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Immune-epithelial interactions in the multilayered model of intestinal homeostasis

Interakce mezi epiteliálními a imunitními buňkami v mnohovrstvém modelu střevní homeostázy

Type of thesis

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Declaration:

I hereby declare that this thesis is a presentation of my original research, where I referenced all the relevant literary sources. I also state that neither this work, nor its substantial part, was used for the award of academic degree or diploma in the past.

Prague, 4.5.2023

Prohlášení:

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Abstract

The intestinal immune system is constantly faced with a vast variety of foreign antigens from food and commensal microbiota on top of intestinal self-antigens. To prevent pathology, the immune system developed multiple mechanisms to tolerate these harmless antigens. These mechanisms use a collaboration of thymic T-cell selection and intestinal homeostatic processes. At the same time, intestinal microbiota must be tightly controlled to prevent its overgrowth, which can lead to pathology. Thus, the intestinal immune system must use a combination of tolerogenic and immunogenic responses and keep them in equilibrium. In this thesis I first provide an overview of the current state of knowledge of these processes and then I present several original studies in which I have participated. First and foremost, in the study that is central to this thesis, we have shown that IL-17-mediated stimulation of Paneth cell antimicrobial functions is one of the important mechanisms of immune-mediated control of commensal microbiota and its perturbation results in intestinal pathology susceptibility. Additionally, I have participated in several other studies, which in combination extend the view of intestinal homeostasis, integrating intestine-specific processes with thymic T-cell selection and reactions to pathobionts. One of these studies identified a novel mechanism that ensures proper thymic T-cells selection, through the transfer of antigens from thymic epithelium to a specific population of $CD14^+$ dendritic cells. In a follow-up study, we described the complexity of the cellular network that participates in this mode of antigen transfer in the thymus. In another study, we identified a population of $Aire^+$ ILC3-like cells that were later found to play a crucial role in immune response to mucosal pathobiont *Candida albicans*. Here I discuss our original findings with previously published results, to deliver a multilayered model of establishment and maintenance of intestinal homeostasis.

Abstrakt (CZ)

Střevní imunitní systém musí neustále čelit obrovskému množství cizorodých antigenů z potravy a komenzální mikroflóry, mimo střevních tělu vlastních antigenů. Imunitní systém vyvinul množství mechanismů pro toleranci těchto neškodných antigenů, aby předešel patologii. Tyto mechanismy využívají spolupráce selekce T-buněk v brzlíku s homeostatickými mechanismy ve střevě. Zároveň je však nutno kontrolovat střevní mikroflóru, která by jinak mohla přerůstat, což může vést k patologii. Proto musí imunitní systém střeva využívat kombinaci tolerogenních a imunogenních odpovědí a udržovat je v rovnováze. V této disertační práci nejdříve shrnuji přehled současného stavu poznání těchto procesů a dále představím několik originálních studií kterých jsem se účastnil. V první řadě, v centrální studii této práce jsme ukázali, že IL-17 zprostředkovaná stimulace antimikrobiálních funkcí Panetových buněk je jedním z důležitých imunitních mechanismů kontroly střevní mikroflóry a porušení tohoto mechanismu vede k náchylnosti ke střevní patologii. Dále jsem se účastnil několika dalších studií, které společně rozšiřují pohled na střevní homeostázu a integrují procesy specifické pro střevo se selekcí T-buněk v brzlíku a reakcemi na pathobionty. Jedna z těchto prací identifikovala nový mechanismus, který zajišťuje správnou selekci T-buněk v brzlíku, skrze předávání antigenů z brzlíkového epitelu na specifickou populaci $CD14^+$ dendritických buněk. V navazující práci jsme popsali komplexitu sítě buněk, které se účastní tohoto způsobu předávání antigenů. V další studii jsme identifikovali populaci $Aire^+$ buněk podobných ILC3, které byli později popsány jako zásadní regulátoři imunitní odpovědi proti slizničnímu pathobiontovi *Candida albicans.* V této práci diskutuji naše výsledky s dříve publikovanými studiemi a představuji mnohovrstevný model ustavení a udržování střevní homeostázy.

Table of contents

Acknowledgments 4
Abstract 5
Abstrakt (CZ) 6
Table of contents 7
List of abbreviations 8
Introduction 9
Current state of knowledge 10
Immune tolerance 10
Central tolerance 10
Tolerance to food and commensal antigens 16
Tolerate or respond? 25
Spatiotemporal map of intestinal homeostasis 31
Breakdown of intestinal homeostasis 34
Summary 37
Thesis aims 39
Results 40
IL-17 driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum 41
Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation. 42
A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer. 43
A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire 44
Aire-expressing ILC3-like cells in the lymph node display potent APC features 45
Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice 46
Discussion and conclusions 47
References 50

List of abbreviations

aDCs	Activated dendritic cells
APCs	Antigen presenting cells
APS-1	Autoimmune polyglandular syndrome type-1
CAT	Cooperative antigen transfer
CD	Crohn's disease
cDC	Conventional dendritic cells
cDC1	Conventional dendritic cells type-1
cDC2	Conventional dendritic cells type-2
CMC	Chronic mucocutaneous candidiasis
DCs	Dendritic cells
FAE	Follicle-associated epithelium
GAPs	Goblet cell associated passages
GWAS	Genome-wide association study
IBD	Inflammatory bowel diseases
ILC3s	Innate lymphoid cells type-3
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
M-cells	Microfold cells
MHCI	Major histocompatibility complex I
MHCII	Major histocompatibility complex II
mLNs	Mesenteric lymph nodes
mTECs	Medullary thymic epithelial cells
pDCs	Plasmacytoid dendritic cells
PPs	Payer's patches
PCs	Paneth cells
scRNA-seq	Single cell RNA sequencing
SFB	Segmented filamentous bacteria
TCR	T-cell receptor
TEDs	Trans-endothelial dendrites
Th17	Helper T-cell type-17
TLRs	Toll-like receptors
TRAs	Tissue restricted antigens
Tregs	Regulatory T-cells
UC	Ulcerative colitis

Introduction

The primary function of the immune system is to protect the organism from invading pathogens. However, to perform this function without hurting the host, it is essential to distinguish pathogens from body-own tissues. On top of body-own tissues immune system needs to tolerate plethora of other antigens coming from harmless sources such as food, air-born particles, and commensal microbiota. Crucial importance of these process can be illustrated by the development of severe autoimmune disorders when tolerance to self-antigens is disrupted, food allergies when tolerance to food antigens is disrupted, and inflammatory bowel diseases (IBD) when tolerance to intestinal microbiota is disrupted.

In the case of commensal microbiota, the situation is extremely complex, since immune system needs to distinguish between microbes able to cause damage to the host (pathogens), from those that are harmless (commensals), despite the fact that those two groups share majority of their molecular patterns. Furthermore, unlike antigens from body-own tissues and food which pose no danger to the host, antigens from commensal microbiota cannot be simply ignored since this could lead to the overgrowth of the microbe even in the case of commensals (Kumar et al., 2016). Thus, immune homeostasis in the intestine, which contains the largest reservoir of foreign antigens coming from harmless sources on top of intestinal self-antigens, is arguably one of the most complex topics in the immunology field. While most food antigens are localized there, the intestine also contains the largest variety of commensal microbiota, unmatched anywhere else in the body.

Throughout my Ph.D. studies I have focused on various aspects of intestinal immune homeostasis, ranging from thymic T-cell selection to antimicrobial effector mechanisms executed by Paneth cells (PCs). I participated in several studies that highlighted the central role of epithelial-immune interactions in this process, starting from the cooperation of thymic epithelial cells with thymic dendritic cells (DCs) and ending with the immune-mediated stimulation of PC antimicrobial functions. In this thesis, I will first summarize scientific knowledge of processes that enable immune homeostasis in the intestine, then I will present studies in which I participated and finally I will discuss the overall context of my results in the broader scope of the field.

Current state of knowledge

Immune tolerance

Immune tolerance is a state in which the immune system does not respond to a potential trigger either by active inhibition of immune responses or by simple ignorance. Since the antigen receptors, i.e. T-cell receptors (TCR) and B-cells receptors are generated by a stochastic process (Klein et al., 2014), it is crucial to eliminate or suppress clones recognizing antigens from harmless sources. Vertebrates evolved a multistep mechanism that mediates this process. The intestine contains both self- and food/commensal-derived antigens, which need to be tolerated by the immune system, thus it needs to utilize additional tolerance mechanisms to those used in other tissues. I will describe these mechanisms in a stepwise manner, starting from the central tolerance, followed by the description of intestine-specific mechanisms.

Central tolerance

Medullary thymic epithelial cells

Central tolerance takes place in the thymus, where T-cells develop and its primary aim is to eliminate T-cell clones, which recognize antigens from body-own tissues. The principal problem of thymic T-cell selection lies in the necessity to physically present all self-antigens directly in the thymus, in order to ensure deletion of self-reactive T-cells. This issue is particularly important for antigens that are typically restricted to specific tissues in the immune periphery, so called tissue restricted antigens (TRA), such as pancreatic insulin. It was long a mystery, how thymic selection can eliminate TRA-reactive T-cells. Strikingly, it was discovered that medullary thymic epithelial cells (mTECs) can express almost complete protein coding genome, thus they generate a "shadow" of the immune periphery directly inside the thymus (Anderson et al., 2002). This incredible gene expression property of mTECs is largely enabled by a unique transcription regulator protein Autoimmune regulator (Aire) (Anderson et al., 2002; Consortium, 1997; Nagamine et al., 1997), which stochastically activates expression of suppressed genes, leading to promiscuous expression of silenced genes (Bansal et al., 2017; Brennecke et al., 2015; Meredith et al., 2015).

