Chowdhary et al., 2023). One particular study assessed a time window during the weaning period, in which microbiota-specific long-lived Tregs are induced (Knoop et al., 2017). Microbiota-specific Treg population expansion in the colon was measured around day 18, not earlier than day 8, and not later than day 28. While the microbiota-specific Treg induction is not exclusive to the mentioned time window, Tregs induced after this period were later proven more transient (Knoop et al., 2020). This study also highlighted the anti-allergenic effect of breastfeeding and showed that lowered levels of Rorγ pTreg after the use of antibiotics in early life resulted in hindered tolerance to oral antigens. An interesting mode of heritability, influenced by the mother, was discovered upon tracing the Rorγ Treg phenotypes across multiple mice generations (Ramanan et al., 2020). The results show that the IgA antibodies passed via mother milk coat specific strains of bacteria in the intestine, effectively modifying their ability to introduce Rorγ+ Tregs. This is, however, one of the many mechanisms involved in maintaining the stable populations of Tregs in the GI.

Role of intestinal Tregs

The importance of the interplay between FOXP3+ Tregs and microbiota was demonstrated by the discovery of the self-regulatory IgA-microbiota loop (Kawamoto et al., 2014). The research team has found a significant negative correlation between the amount of IgA produced in germinal centers and the diversity in microbiota, which again resulted in the amount of IgA and induced FOXP3+ cells. This notion was later reformulated die to the previously mentioned

work revolving around maternal inheritance, which proposed a double-negative feedback loop, based on the measured negative correlation between Rorγ+ Tregs and IgA-coated microbiota (Ramanan et al., 2020). Later it was proven that Rorγ+ pTregs protect symbiotic microbiota by suppressing Teff cells which are specific for the same epitope (Xu et al., 2018).

Another essential function of intestinal Tregs is the secretion of IL-10, which promotes intestinal stem cell renewal (Biton et al., 2018). Considering the raised levels of Tregs after inflammation, it is reasonable to assume that Tregs play a role in reestablishing gut tissue homeostasis after inflammatory events. Using a pTreg-deficient mouse model, it has been shown that Rorγ+ Treg play also a critical role in the suppression of mastocytosis and type 2 immune responses, which are facilitated by Th2 cells (Campbell et al., 2018). In pathological conditions like human colorectal cancer or mouse polyposis, where the disbalance in microbiota is present, Rorγ+ Tregs failed to suppress inflammation and in turn supported the tumor growth by inhibiting CD8+ T cell anti-tumor surveillance (Osman et al., 2021). This discovery lend a support to the speculative idea of transiently removing Rorγ+ Tregs to aid cancer therapy.

Regarding food antigens, a particular population of Rorγ- pTregs was found to be stable in the absence of microbiota but drastically reduced in the absence of antigens in the diet (Kim et al., 2016). This population was later labeled as food antigen-specific because its deletion resulted in allergy to food. The analysis of human fecal microbiota has also highlighted an essential link between microbiota-specific Tregs and food allergy (Abdel-Gadir et al., 2019). Rorγ+ Treg-deficient mice were more sensitive to food allergies and a significant microbiota dysbiosis was present in patients with food allergies. The authors also proposed a MyD88-Rorγt regulatory axis, which creates a disbalance between IgA and IgE responses to symbiotic microbiota during dysbiosis.

Intestinal Treg sources

While the exact molecular marker used for the distinction between tTregs and pTregs in the intestine has not been completely established yet, Helios+ GATA3+ Tregs are generally considered to be of thymic origin, while Rorγ+ c-MAF+ Tregs are considered to be peripherally induced. However, the evidence confirming this notion is far from absolute, and arguments against this distinction still exist. One of them is the fact that Helios and Neuropilin-1 are not reliable discriminators between thymic or peripheral origin of Tregs (Szurek et al., 2015). On the other side, Rorγ is also not the exclusive marker of pTregs, because of documented

instances of thymus-derived Tregs expressing Rorγ under inflammatory conditions (J. Yang et al., 2018). However, genetic tracing after the introduction of microbiota and food antigens was able to visualize that the majority of induced pTregs were in fact Rorγ+ (van der Veeken et al., 2022).

As mentioned in the chapter about negative selection, thymic APCs play an indispensable role in tTreg induction. Less obvious is the nature of APC population responsible for Treg induction in the periphery. The most serious candidates were the DCs present in LP, because of their ability to generate Tregs in vitro, via the TGF-β-–mediated conversion in the presence of retinoic acid (Sun et al., 2007). Interestingly, a breakthrough discovery revealed that disabling MHC II antigen presentation on Rorγ+ cells results in mice unable to establish microbiota tolerance, which results in severe colitis (Hepworth et al., 2013). This observation led to the conclusion that Rorγ+ APCs facilitate microbiota-specific T cell deletion via MHCII-dependent interactions, thus preventing intestinal inflammation.