Once, T-cell recognizes an antigen presented in the context of major histocompatibity complex class I and II (MHCI and II) on mTEC surface, it is either directly eliminated (Hinterberger et al., 2010; Liston et al., 2003; Surh and Sprent, 1994) or it is diverted to regulatory T-cell lineage (Treg) (Aschenbrenner et al., 2007; Malchow et al., 2016), which suppress inflammatory immune responses in the periphery (Bennett et al., 2001; Fontenot et al., 2005; Kim et al., 2007). In either case, self-specific T-cell is thus eliminated from the repertoire of conventional T-cells that are allowed to exit the thymus and participate in the immune patrolling of the peripheral tissues. Since mTECs produce TRAs endogenously, it enables them to eliminate self-specific $CD8^+$ T-cells by presentation of TRA peptides

on MHCI. However, mTECs are also endowed with the ability to utilize endogenously expressed TRAs for the MHCII-dependent presentation to the developing $CD4^+$ T-cells, even though MHCII classically presents exogenous antigens. Mechanistically, this is achieved by autophagy, which enables mTECs to deliver endogenous proteins to the endosomal compartment, where MHCII is being loaded with antigens. This enables mTECs to directly eliminate self-specific $CD4^+$ T-cells or to divert them to Treg lineage (Nedjic et al., 2008; Wu et al., 2013).

Avidity model. It was postulated that the decision between clonal deletion, diversion to Treg linage and classic conventional T-cell development is achieved by the combination of TCR affinity to self-antigen-MHC complex and the abundance of the antigen in thymus, which together constitute antigen-MHC-TCR avidity. In this model self-reactive clones with intermediate affinity and/or low antigen availability are destined to become regulatory T-cells (Tregs), while high affinity clones reactive to antigens ubiquitously expressed in the thymus are destined to be clonally deleted (Klein et al., 2014). Indeed, it was experimentally proven that: 1) TCR affinity dictates negative selection vs. generation of Treg (Jordan et al., 2001; Relland et al., 2009; Simons et al., 2010), 2) tolerance to ubiquitous antigens is mediated by clonal deletion, while tolerance to TRAs is rather mediated by the generation of Tregs (Legoux et al., 2015; Malhotra et al., 2016). On the top of the avidity, other mechanisms, such as costimulatory molecules context, participate in fate decision between clonal deletion and Treg selection (Klein et al., 2019). Thus, T-cells that pose the highest danger to cause autoimmunity (high affinity and/or reactive to ubiquitous antigens) are never allowed to leave the thymus, while T-cell with mild and/or tissue-restricted self-reactivity are exploited to prevent autoimmune reactions by their diversion to Treg lineage. In the periphery Tregs use a number of immunosuppressive mechanisms, such as the production of immunosuppressive cytokines (IL-10, TGFβ) and direct suppression of antigen presenting cells.

Cooperative antigen transfer

Although mTECs are crucial players in the thymic selection of self-specific T-cells their numbers are limited. Furthermore, it was shown that although mTECs as a population indeed express almost all the possible TRAs, one mTEC expresses only a small fraction of TRA repertoire and as a result each individual TRA is expressed only by 1-3% of mTECs at a given point of time (Brennecke et al., 2015; Meredith et al., 2015). Thus, the availability of TRA antigens would be very low if only mTECs would carry out TRA presentation. On the top of this scattered TRA expression, recent studies demonstrated that mTECs are able to generate so called “mimetic” lineages in the thymus. These distinct yet rare cells represent thymic counterparts of specialized cell types in the periphery. Thus, thymus contains for example cells resembling skin corneocytes, intestinal microfold cells (M-cells) or intestinal tuft cells (Bornstein et al., 2018; Michelson et al., 2022; Miller et al., 2018). Nevertheless, the ability of these

cells to process and present antigens and thus their direct contribution to the T-cell selection remains unclear.

To compensate for low availability of TRAs, mTEC-produced antigens are recycled in a process called cooperative antigen transfer (CAT) (Koble and Kyewski, 2009). In this process, thymic DCs acquire mTEC-produced antigens and present them to developing thymocytes. Importantly, it was demonstrated that CAT functionally contributes to central tolerance (Leventhal et al., 2016; Perry et al., 2014; Perry et al., 2018). While not studied so far, the above described mimetic mTECs could serve as an ideal source of antigens for CAT, since they accumulate peripheral antigens in high amounts, but their intrinsic ability to present antigens is unclear. However, molecular mechanisms which regulate CAT as well as the complexity of cellular network, which participates in CAT are poorly understood. To demonstrate this issue, I will start by the description of thymic DC populations, which serve the purpose of putative CAT recipients. Next, I will summarize the current knowledge of mechanisms regulating CAT.

DC heterogeneity in the thymus. DCs were classically divided to plasmacytoid (pDC) and classical (cDC) ones. However, it should be noted that the identity of pDCs as members of DC lineage is disputed (Reizis et al., 2023; Ziegler-Heitbrock et al., 2023a; Ziegler-Heitbrock et al., 2023b). cDCs are further subdivided to type-1 (cDC1) and type-2 (cDC2). cDC1 are marked by their unique expression of XCR1 chemokine receptor and they possess a unique ability to perform cross presentation, which enables them to present exogenous antigens to $CD8^+$ T-cells in the context of MHCI (Belz et al., 2002; den Haan et al., 2000). cDC2 on the other hand are functionally and phenotypically closer to macrophage lineage and they share many of classic monocyte/macrophage markers, such as CD11b and Sirpα. While phenotypically similar, another category of so called monocyte-derived DCs (moDC) can be defined (Fig. 1) (Liu et al., 2021). These cells carry a combination of bona fide monocyte/macrophage markers, such as CD14, Cx3cr1 and Csf1r, together with markers that define cDC lineage, such as Flt3. We were among the first to define the heterogeneity of thymic DCs in an unbiased single cell RNA sequencing (scRNA-seq) approach. Importantly, this enabled us to distinguish moDCs from cDC2 lineage both phenotypically and functionally (Vobořil et al., 2020).

We and others also identified another population of so called activated cDCs (aDCs), which are marked by the expression of Ccr7 (Breed et al., 2022; Park et al., 2020; Vobořil et al., 2020). We were able to identify two subpopulations of these aDCs, $Xcr1^+$ and $Xcr1^-$ (Vobořil et al., 2022), in agreement with other studies which used scRNA-seq approach (Breed et al., 2022; Park et al., 2020). While the origin of these cells is currently unknown, it seems likely that $Xcr1^+$ aDCs are ontogenically related to cDC1, due to their shared expression of Xcr1. In contrast, cDC2s likely contribute to thymic $Xcr1^-$ aDC pool, since the depletion of cDC2 leads to a drop in numbers of thymic $Xcr1^-$ aDCs (Breed et al., 2022).

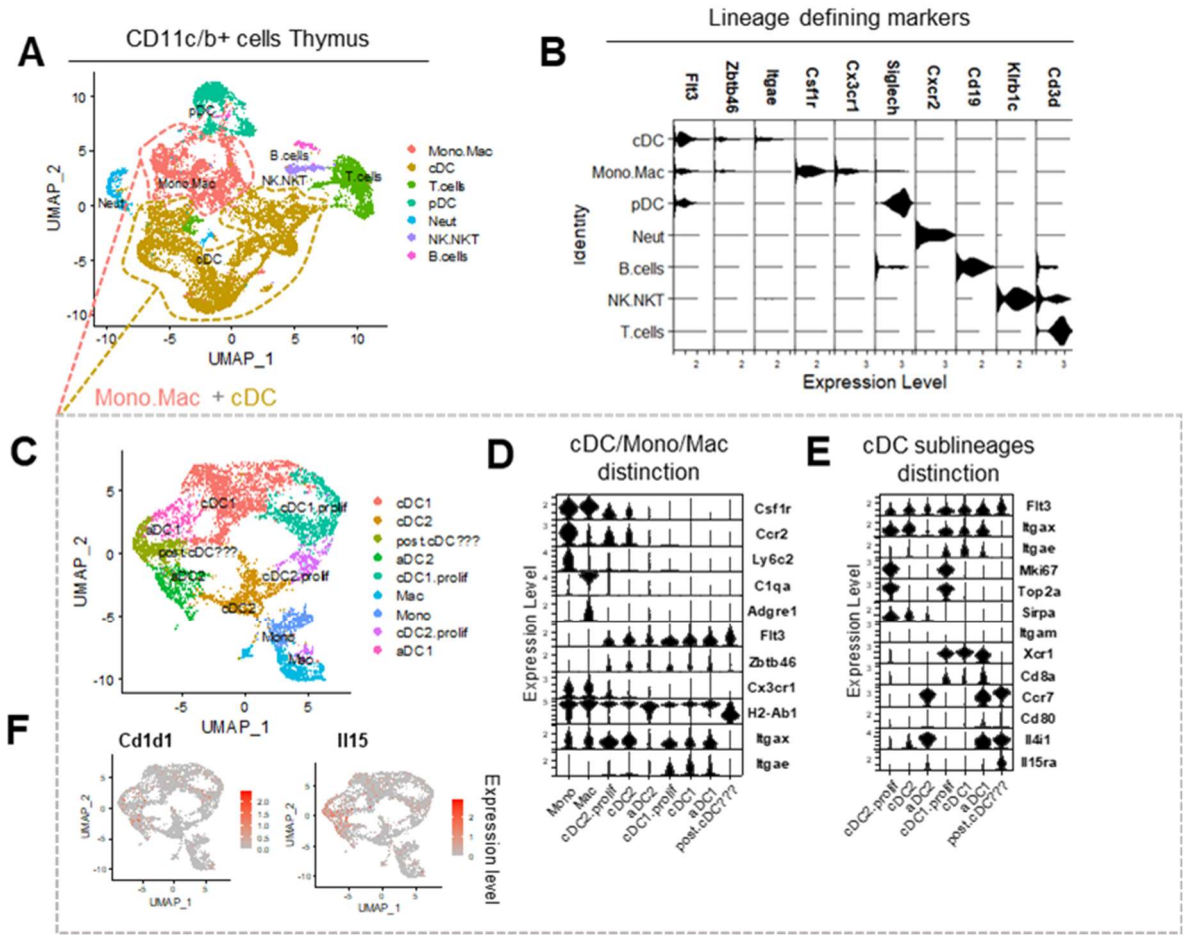

Figure 1. Heterogeneity of thymic APCs. Reanalysis of publicly available sc RNA sequencing dataset (GEO database: GSE198247, Breed et al., 2022). Authors FACS sorted thymic APCs based on their positivity for either CD11c, CD11b or both. For the details of sample preparation see the original study. Here I show reanalysis of the original data using Seurat package (v 4.0.4) in R (v 4.1.1). **A,** UMAP visualization of all the cells after filtering of death cells (mitochondrial genes threshold 5%) showing basic cell type annotation. **B,** Violin plot showing expression of canonic cell type markers for basic cell types shown in A. C-F, Subclustering of cDC and Monocyte/Macrophage clusters. **C,** UMAP visualization showing annotation of subtypes of Monocyte/Macrophage and cDC lineages. **D,** Violin plot showing expression of marker genes distinguishing between Monocyte/Macrophage lineage and cDC lineage. Note ubiquitous expression of *Itgax* (encoding CD11c) in both of those lineages. **E,** Violin plot showing expression of marker genes distinguishing between cDC sublineages. Note drop in the expression of classic cDC markers (*Itgax* – CD11c, *Itgae* – CD103) in a putative post-cDC subpopulation in contrast to their maintained expression of *Flt3*, arguing for their cDC identity. F, Scatter heatmaps of *Cd1d1* and *Il15* expression. Note the accumulation of the signal in aDC2 population.

To illustrate the heterogeneity of thymic DCs and to demonstrate the power provided by recent advances in scRNA-seq method, I have performed reanalysis of publicly available dataset, originating from the aforementioned study (Breed et al., 2022) (Fig. 1). Interestingly, aDCs transcriptionally closer to cDC2 lineage show high expression of genes stimulated by IL-4 (Breed et al., 2022) (Fig. 1). As it was shown that both cDC2 and a portion of aDCs are dependent on thymic helper T-cell type-2 (Th2) cytokines (Breed et al., 2022), it seems plausible that this type of stimulation might contribute to the

generation of Xcr1$^-$ aDCs from cDC2. Additionally, aDCs transcriptionally closer to cDC2 lineage also express high levels of CD1d (Fig. 1), nonclassical MHC molecule necessary for the presentation of lipid antigens to natural-killer (NK) T-cells (Sköld and Behar, 2003). Interestingly, the same population expresses high levels of IL-15, the major cytokine necessary for NKT development (Fig. 1). Since, thymic NKT cells are the major producers of IL-4 (Wang et al., 2019), it is likely that cDC2 and thymic NKT cells form a regulatory loop that ensures both NKT differentiation/maintenance and the activation of cDC2 that acquire aDC phenotype.

Furthermore, a population of DCs that downregulate classic DC markers (CD11c, CD103 encoded by *Itgax* and *Itgae*) can be defined (Fig. 1). While the function of these cells is currently unknown it is possible that these cells are the terminal differentiation of state of both cDC1 and cDC2 lineage. Importantly, due to the downregulation of markers, classically used for cDC definition, these cells were likely overlooked in most of the studies. Notably, a cell populations with similar expression patterns can be identified also in the intestine and intestine-draining lymph nodes (compare Fig. 1 and Fig. 2, also see section *Antigen presentation* in *Tolerance to food and commensal antigens*).

On the top of discrimination of aDC subpopulations and their expression patterns described above, the re-analysis, which I have performed allows the comparison of markers classically used for the discrimination between DC populations with other markers, that are expressed at higher levels and/or with higher specificity for the desired population (Fig. 1). This issue is discussed in detail on the example of the intestinal antigen presenting cells (APCs) in BOX2.

Mechanisms regulating CAT. CAT can be regulated on several levels. First, mTECs could actively attract potential antigen recipients by chemokines secretion. Second, DCs can express molecules that would allow them to efficiently uptake mTEC-derive antigens. However, as mentioned above, the knowledge of these mechanisms is currently only fragmental.

Importantly, in a study shown below, we identified one of the first described mTEC-intrinsic mechanisms that control CAT. We were able to show that mTECs use Toll-like receptors (TLRs) to increase their production of chemokines, which attract CD14$^+$ DCs to the thymus, where they acquire mTEC-derived antigens. This mechanism is then utilized to drive the generation of Tregs from developing thymocytes. Importantly, the disruption of mTECs' ability to signal through TLRs leads to generation of altered T-cell repertoire, which drives the development of colitis upon transfer to lymphopenic recipients (Vobořil et al., 2020).

Regarding the DC-intrinsic regulation of CAT, the only molecule participating in this process described so far is CD36, a scavenger receptor used specifically by thymic cDC1 to acquire mTEC-derived antigens (Perry et al., 2018). However, the deletion of CD36 on DCs leads only to a partial decrease in the acquisition of mTEC-derived antigens in cDC1 (Perry et al., 2018). Furthermore, while CD36 expression is restricted to cDC1 lineage, other thymic DC lineages are also able to acquire mTEC

derived antigens (Vobořil et al., 2020; Vobořil et al., 2022). Interestingly, depletion of thymic cDC2 and a portion of Xcr1$^-$ aDCs impacts thymic negative selection (Breed et al., 2022). However, it remains to be addressed if this effect is dependent on antigen acquisition from mTECs.

aDCs in CAT. In the aforementioned study we also found that not classically considered cDC1 or cDC2 population, but rather thymic aDCs are the most efficient DC population in antigen uptake from mTECs (Vobořil et al., 2022). Although both the ontogeny and function of aDC populations warrant further studies, it is tempting to hypothesize that aDCs are the progeny of cDCs, which acquired antigens from mTECs. If proven, this would pose aDCs as a principal player in the indirect presentation of mTEC-derived antigens. In support of this hypothesis, it was shown that phenotypically similar DC phenotype is mounted in the tumor after the engulfment of apoptotic cancer cells (Maier et al., 2020).

Delivery of peripheral antigens. Aside from mTEC-produced antigens, DCs can also deliver self-antigens from the immune periphery and utilize these antigens for T-cell selection in the thymus (Bonasio et al., 2006). Interestingly, pDCs express high levels of chemokine receptor CCR9, which allows them to migrate both to the intestine (Wendland et al., 2007) and to the thymus (Hadeiba et al., 2012). Although it was not directly shown, where in the body pDCs uptake antigens for thymic delivery, they are able to promote central tolerance (Hadeiba et al., 2012). While the specific role of cDC1 or cDC2 in the delivery of peripeheral antigens to the thymus is unclear, it was shown that Cx3cr1$^+$ APCs, presumably moDCs or marcophages, are able to uptake blood born antigens, by extending their dendrites though the endothelium of thymic blood vessels (Vollmann et al., 2021). Cx3cr1$^+$ APCs are also able to migrate to the thymus from the intestine and participate in the activation of microbiota specific T-cells (Zegarra-Ruiz et al., 2021) (for more details see section *Induction of SFB-driven T-cell responses*).

In summary, central tolerance is a crucial process to set a baseline for intestinal homeostasis. mTECs and their ability to express a vast array of TRAs, which can be utilized to select developing T-cells, play a cardinal role in central tolerance. Although mTECs are able to directly present TRAs and select developing T-cells, indirect presentation of TRAs contributes to thymic T-cell selection. This process is executed by thymic DCs, through the acquisition of mTEC-produced antigens via CAT. Furthermore, DCs can deliver antigens from the immune periphery. Both direct and indirect presentation of mTEC-produced antigens, as well as the presentation of peripheral antigens, shape T-cell repertoire and protect the body from autoimmunity, thus preventing the disruption of homeostasis in all the tissues, including the intestine.

Tolerance to food and commensal antigens

Although majority of protein antigens in the intestinal lumen are digested to amino acids or dipeptides, considerable amount of undigested peptides and proteins translocate to the surrounding tissues (Brandtzaeg, 1998). Therefore, these antigens cannot be simply ignored, and immune tolerance has to be actively established in order to prevent pathologic immune reactions to these antigens. It is known for more than a hundred years that oral delivery of antigen has the potency to induce tolerance to this antigen (Wells and Osborne, 1911). This phenomenon known as oral tolerance serves a complementary purpose to thymic central tolerance, enabling the establishment of tolerance to food and commensal microbiota antigens. During the last few decades, many processes of tolerance to microbiota and food antigens were described. In this section I will review the current state of knowledge in this field. Regardless of the chronology of these findings I will describe the process of oral tolerance mechanistically, starting from the antigen uptake and ending with the effector mechanisms.

BOX 1. Organization of intestinal lymphoid tissues. The intestine contains both large numbers and a great variety of immune cells and it is even considered the largest immune organ in the body by some researchers. To control such a complex system, the intestine developed an intricate network of lymphoid organs, collectively termed as gut-associated lymphoid tissue. These include mesenteric lymph nodes (mLNs), Payer's patches (PPs) and a variety of smaller immune cell clusters in intestinal lamina propria, such as isolated lymphoid follicles. However, large number of immune cells also reside dispersed in lamina propria and intraepithelial space, where they perform immune surveillance by constant patrolling. PPs are located directly under the epithelial layer in the small intestine and the cecum and the epithelium covering them from the luminal side is termed follicle-associated epithelium (FAE). In contrast, mLNs are connected with the intestine indirectly, by the lymphatic drainage. (Mörbe et al., 2021). See also Fig. 4.

Antigen acquisition

In contrast to the thymic tolerance to self-antigens, which are expressed endogenously, antigens in the intestinal lumen must be acquired by the intestinal APCs that can present them to cognate T-cells and drive immune tolerance. Several pathways responsible for the delivery of antigens from intestinal lumen have been identified. The fact that most of these pathways are executed by epithelial cells further highlights the fundamental role of epithelial cells in the organization of the immune responses.

M-cells. M-cells are a specialized epithelial cell type residing in FAE (see BOX 1). Their primary function is to uptake luminal antigens and transport them to the underlying APCs in PPs. To perform this function, M-cells are equipped with unique set of molecules, hallmarked by Gp2, protein that facilitates uptake and transcytosis of luminal antigens (Hase et al., 2009; Kadaoui and Corthésy, 2007;

Rey et al., 2004; Verbrugghe et al., 2006). From the basolateral side, M-cells form a pocket, where APCs reside and take up antigens from M-cells (Mabbott et al., 2013). FAE epithelium actively organizes immune responses in PP by attracting $CD11c^+$ $CD103^+$ DCs (see section BOX 2 and Fig. 2) through M-cell-produced CCL9 (Zhao et al., 2003) and T-cells though Cxcl16 expressed throughout FAE (Hase et al., 2006).