The idea of T cell deletion in the intestinal environment later inspired the thought that Rorγ+ Tregs are induced by Rorγ+ APCs, with innate lymphoid cells type 3 (ILC3) being the main candidates. This was mainly because of the assumption that ILC3s are the only Rorγ+ professional APCs expressing MHC II (Eberl et al., 2004). However, this argument was slowly losing validity as the new cDC2 subset expressing Rorγ was discovered (C. C. Brown et al., 2019). An interesting addition was also the description of a new AIRE+ Rorγ+ ILC3-like cell population capable of interacting with CD4+ T-cells (Yamano et al., 2019). These ILC3-like cells were found in peripheral lymph nodes and resembled the ILC3s, but also DCs. AIRE expressed in these cells led to speculations about the presentation mechanism similar to the thymic negative selection, but it seems that the role of AIRE in this mechanism is dispensable (Akagbosu et al., 2022).

The importance of Rorγ+ APCs was highlighted in a study that also described their CCR7-dependent homing mechanism resulting in the induction of Rorγ+ Tregs in mesenteric lymph nodes (Kedmi et al., 2022). This study also proposed that the mechanism is not APC-specific, i.e., that various APCs display somewhat different capacity to induce Tregs. The authors also proposed that the APCs which are responsible for the generation of Rorγ+ Treg could be the earlier discovered Janus cells or ILC3s, but this remained without direct evidence. In line with this study came the results of experiments using *Helicobacter hepaticus* and single-cell RNA sequencing (sc-RNA-seq), which indicated that ILC3-like cells are the real Rorγ+ Treg inducers via ITGAV–ITGB3 integrin-mediated processing of TGFβ (Lyu et al., 2022). This study suggested that the ILC3 population alone is capable of inducing a sufficient population

of Rorγ+ Tregs in the large intestine and MLNs. To further highlight the importance of ILC3s, the authors also observed a disbalance in the numbers of ILC3s and Rorγ+ Tregs during IBD. The notion of Rorγ+ APCs being the ultimate T cell-mediated tolerance inducers was also successfully confirmed by repeating the experiment with MHC II presentation impairment (Akagbosu et al., 2022). Apart from colitis and loss of Rorγ+ Tregs, authors also report increased numbers of Th17 cells, which supports the idea of competition between Th17 cells and Rorγ+ Tregs. By temporally manipulating the expression of MHCII on Rorγ+ APCs and using antibiotics, authors have also proven the importance of a short time window, in which the majority of pTreg population is induced.

An important discovery of a specific cell type of APC came with scRNAseq of MLN cells. Authors were able to distinguish four APC cell types with similarities to mTECs and DCs, also expressing a regulator p63, which is typical for epithelial cells. These newly discovered AIRE+ cells were also analyzed via electron microscopy, which further concluded their phenotypical difference from mTECs, DCs, and ILC3s, and their resemblance to the myeloid lineage. Further experiments have shown that unlike the dispensable role of DCs and ILC3s in pTreg induction, the role of these newly-called Thetis cells (TCs) is vital for establishing oral tolerance in early life. Taken together with the results of the previous 2 studies, the contradictory hints of either ILC3s, Janus, or TCs being the key Rorγ+ APC cell type caused significant confusion in the field of Treg-mediated dominant immune tolerance. It is crucial to mention the age difference in mice used in the two studies heading in the direction of ILC3s and Janus cells and the ones focused on TCs.

Among the four populations of TCs, TC IV, expressing Itgb8, crucial for TGFβ processing, was also assigned a role in food antigen-specific Treg induction (Parisotto et al., 2024). The authors of this study also challenged the earlier idea of DCs being the subset responsible for the establishment of food tolerance. Using scRNAseq, another group was able to localize 2 distinct populations of Rorγ+ APC candidates, one with characteristic of TC I transcriptomics, and the other expressing the transcription coregulator Prdm16 (Fu et al., 2024). The second population also expressed AIRE and CCR7 more than the TC I subset, suggesting that it can be a migratory subset and/or functioning in an AIRE-dependent manner. Interestingly, upon inspection of a human dataset, authors found a very similar Prdm16+ subset of cells, differing only in the absence of AIRE. Despite the field leaning towards TCs being the crucial APC required for Rorγ+ Treg induction, some venues remain unexplored, and the answer is still not definitive.

Tregs and microbiota in disease

Since their discovery, Tregs have remained at the center of the attention of scientists searching for therapy for various immune diseases. Intriguing is the field of gastrointestinal immune diseases, like IBD, commonly divided into Crohn's disease (CD) and ulcerative colitis (UC). Due to their impressive immunosuppressive and regenerative effect, Tregs have been used in cell therapy not long after their discovery. Regarding IBD, a single transfer of CD4+ CD25+ T cells was enough to reverse histological pathological abnormalities in colitis model mice colon after 10 weeks (Mottet et al., 2003). Interestingly, Tregs which are present in the intestine of IBD patients, showed an unusual IL-17+ phenotype, which is presumed to be responsible for the inflammation despite its immunosuppressive ability (Kryczek et al., 2011). Using flow cytometry, scientists were able to measure increased rates of intestinal and circulating Tregs in pediatric patients suffering from IBD (Sznurkowska et al., 2020). This finding prompts the question of what mechanism leads to the malfunction of immune regulation, despite the higher number of suppressor cells. One theory trying to explain this phenomenon relies on data showing that under inflammatory conditions, FOXP3+ Tregs can switch their phenotype, downregulating FOXP3 and acquiring Th17 phenotype, a typical proinflammatory feature (X. O. Yang et al., 2008). It is also likely that FOXP3+ Tregs simply acquire the IL-17 phenotype and therefore contribute to inflammation despite their conserved immunosupressive abilities. The second theory proposed that rather than Tregs, it is the Teff cells that evade dominant tolerance by expressing Smad7, which hinders the TGFβ-dependent Treg signaling (Fantini et al., 2009). Immunochemistry analysis of biopsy samples from patients suffering from CD has shown that Smad7 upregulation occurs in early phases of this inflammatory disease (Zorzi et al., 2020).