Although it was shown that artificial targeting of dietary antigens to M-cells can lead to oral tolerance induction (Rynda et al., 2008; Suzuki et al., 2008), no such phenomenon was demonstrated for naturally occurring food antigens. On the other hand, M-cell-mediated antigen delivery plays a crucial role in the initiation of IgA responses to intestinal microbes (Rios et al., 2016). Thus, M-cell-dependent antigen uptake likely serves immunogenic purposes rather than the induction of oral tolerance. Alternatively, M-cells mediated antigen delivery might specifically drive immune responses (possibly both tolerogenic and immunogenic) to microbial antigens and not those derived from food, since M-cells seem rather specialized to uptake antigens from microbiota (Hase et al., 2009; Rey et al., 2004). This important issue requires further studies since it is currently unknown which antigen acquisition pathway is used to drive tolerance to intestinal microbiota (see *$MHCII^+$ ILC3* in *Antigen presentation* section).

Interestingly, some studies showed that aside from M-cells residing in FAE, phenotypically similar cells can be detected in villous epithelium of the small intestine (Jang et al., 2004; Kanaya et al., 2012; Knoop et al., 2009). However, since goblet cells associated passages (GAPs) were not known at this time, it is possible that villous M-cells can overlap with goblet cells performing GAPs. This hypothesis is supported by the fact that villous M-cells were primarily detected using UEA-I staining (Jang et al., 2004), for which goblet cells show high positivity (Gustafsson et al., 2021). Furthermore, FAE M-cells are dependent on Rank ligand signals (Knoop et al., 2009), which are delivered by underlaying PP stromal cells (Katakai et al., 2008; Taylor et al., 2007). In contrast, bona fide $Gp2^+$ M cells can be detected on the villi only after the treatment with exogenous Rank ligand (Knoop et al., 2009).

Goblet cells associated passages. Goblet cells are the primary producers of intestinal mucins, thus creating the protective mucus layer segregating intestinal microbes from epithelium. However, it was found that on the top of mucus production, goblet cells play a major role in the antigen uptake in the intestine by creating GAPs (McDole et al., 2012). Similar to M-cells, GAPs deliver antigens primarily to $CD103^+$ DCs (McDole et al., 2012), however $CD103^-$ $CD11b^+$ APCs are also able to acquire antigens through this route (Kulkarni et al., 2020) (see BOX 2 and Fig. 2). Importantly, GAPs-mediated antigen delivery was shown to drive oral tolerance to naturally occurring food antigens (Kulkarni et al., 2020). Although the functional outcome of GAPs- and M-cell mediated antigen delivery routes were not formally compared in same conditions, it is possible that GAPs induce tolerogenic responses through

the co-transport of mucin, which is produced by goblet cells. This hypothesis is supported by findings, that mucin is able to induce tolerogenic phenotype in DCs (Rivera et al., 2022; Shan et al., 2013). Thus, the co-transport of antigens from intestinal lumen together with mucins might be one of the mechanisms that enforce oral tolerance to antigens acquired though GAPs.

Interestingly, it was shown that goblet cells express an array of chemokines, hallmarked by Ccl6 and 9, which are absent in other epithelial cells of the villous epithelium (Haber et al., 2017). While the functional importance of goblet cell-produced chemokines was not experimentally tested, it is possible that goblet cells use these chemokines to attract recipients of GAP-delivered antigens, similar to M-cells (Zhao et al., 2003).

Enterocytes. It was suggested that classical absorptive enterocytes, which compose the vast majority of intestinal epithelium, are also able to uptake and transfer luminal antigens. Alternatively, antigens can be transferred in a paracellular fashion, translocating between the neighboring cells (Snoeck et al., 2005). While these antigen uptake routes may play a role in pathologic conditions, when intestinal barrier is disrupted (Perrier and Corthésy, 2011), their contribution to the oral tolerance induction in the unperturbed conditions is unclear.

On the other hand, direct uptake of antigens by enterocytes might be crucial for the induction of homeostatic immunogenic responses. It was shown that small vesicles of epithelium-attaching bacteria are taken up and transferred by enterocytes, to which these bacteria attach. Ultimately, this process facilitates the induction of helper T-cell response type-17 (Th17) response, which targets epithelial cell-attaching bacteria (Ladinsky et al., 2019). For more details on homeostatic immunogenic responses in the intestine see section *Tolerate or responde?*.

Trans endothelial dendrites. Several studies showed that intestinal phagocytes are able to extend appendages through the intestinal epithelial layer, forming so called trans endothelial dendrites (TEDs) (Chieppa et al., 2006; Niess et al., 2005; Rescigno et al., 2001). Although both Cx3cr1-GFP and CD11c-GFP reporter positive cells were found to have this capacity (Niess et al., 2005; Rescigno et al., 2001), it is unclear if this includes only macrophages or also intestinal DCs, since CD11c expression is shared by both these populations (See BOX2 and Fig. 2). Furthermore, the impact of this phenomenon on the immune responses targeting luminal antigens was not determined. Importantly, a recent study showed that in unmanipulated intestine TEDs are present at much lower frequencies than demonstrated previously as TED formation is induced by tissue processing methods used in previous studies (Kulkarni et al., 2020).

BOX 2. Intestinal APCs in the age single cell RNA sequencing. Much like the thymus, intestine contains a great variety of APCs. While older studies largely depended on the usage of CD11c (encoded by *Itgax*), which was deemed a unique DC marker, it is now clear that many other cell types express CD11c (Fig. 1 and 2). The research of the intestinal APCs however, largely utilized CD103 marker (encoded by *Itgae*), which seems to be rather specific for conventional DCs at least among intestinal myeloid cells (Fig. 2). Due to a dramatic scientific advance introduced by scRNA-seq method, much more precise definition of APC populations is now available (Fig. 2). Since this method allowed comparison with classic marker sets, we can back-annotate classically used cell type definitions with newly discovered complexity of cell type heterogeneity. Thus, it is now clear that in the intestine $CD103^+$ DCs represent bona fide cDC population, that can be further subdivided to $CD11b^+$ (encoded by *Itgam*) cDC2 and $CD11b^-$ cDC1, although this definition of cDC1 population likely leads to some contamination with non-cDC1 cells (Fig. 2). Thus, the usage of other cDC1 markers such as XCR1 would likely increase the specificity of their definition. On the other hand, $CD103^-$ $CD11b^+$ cells classically termed as DCs based on their CD11c positivity likely represent macrophages (Fig. 1 and 2). On the top of those APCs, which are all of the myeloid origin, the intestine contains B cells, MHCII+ ILC3 cells, plasmacytoid DCs (pDCs), as well as intestinal epithelial cells, which can induce MHCII expression.

In summary, several pathways of antigen acquisition were described. Among those, GAPs seem to be the major drivers of oral tolerance, while M-cell-mediated antigen transfer likely plays a major role in the induction of intestinal immunogenic responses (see also Fig. 3).

Antigen presentation

Once luminal antigens are transferred through the epithelial barrier, they are delivered to intestinal APCs. Intestines contain similar populations of APCs as the thymus (see *Central tolerance* section, BOX2, Fig. 1 and Fig. 2), with an important addition of innate lymphoid cells type-3 (ILC3) subpopulation, which expresses high levels of MHCII and performs APC functions. Among these subsets, it was shown that $CD103^+$ cDCs play a major role in the induction of tolerance to food antigens (Coombes et al., 2007; Esterházy et al., 2016; Sun et al., 2007), while $MHCII^+$ ILC3 are largely responsible for the induction of tolerance to intestinal microbiota (Hepworth et al., 2015; Hepworth et al., 2013; Kedmi et al., 2022; Lyu et al., 2022).

cDCs. In the current model of tolerance to food antigens, intestinal cDCs acquire antigens through one of the processes described above (see section *Antigen acquisition*). After antigen acquisition, cDCs mature, upregulate Ccr7 and migrate to mesenteric lymph nodes, where they induce tolerance to food antigens through the induction of gut homing Tregs. This model was built on a number of studies that identified crucial points of this process. First, $CD103^+$ $Cx3cr1^-$ cDCs were identified as the primary inducers of tolerance to food antigens (Coombes et al., 2007; Sun et al., 2007). Second, the surgical

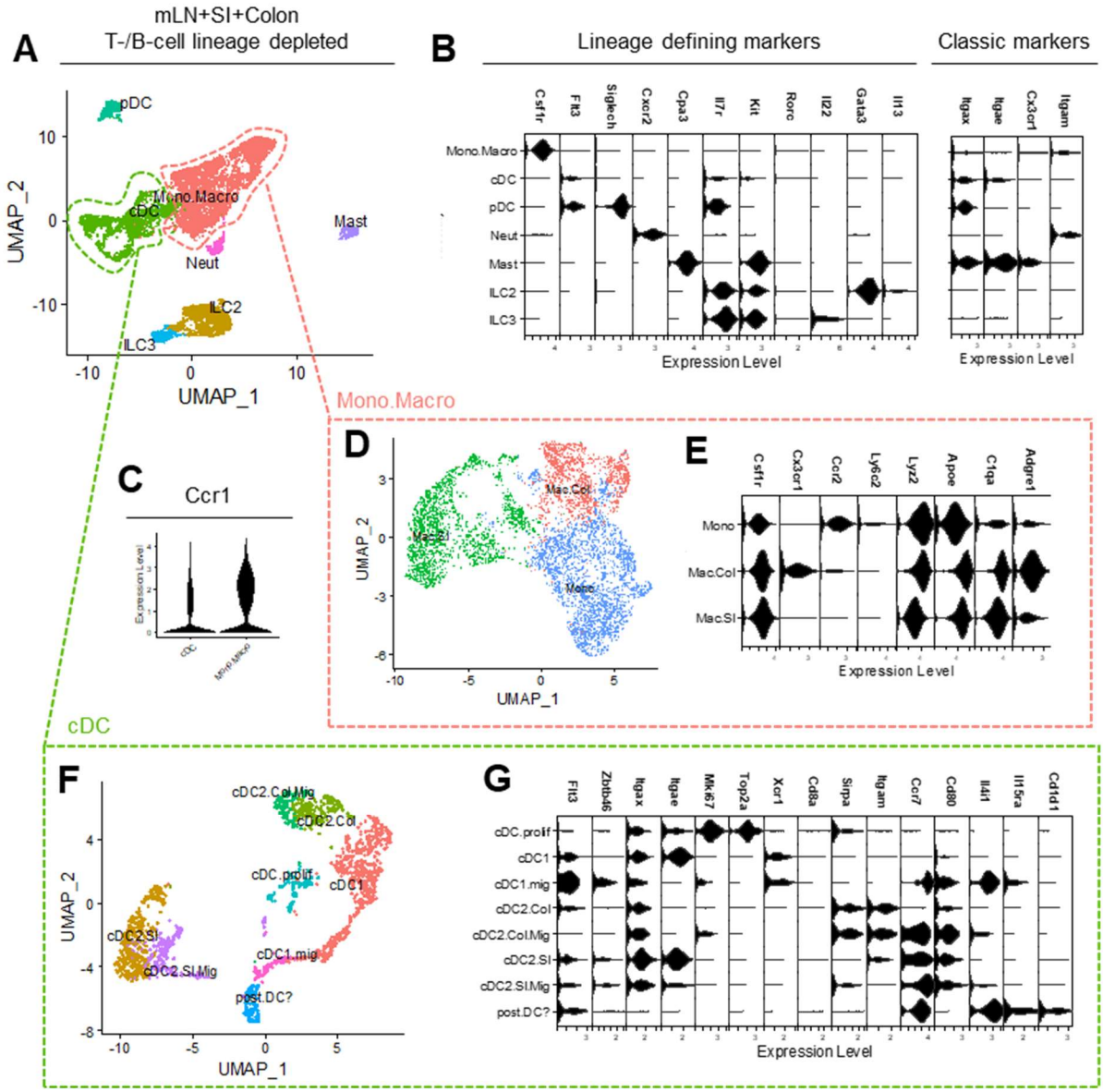

Figure 2. Heterogeneity of intestinal APCs. Reanalysis of publicly available scRNA-seqdataset (GEO database: GSE183885). Authors FACS sorted CD45+ cells from mLN, and both small and large intestinal lamina propria and intraepithelial space. For the details of sample preparation see the original study. Here I show the reanalysis of the original data using Seurat package (v 4.0.4) in R (v 4.1.1). **A**, UMAP visualization of all the cells after bioinformatic filtering of T- and B-cell lineages showing basic cell type annotation. **B**, Violin plot showing expression of cell type-defining markers (left) and to the ones used classically (right) for basic cell lineages shown in A. Note expression of *Itgax* (encoding CD11c) in both cDC and monocyte/macrophage lineages. **C**, Violin plot comparing Ccr1 expression between cDC and Monocyte/macrophage lineages. **D-E**, Subclustering of Monocyte/Macrophage cluster. **D**, UMAP visualization showing annotation of subtypes of Monocyte/Macrophage lineage. **E**, Violin plot showing expression of marker genes distinguishing between Monocyte/Macrophage subtypes. **F-G**, Subclustering of cDC cluster. **F**, UMAP visualization showing annotation of cDC subtypes. **E**, Violin plot showing expression of marker genes distinguishing between cDC subtypes. Note a population of cells negative for most cDC sub lineage markers, despite their retention of *Flt3* expression termed here as post.DC?.

removal of mesenteric lymph nodes, as well as the inability of DCs to migrate to mLN in $Ccr7^{-/-}$ mice abrogates the induction tolerance to food antigens (Worbs et al., 2006). In this regard it is interesting to note that PPs seem to be dispensable for tolerance to food antigens (Spahn et al., 2002), arguing for FEA-associated M-cell independent mechanism of antigen acquisition in this process.

Mechanistically, cDCs in mLN induce Treg differentiation through their expression of TGFβ (Coombes et al., 2007), which is secreted in an inactive form that has to be activated by complex of integrins, composed of Itgav and Itgb8 subunits (αvβ8) (Worthington et al., 2011b). It was shown that mLN cDCs express high levels of αvβ8, which they use to generate Tregs (Worthington et al., 2011a). The action of TGFβ is complemented by mLN cDCs' expression of indoleamine 2,3-dioxygenase (IDO). This enzyme mediates the katabolism of tryptophan to kynurenines, which contribute to Treg differentiation (Matteoli et al., 2010). In parallel, mLN cDCs express high levels of retinol metabolizing enzymes, leading to retinoic acid production, which is the major inducer of gut homing phenotype in T cells (Iwata et al., 2004) and it also contributes to the ability of cDCs to induce Treg differentiations (Benson et al., 2007; Coombes et al., 2007; Sun et al., 2007). Interestingly, cDCs cooperate with mLN stromal cells to induce gut homing phenotype in T-cells (Hammerschmidt et al., 2008). Conversely, both gut homing molecules (Ccr9 and α4β7 integrin) and Treg generation are crucial for the induction of tolerance to food antigens (Cassani et al., 2011). Notably, it was shown that DC-epithelial interactions are important for DCs to drive Treg differentiation (Iliev et al., 2009), further highlighting the importance of immune-epithelial interaction in the intestinal homeostasis.

Although it was shown that cDCs in general are essential for the induction of tolerance to food antigens (Esterházy et al., 2016) the distinct contribution of individual DC subsets to this process is still enigmatic. While $CD11b^{+}$ cDC2 are more abundant in the intestine and draining lymph nodes (Sun et al., 2007) and they are able to induce intestinal Treg generation (Welty et al., 2013), $CD11b^{-}$ cDC1 cells were found to induce food specific Tregs, however functional induction of oral tolerance was maintained in their absence (Esterházy et al., 2016). Thus, it seems that there might be some level of redundancy between intestinal cDC subsets in their ability to induce tolerance to food antigens. Interestingly, it was also shown that cDC1s bear a unique ability to cross-present exogenously acquired self-antigens from the intestine on their MHCI, which allows them to drive $CD8^{+}$ T-cell responses (Cerovic et al., 2015). Thus, the mechanisms used by each of the cDC subsets to drive tolerance to intestinal antigens might be different, which could be reflected in a more complex experimental system, that would reflect natural situation and not just one model food antigen.

Notably, it was found that in absence of mLN, $CD103^{-}$ $Cx3cr1^{-}$ cells accumulate in the lymph (Cerovic et al., 2013). Since these cells were presumably destined to enter mLN from the intestine, it is likely that these cells are progeny of migratory intestinal cDCs. In agreement with this hypothesis, using publicly available scRNA-seq dataset integrating cells from colon, small intestine and mLN, I was able to identify subset of $CD103^{-}$ $Cx3cr1^{-}$ $Flt3^{+}$ cells which are transcriptionally close to $Ccr7^{+}$ migratory

cDCs (Fig. 2). These cells express high levels of IL-15 receptor and IL-4 stimulation signature as well as the highest levels of CD1d among intestinal APCs (Fig. 1). Thus, they likely represent intestinal counterparts of putative thymic post-DCs (see section *Cooperative antigen transfer* in *Central tolerance*) and it is possible that they participate in the activation or maintenance of intestinal NKT-cells.

Macrophages. As described above, cDCs are able to induce tolerance to food antigens starting from antigen uptake and ending with induction of Treg differentiation. Thus, it was with a great surprise when it was found that tolerance to food antigens is abrogated in $Cx3cr1^{-/-}$ mice (Hadis et al., 2011), as cDCs do not express Cx3cr1 (see BOX2 and Fig. 1 and 2). There are two possible explanations of this phenomenon: 1) While $Cx3cr1^{+}$ macrophages do not migrate to mLN after antigen acquisition (Schulz et al., 2009) and thus likely do not play a role in the induction of Treg differentiation, it is possible that local antigen presentation by intestinal macrophages is needed to maintain and/or expand Tregs once they migrate to this tissue (Hadis et al., 2011). This scenario would indicate a division of labor between intestinal APCs, where cDCs would play a role of Treg inducer and macrophages the role of local promoter of Tregs. 2) It was found that intestinal macrophages form gap junctions with intestinal cDCs (Mazzini et al., 2014). These junctions enable intestinal macrophages to transfer antigens to intestinal cDCs. Furthermore, the abrogation of gap junctions' formation between intestinal cDCs and macrophages leads to the accumulation of food antigens in macrophages, while in unperturbed conditions these antigens are largely restricted to cDCs (Mazzini et al., 2014). Thus, it is possible that in fact macrophages are primary recipients of food antigens and cDCs acquire these antigens from macrophages. This hypothesis would be supported by higher expression of Ccr1 in monocyte/macrophage populations (Fig. 2), as Ccr1 is the receptor for Ccl6 and Ccl9, the main chemokines expressed by both M cells and goblet cells (see previous section). Furthermore, these two hypotheses are not mutually exclusive, and both these mechanisms can act in parallel.

$MHCII^{+}$ ILC3. While myeloid APCs play a crucial role in the tolerance to food antigens, their role in the tolerance to intestinal microbiota is unclear. In contrast, it was shown that specific population of ILC3s, which bear high levels of MHCII, plays a crucial role in tolerance to intestinal microbiota (Hepworth et al., 2015; Hepworth et al., 2013). The depletion of MHCII on ILC3s leads to splenomegaly, mLN enlargement and colitis (Hepworth et al., 2013). This pathology is dependent on microbiota and is executed by $CD4^{+}$ T-cells (Hepworth et al., 2013). Using Cbir TCR transgenic mouse, in which T-cells recognize ubiquitous microbial antigen flagellin (Lodes et al., 2004), it was shown that $MHCII^{+}$ ILC3s are both necessary and sufficient to drive the tolerance to microbiota in an antigen specific manner (Hepworth et al., 2015). In these studies, it was suggested that $MHCII^{+}$ ILC3s are able

to delete microbiota reactive T-cells, possibly due to the lack of costimulation, since in contrast to cDCs, MHCII$^+$ ILC3 do not express CD80 and CD86 (Hepworth et al., 2015; Hepworth et al., 2013).