To mitigate IBD, several studies have tried to implement FOXP3+ iTreg in vitro induction, but have failed due to iTregs switching back to proinflammatory phenotype upon secondary exposure to antigen (Beres et al., 2011). Further Treg-based therapeutic research has been dealing with problems of iTreg instability, because of the inability to mimic proinflammatory conditions ex vivo. Upon transfer, both in vitro and ex vivo iTregs lose FOXP3+ expression and turn into proinflammatory exFOXP3 Teff cells (Hua et al., 2018). CRISPR-mediated FOXP3+ knockin has shown that mature Tregs do not rely solely on FOXP3 to maintain their immunosuppressive abilities and has remained a go-to method in exploring this way of immunotherapy (Lam et al., 2021). Contrary to iTreg therapy, a part of the immunotherapy field has focused more on in vivo Treg cell expansion as the means of immunosuppression.

The administration of a low-dose (LD) of IL-2 was effective in mitigating the induced mouse colitis humanized model (Goettel et al., 2019). Early clinical trials have proven the safety of LD IL-2 treatment of CD and UC, but the efficiency of Treg induction remains in question (Rosenzwajg et al., 2019). Some scientists have proposed a combined therapeutic approach of Treg transfer with their IL-2 mediated expansion, but additional research is needed to provide greater context and insight into this area.

Discussion

Since the discovery of FOXP3+ Tregs, the field of dominant tolerance has received a great amount of attention, which resulted in many valuable discoveries and seminal papers. This thesis aimed to review this critical component of the immune system. First chapters were dedicated to the stages of T cell development, which begin in the bone marrow, through VDJ recombination, positive and negative selection occuring in the thymus. It is important to notice that neither of these processes is perfect, so some autoreactive T cells escape to the periphery and pose risk of autoimmunity. The evolution came up with a mechanism to suppress these autoreactive cells, through peripheral deletion as well as suppression using Tregs. As a functional outcome of Treg evolution, the immune system also evolved a process to suppress immune responses to food- and symbiotic microbiota-derived antigens.

With the overlap to gastrointestinal biology, Tregs quickly caught attention of many immunologists as the description of their role could be the key to explain the mutually beneficial interplay between the immune system and commensal microbiota. At the moment there is a consensus in two main intestinal Treg populations, Helios+ tTregs and Rorγ+ pTregs, with the first residing mainly in the small intestine and second in the colon. While this separation is useful for overall simplification of the real situation, there are many reasons to believe that the situation is much more complicated. Intestinal Tregs are considered, in general, somewhat less stable compared to tTregs, with microbiota-specific Rorγ+ pTregs being more stable than food-antigen-specific ones. In addition, both Helios and Rorγ were shown to be unreliable markers under certain circumstances. It seems that several research groups are in the initial stages of describing new types of Treg populations, so today's view is likely going to change with future research. It is also important to take into consideration methods used in assessing the overall impact of Tregs on microbiota because many assumptions have been made due to the utility of germ-free mice, which are known to be physiologically very distinct from their conventional counterparts.

When looking at pTregs, it is crucial to define the APC population responsible for their induction. At first, the theory was that Rorγ+ ILCs delete microbiota-specific T cells in the periphery. As the number of newly discovered subsets of Rorγ+ APCs is gradually increasing, a debate emerged, about whether ILC3s, Janus or Thetis cells are the key population for pTreg induction. The publically available studies discussed in this work suggest that TCs act as the main Rorγ+ Treg-inducing APC subset in 2-3 week old mice, which adequately aligns with the short window of microbiota-specific Treg induction compared to results of other studies which used adult mice. Indeed, at the moment it seems that TCs could be the main contributing population, but further research is ongoing in to clarify this issue.

The last part of this thesis focuses on clinical research exploring Treg therapy. Using the current knowledge, it is reasonable to assume that Treg pathologies manifest in many autoimmune diseases, and thus the understanding of underpinning causes could lead to efficient therapeutic protocols of effective intervention. However, current research struggles with several problems, the most obvious being the transferred iTreg instability. On the bright side, there are ongoing clinical studies with Tregs that potentially could, if successful, bring a new hope into the lives of patients suffering from autoimmune conditions like IBD, celiac disease, or gastritis.

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(*=review article)

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