Recent studies showed that MHCII$^+$ ILC3s also play a major role in the generation of microbiota specific Rorγt$^+$ Tregs (Akagbosu et al., 2022; Kedmi et al., 2022; Lyu et al., 2022) (for more details on intestinal Treg subpopulations see *Rorγt$^+$ and Gata3$^+$ Tregs* section). Findings that ILC3 use Ccr7 to traffic from the intestine to the mLN (Mackley et al., 2015) and that this process is needed for the generation of microbiota specific Rorγt$^+$ Tregs (Kedmi et al., 2022) suggest that MHCII$^+$ ILC3s act in a similar fashion as cDCs (see *DCs* subsection in this section). I.e., they acquire antigens in the intestine and migrate to the mLN, where they present these antigens to cognate T-cells and induce their conversion to Tregs. It was also shown that ILC3s mediated Treg induction requires Itgav and Itgb8 (Akagbosu et al., 2022; Kedmi et al., 2022; Lyu et al., 2022), suggesting that ILC3 use TGFβ to drive the differentiation of microbiota specific Tregs, similar to cDC-mediated induction of Tregs specific for food antigens. However, it is currently unclear if MHCII$^+$ ILC3s produce IDO or RA converting enzymes, which are known to modulate Treg generation and intestinal homing phenotype in T-cells by intestinal cDCs (see *DCs* subsection in this section). Furthermore, although it was shown that MHCII$^+$ ILC3s can directly uptake, process and present antigens (Dobeš et al., 2022; Hepworth et al., 2013), it is currently unclear which pathway of antigen acquisition they use to drive tolerance to intestinal microbiota. Interestingly, a recent study found that MHCII$^+$ ILC3s limit IgA induction in response to intestinal microbiota (Melo-Gonzalez et al., 2019). Although this topic will require further studies, this finding would support the notion that induction of tolerogenic T-cell responses acts in opposition to the generation of IgA.

While classical MHCII$^+$ ILC3 play a major role in the induction of immune tolerance to commensal microbiota, we have demonstrated the existence of another subpopulation of MHCII$^+$ cells that are phenotypically very close to ILC3s, but they are defined by their expression of Aire (Yamano et al., 2019). In contrast to Aire function in mTECs, in Aire$^+$ ILC3-like cells Aire does not drive production of TRAs (for more details on Aire function in the thymus see section *Central tolerance*). Furthermore, these cells are dispensable for the induction of microbiota specific Tregs (Kedmi et al., 2022; Lyu et al., 2022), although they bear strong antigen presentation capabilities (Yamano et al., 2019). Strikingly, it was later shown that Aire$^+$ ILC3-like cells play a central role in the induction of the Th17 response to *Candida albicans* (Dobeš et al., 2022), a commensal fungus living throughout the whole gastrointestinal tract that can however cause severe pathology if not controlled by the immune system (for more details see *Breakdown of intestinal homeostasis* section). Thus, a population ILC3-like cells is specialized for the induction of proinflammatory immune responses to a specific group of fungal commensals, highlighting the crucial role of MHCII$^+$ ILC3s in the maintenance of intestinal homeostasis (for more details of homeostatic proinflammatory see *Tolerate or respond?* section).

Effector mechanisms of tolerance to intestinal antigens

Once acquired, processed, and presented, intestinal antigens shape the repertoire of conventional T-cells to induce oral tolerance. Similar to the thymic T-cell selection, tolerance to food and microbiota antigens can be achieved either by clonal deletion or by the conversion of cognate T-cells to Tregs in the intestine and draining lymph nodes. It was also suggested that oral tolerance can be achieved by the induction of T-cell anergy, a state in which T-cells persist but are functionally suppressed (Friedman and Weiner, 1994; Van Houten and Blake, 1996). Recent study, mapping the complexity of food antigen targeting T-cell, showed that low-grade T-cell proliferation and activation is mounted. However, these T-cells do not differentiate to proinflammatory phenotype, due to the presence of Tregs (Hong et al., 2022), suggesting that it is the combination of all three cell fates: clonal deletion, Treg differentiation and T-cell anergy that governs oral tolerance.

Like in the thymus, the abundance of the antigen seems to be the deciding factor for the T-cell fate decision, since low dose of food antigens rather directs the conversion of cognate T-cell to Treg lineage, while high dose of food antigens preferentially induce T-cell deletion (Chen et al., 1995; Chen and Weiner, 1996; Friedman and Weiner, 1994). While not studied in detail, similar mechanism might operate for antigens from commensal microbiota. In support of this notion, responses to antigens broadly conserved among microbial taxa, such as flagellin, mostly induce deletion of cognate T-cells (Hepworth et al., 2015). In contrast, responses to antigens restricted to single or few bacterial taxa rather lead to Treg generation (Kedmi et al., 2022; Lyu et al., 2022). However, since intestinal microbiota actively modulates T-cell responses (see section *Microbiota mediated immunoregulation*), mechanisms of tolerance to their antigens are likely much more complicated.

Once generated, Tregs use a combination of mechanisms to suppress inflammatory responses and promote intestinal homeostasis. Briefly, these mechanisms include the production of inhibitory cytokines, such as TGFβ and IL-10 and the ability to induce T-cell apoptosis by IL-2 consumption and secretion of granzymes (Shevach, 2009).

Rorγt$^+$ and Gata3$^+$ Tregs

Over the past decade studies identified several types of Tregs that express transcription factors typically considered a hallmark of other Th subtypes. This includes the identification of Gata3$^+$ Tregs (Wohlfert et al., 2011) and Rorγt$^+$ Tregs (Ohnmacht et al., 2015; Sefik et al., 2015). Interestingly, in the intestine, the positivity for either Gata3 or Rorγt defines two distinct Treg populations (Sefik et al., 2015).

Both Gata3$^+$ and Rorγt$^+$ Tregs are able to differentiate in the periphery (Pratama et al., 2020). Although Gata3 is necessary for Tregs to provide protection from colitis (Wohlfert et al., 2011), it also plays an important role in the maintenance of thymus derived Tregs (Ding et al., 2022; Wang et al., 2011). In contrast, the development of Rorγt$^+$ Tregs is almost completely dependent on the intestinal microbiota (Ohnmacht et al., 2015; Sefik et al., 2015). Furthermore, some microbial species such as

Helicobacter hepaticus are potent inducers of this Treg fate (Xu et al., 2018). Thus, Rorγt$^+$ Tregs likely represent a *bona fide* microbiota-reactive and microbiota-induced peripheral T-cell fate. Recent studies found that these cells are completely dependent on MHCII$^+$ ILC3 and, their expression of Ccr7 and TGFβ-converting enzymes (Kedmi et al., 2022; Lyu et al., 2022) (for more details see above). Importantly, Rorγt$^+$ Tregs play an indispensable role in the tolerance to the intestinal microbiota since the deletion of Rorγt specifically in the Treg population leads to microbiota dependent intestinal pathology (Al Nabhani et al., 2019; Ohnmacht et al., 2015; Sefik et al., 2015). While Gata3 is needed to stabilize Treg fate through the activation of Foxp3 locus (Wang et al., 2011), specific effector immunoregulatory mechanism operating in Rorγt$^+$ Tregs in comparison to other Tregs has not been identified so far. For further information regarding role of Rorγt$^+$ Tregs in the development of intestinal immune responses see section *Spatiotemporal map of intestinal immune responses*.

In summary, immune tolerance to intestinal antigens is a complex process starting in the thymus, where tolerance to intestinal self-antigens is established. In contrast, the tolerance to food- and commensal microbiota-derived antigens is established in the intestine and draining lymphoid organs. cDCs, and their cooperation with intestinal macrophages, play a major role in the tolerance to food antigens, which they acquire mainly via GAPs. After antigens acquisition, cDCs migrate to mLN, where they present these antigens to cognate T-cells and induce their conversion to Tregs. In contrast, tolerance to intestinal microbiota is largely done by MHCII$^+$ ILC3s, which seem to operate in similar fashion to cDCs, i.e. they migrate to mLN after antigen acquisition and induce Treg differentiation, but their route of antigen acquisition is currently unclear. Tregs are the major effector cells that enforce tolerance in the intestine, using a battery of immunosuppressive mechanisms. For a schematic summary of stepwise selection of T-cells specific for intestinal antigens see Fig. 3 and 4.

Tolerate or respond?

Self-antigens and food-derived antigens pose no threat to the host (leaving aside cancerous host cells and ingested toxins). The situation is very different when the immune system reacts to commensal microbiota. While commensal microbes pose no threat under homeostatic conditions, they can overgrow and cause severe pathology when not kept in check. Although more examples can be found, this phenomenon can be illustrated by a recent finding that commensal strain of Cryptosporidium is well tolerated in WT mice, but it causes death in mice where cDC1 lineage and their ability to induce Th1 responses is disrupted (Russler-Germain et al., 2021). Thus, the immune system must keep the equilibrium between tolerance to commensal microbes and the homeostatic (proinflammatory) immune responses that enable the homeostatic cohabitation of host and commensal microbiota. In this section I will aim to provide the mechanistic insight into these homeostatic immune responses and highlight their indispensable role in the maintenance of intestinal homeostasis.

Segmented filamentous bacteria

Segmented filamentous bacteria (SFB) (alternatively termed *Candidatus Arthromitus* or *Candidatus Svagella*) are spore-forming, gram-positive commensals of mice and other vertebrates (Hedblom et al., 2018). They attach to enterocytes of small intestine, with the preference to the ileum (Atarashi et al., 2015; Sano et al., 2015). Their attachment leads to the induction of strong Th17 response, marked by the production of IL-17 and IL-22, which however does not cause intestinal pathology (Atarashi et al., 2015; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). This intriguing contradiction led to massive interest of immunologic community in these microbes. Thus, we currently have a deep understanding of SFB-driven immune responses, which I will use to exemplify pro-inflammatory homeostatic non-pathogenic immune responses that intestinal immune system uses to maintain homeostatic host-microbiota relationship.

Uptake and presentation of SFB-derived antigens

Immune response to SFB starts by the attachment of SFB to the intestinal epithelium. To ensure their attachment, SFB use hook-like protrusions, which are submerged into the infected enterocyte, without the perforation of enterocyte plasma membrane (Ladinsky et al., 2019). Due to a long coevolution with their host, SFB are specialized to a particular species, which they infect. For instance, SFB from rats are unable to attach to mice enterocytes. This enabled researchers to determine that the attachment of SFB to the enterocytes is necessary for the induction of Th17 response by SFB (Atarashi et al., 2015). Once attached, an interface between the enterocyte and SFB is established. At this interface, enterocytes perform endocytosis dependent on actin regulator CDC42, enabling them to uptake SFB-derived antigens and transport these antigens to intestinal APCs (Ladinsky et al., 2019). After transport, SFB antigens are utilized by $Cx3cr1^{+}$ $MHCII^{+}$ intestinal monocyte-derived APCs, which ultimately drive Th17 response to SFB (Goto et al., 2014; Panea et al., 2015). It should be noted that it was initially thought that cDCs are responsible for SFB-driven Th17 induction, based on the usage of CD11c-Cre system (Goto et al., 2014), but as explained above, CD11c is also expressed by monocyte/macrophage lineage (please see BOX2 and Fig. 2).

Induction of SFB-driven T-cell responses

It was disputed, if SFB driven responses are induced locally in the intestine or if antigen delivery to lymphoid organs is necessary. Although the general amount of SFB-induced $IL\text{-}17^{+}$ $CD4^{+}$ T-cells is not altered in by the absence of mLNs and PPs (Geem et al., 2014), antigen-driven production of IL-17 in response to SFB is largely dependent on the presence of secondary lymphoid organs (Lecuyer et al., 2014). A possible explanation of these results could be that Th17-inducing milieu is established locally in the tissue, while lymphoid organs are necessary for the recruitment of SFB-specific T-cells. In this

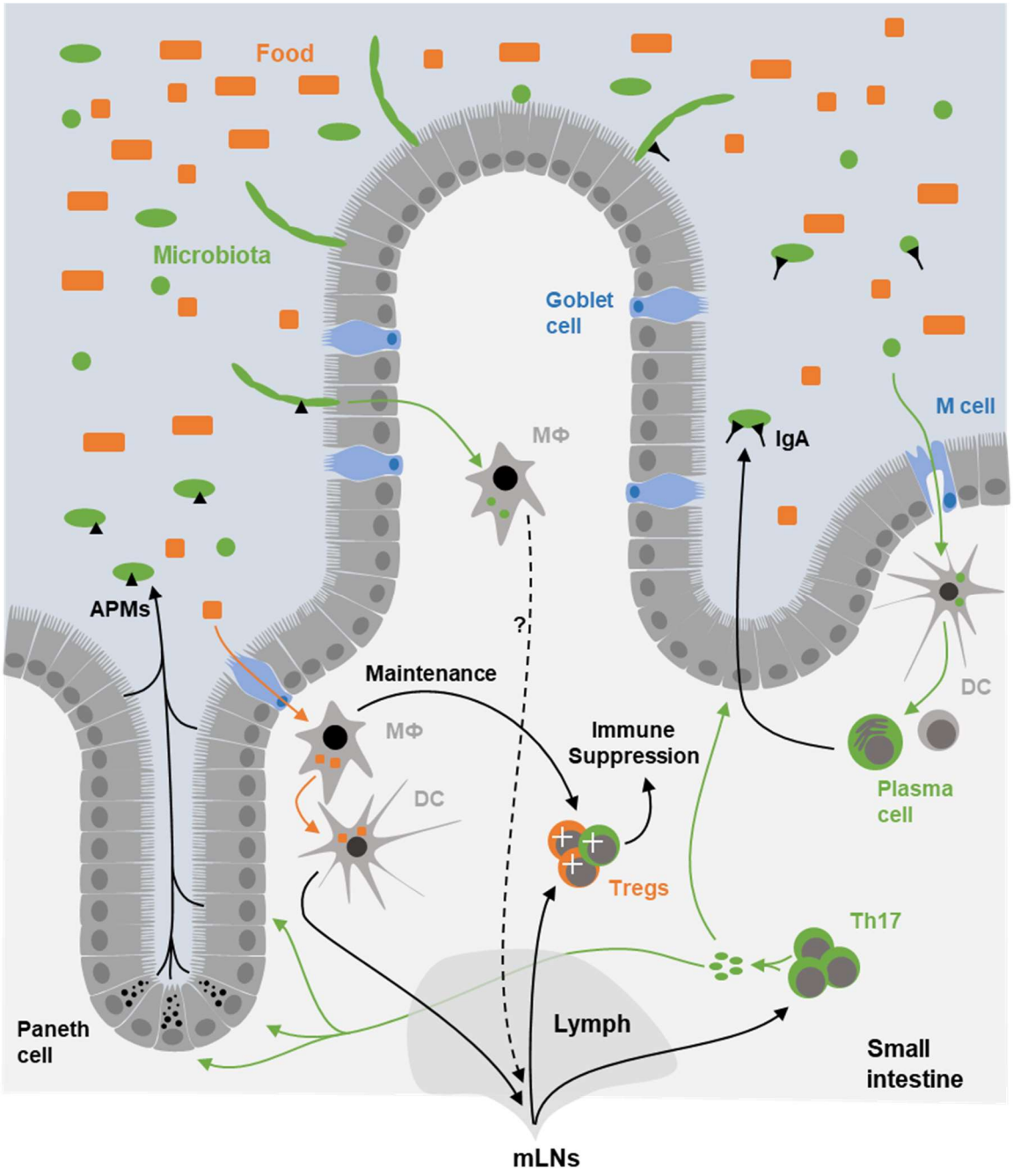

Figure 3. Schematic representation of processes ensuring small intestinal homeostasis. While macrophages and cDCs uptake food antigens from goblet cells, to drive tolerance to dietary antigens, macrophages (MΦ) acquire microbial antigens from enterocytes, to drive homeostatic immunogenic T-cell responses, such as Th17. On the other hand, cDCs acquire microbial antigens from Payer's patch-residing M cells, ultimately leading to microbiota-specific IgA production. While food antigen-loaded DCs migrate to mLNs to drive deletion and Treg generation of food-specific T-cells, it is unclear if microbiota antigen-loaded macrophages migrate to mLNs do induce Th17 (although Th17 targeting microbiota is initiated in mLNs). Both Tregs and Th17 cells migrate to small intestine from mLNs and once in the intestine, they enforce intestinal homeostasis. While Tregs suppress proinflammatory immune responses, Th17 cells induce production of antimicrobial peptides (AMPs) in Paneth cells and enterocytes and support the transport of IgA to the lumen. Both IgA and AMPs regulate microbiota to support balanced host-microbiota relationship.

scenario, T-cells not specific for SFB could differentiate to Th17 by a bystander effect to fill the niche that enables Th17 differentiation, in the absence of lymphoid organs. Furthermore, it was shown that SFB induce de novo generation of tertiary lymphoid organs, which may partially compensate the role of mLN and PPs in Th17 induction (Lecuyer et al., 2014).

Although it was initially though that PPs might be the primary site of SFB-specific Th17 response, reasoning that SFB attachment to the epithelium covering PPs could facilitate SFB antigen delivery to PP (Gaboriau-Routhiau et al., 2009; Lecuyer et al., 2014), it was later shown that SFB-specific T-cells mount Th17 response primarily in mLNs (Sano et al., 2021). In light of these findings, it is unclear how SFB antigens are delivered to mLN, since intestinal macrophages are typically considered resident, and it was shown that they do not migrate to mLN (Schulz et al., 2009). However, macrophage migration to mLN was not studied in the context of SFB colonization. Furthermore, a recent study showed that during weaning, but not later in life, intestinal $MHCII^+$ $Cx3cr1^+$ cells are able to migrate not only to mLN but also to the thymus. While in mLN intestine-originating $Cx3cr1^+$ cells are outnumbered by other $MHCII^+$ $CD11c^+$ (further undefined) cells even at weaning, in the thymus almost all cells coming from the intestine are $Cx3cr1^+$. In parallel, SFB-specific T-cells accumulate in the thymus during weaning, in manner dependent on $Cx3cr1^+$ cells (Zegarra-Ruiz et al., 2021). Despite this observation, re-immigration of peripherally expanded SFB-specific T-cell to the thymus was not excluded in this study and thus the physiological relevance of thymic (pre-)priming of SFB-targeted T-cell responses is currently unclear. Nevertheless, the accumulation of intestine-originating $Cx3cr1^+$ APCs in mLNs and the thymus suggests the possibility that initial induction of Th17 response to SFB might be occur specifically during weaning and that intestinal macrophages bear greater migratory potential in this specific timepoint than previously anticipated. For more details on the dynamics of intestinal immune responses see section *Spatiotemporal map the intestinal homeostasis*.

The molecular mechanism behind the SFB-driven Th17 induction is rather complex. The initiation of Th17 program in mLNs, through the induction of Rorγt expression in $CD4^+$ T-cells, is governed by the combination of IL-6, IL-21 and IL-23 (Sano et al., 2021). On the other hand, the production of IL-17 is induced locally, once pre-Th17 cells leave mLNs and migrate to the intestine (Sano et al., 2015). In the ileum, SFB drive induction of IL-23 (Sano et al., 2015), presumably in intestinal cDCs, which are potent producers of this cytokine (Kinnebrew et al., 2012). IL-23 induces the expression of IL-22 in intestinal ILC3s and IL-22 in turn acts on the intestinal epithelium to initiate the production of serum amyloid proteins (SAA) (Sano et al., 2015). As a result, potent, fully maturated, Th17 cells are induced by the combination of signals delivered in mLN and local signals in the intestine.

Induction of B-cell responses to SFB

Aside from Th17, SFB are also potent drivers of IgA responses (Talham et al., 1999; Umesaki et al., 1999). SFB drive B-cell maturation, formation of germinal centers, class switch of B-cells to IgA and

differentiation of B-cells to IgA-producing plasma cells (Lecuyer et al., 2014; Talham et al., 1999). Similar to T-cell responses, SFB induce IgA specific for SFB (Jiang et al., 2001; Lecuyer et al., 2014). In contrast to SFB-induced Th17 responses, SFB-induced IgA is primarily mounted in PPs (Lecuyer et al., 2014), arguing for the primary role of PPs to drive B-cell responses to intestinal microbes rather than T-cell tolerance (see section *Antigen acquisition* in *Tolerance to food and commensal antigens*). However, even in the absence of PPs, SFB-induced tertiary lymphoid organs, which form germinal centers and are able to compensate to some extent (Lecuyer et al., 2014). Interestingly, SFB-specific IgAs are transferred to pups from their mother, presumably in milk (Jiang et al., 2001) (for more details see *Spatiotemporal map of intestinal homeostasis* section). Similar to SFB-specific Th17 response, SFB-induced IgA is also dependent on the epithelial adhesion (Atarashi et al., 2015).

Effector mechanisms

Once SFB-induced T-cell and B-cell responses are established, effector mechanisms are mounted to limit the growth of SFB. Interestingly, T- and B-cell responses exert their effector functions through the intestinal epithelium.

IL-17 and Paneth cells. PCs are specialized epithelial cells residing in the crypts of the small intestine and their primary function is the secretion of antimicrobial molecules, such as α-defensins and lysozyme (Bevins and Salzman, 2011). Importantly, PC-restricted α-defensins were shown to regulate intestinal microbiota composition and limit SFB growth (Salzman et al., 2010). While PCs express many antimicrobial molecules constitutively, their expression and secretion can be modulated by extrinsic stimuli. PCs are able to sense microbes directly by their pattern recognition receptors, such as Nod2 (Petnicki-Ocwieja et al., 2009) and TLRs (Ayabe et al., 2000; Rumio et al., 2012) as well as indirectly through the recognition of IFNγ (Farin et al., 2014; Raetz et al., 2013). However, all these signals primarily induce the extrusion of PC granules and, when the stimulation is delivered in high concentrations, it even induces PC depletion (Raetz et al., 2013). On the other hand, it was shown that IL-17 stimulation of intestinal epithelium leads to the upregulation of expression of PC-specific α-defensins, without the discrimination if the effect is direct to PCs (Kumar et al., 2016). We have recently found that direct IL-17 stimulation of PCs increases their expression of α-defensins (Brabec et al., 2023). Furthermore, this stimulation primarily targets a specific set of α-defensin genes (Brabec et al., 2023), which are primarily expressed in the ileum (Castillo et al., 2019; Haber et al., 2017). Although this issue was not studied in detail, it is interesting that specific set of IL-17 regulated defensins shows regional expression pattern, matching growth of SFB. Recent study also showed that IL-17-mediated stimulation of intestinal stem cells drives preferential differentiation to secretory epithelial lineages, including PCs (Lin et al., 2022). Together, these results establish IL-17-mediated stimulation of PC

antimicrobial functions as one of the fundamental effector mechanisms used by homeostatic immunogenic responses to control intestinal commensals, such as SFB.

IL-22 and Reg3 proteins. SFB induced IL-22 was shown to drive the expression of Reg3 proteins, including Reg3α and Reg3γ, which in turn limit SFB growth (Sano et al., 2015; Shih et al., 2014). In contrast to PC-restricted α-defensins, Reg3 proteins are widely expressed throughout intestinal epithelium (Vaishnava et al., 2011).

IL-17, ROS and neutrophils. On top of the above-described effects, IL-17 stimulation of the intestinal epithelium drives the expression of reactive oxygen species (ROS) generating enzyme Nox1 (Kumar et al., 2016) as well as Cxcl1/2-dependent recruitment of neutrophiles (Flannigan et al., 2017). Both these responses limit SFB growth (Flannigan et al., 2017; Kumar et al., 2016).

IL-17 and IgA. As described above, SFB induce intestinal IgA responses. In turn B-cells, their ability to class switch, as well as IgA production are necessary to limit SFB growth (Jiang et al., 2001; Kumar et al., 2016; Suzuki et al., 2004). Interestingly, the effector function B-cell responses to SFB is also dependent on the intestinal epithelium and modulated by SFB-driven T-cell responses. It was shown that SFB-induced IL-17 increases the epithelial expression of secretory IgA receptor (Pigr), which is necessary for IgA transcytosis to the intestinal lumen, where IgA performs its function (Kumar et al., 2016),

In this section I have used the example of immune response to SFB as the model of commensal driving homeostatic proinflammatory immune responses in the intestine. In summary, immune response to SFB is induced by epithelial cells, which endocytose SFB antigens and transfer them to intestinal APCs. In turn, these APCs induce intestinal Th17 and IgA responses, which ultimately limit SFB growth through their cooperation with intestinal epithelium. Thus, homeostatic responses to epithelial adhesive commensals seem to be largely different from those, which drive intestinal tolerance, including antigen acquisition route, major APCs involved as well as effector mechanisms used (see section *Antigen acquisition* in *Tolerance to food and commensal antigens*). For a schematic representation of these processes see Fig. 3 and 4.

Microbiota mediated immunoregulation

Classically, antigens are viewed as mere targets of the immune system that decides which immune response to mount as well as its magnitude based on the context of PRR ligands delivered to sentinel APC, which encounters the antigen. In contrast to this model, intestinal commensals developed multiple mechanisms to modulate immune responses that are mounted by the host immune system after the

recognition of commensal antigens. One of the major routes of microbial immune regulation is mediated by so-called secondary metabolites, i.e. molecules that are generated by intestinal microbiota through the metabolism of dietary compounds and host products (Geuking and Burkhard, 2020).

An example of immunomodulatory secondary metabolites are short chain fatty acids (SCFA). These molecules, such as acetate, butyrate and propionate, are produced by some intestinal commensals through the fermentation of dietary fiber (Geuking and Burkhard, 2020). It was found that several strains of commensal clostridia use SCFA to drive intestinal Treg responses (Atarashi et al., 2013). In the past few decades, many of other secondary metabolites with immunomodulatory properties were identified (Geuking and Burkhard, 2020). Importantly, generation of these secondary metabolites is largely dependent on the diet composition, which might be one of the explanations of recent increase in the incidence of immunologic disorders observed in western countries, where diet is typically high in fat, salt and sugar, while low in fiber (Bach, 2002).

Thus, intestinal microbes actively modulate the intestinal immune system, which enables them to participate in the decision-making whether proinflammatory or regulatory immune responses are mounted.

Spatiotemporal map of intestinal homeostasis

Throughout this thesis I have focused on the mechanistic description of processes that govern intestinal homeostasis. Although this kind of perception allows the identification of individual processes, it gives us a "cross-section frozen in time". Recent studies demonstrated that the establishment of intestinal immune homeostasis is dynamic in time and compartmentalized to specific regions of the intestine.

Timing

It has been demonstrated that there are several crucial time windows, in which certain aspects of intestinal immune homeostasis are established.

Neonatal window. Firstly, in a neonatal period, intestinal immune system comes to the first contact with complex microbiota that colonizes the intestine shortly after birth. These microbes largely originate from the mother (Mueller et al., 2015) and their presence is beneficial for the infant, among other things, to optimize nutrients uptake (Schwarzer et al., 2023). However, along with the microbiota, mother also provides the infant with her own immune memory of host-microbe interaction, through the delivery of IgA in breast milk (Jiang et al., 2001; Ramanan et al., 2020). Thus, the inexperienced immune system of the infant is given a period, in which it can establish its own homeostatic relationship with its own intestinal microbiota. It was recently found that breast milk delivered IgA determines the setpoint of intestinal homeostasis in the infant through the modulation of the amount of Rorγt^{+} Tregs, which differentiate in response to microbiota (Ramanan et al., 2020). Strikingly, milk-born antibodies

targeting food antigens also protect the infant from the development of food allergy, through the induction of food antigen-specific Tregs (Ohsaki et al., 2018). Thus, transfer of antibodies from the mother in breast milk provides the infant with the shadow of experienced maternal oral tolerance.

It seems that even immunogenic memory of homeostatic microbiota-regulatory responses is delivered to the infant since SFB-specific IgA delivered through breast milk limits the growth of SFB in the pre-weaning period (Jiang et al., 2001). While this route of SFB-specific IgA acquisition is induced shortly after birth and peaks around weaning, it is overtaken by endogenous production of SFB-specific IgA later in life (Jiang et al., 2001). Although the functional outcome of SFB-specific IgA transfer from mother is not clear, it is tempting to speculate that it might play a role in SFB-targeted Th17 response, similar to the effect of maternal IgA on the development of Rorγ^{+} Tregs (Ramanan et al., 2020).

Interestingly, at the same timepoint, infant thymus generates a distinct population of Aire-dependent neonatal Tregs, crucial for self-tolerance (Yang et al., 2015). Thus, in a preweaning period, mother provided immune memory and the infant-intrinsic mechanisms collaborate to generate a setpoint of immune tolerance to intestinal self-antigens as well as to those from food and microbiota.

Weaning. Another crucial time window is the weaning. It is a period when the infant starts eating solid food, which leads to massive changes in the intestinal microbiota composition (Al Nabhani et al., 2019). At this timepoint intestinal immune system mounts nonpathogenic proinflammatory immune responses (Al Nabhani et al., 2019) which are dependent on the intestinal microbiota and they target microbial antigens (Al Nabhani et al., 2019; Knoop et al., 2017). Importantly, the disruption of these immune responses abrogates tolerance to commensal microbiota (Knoop et al., 2017) and predisposes the host to many severe pathologies, such as colitis, colorectal cancer and allergy, later in life (Al Nabhani et al., 2019). Interestingly, the time window for the so call “weaning reaction” seems to be rather limited as the shift in time of microbiota introduction results in pathology (Al Nabhani et al., 2019). The initiation of weaning reaction is induced by the drop of breast milk delivered EGF (Al Nabhani et al., 2019; Knoop et al., 2017), which controls the goblet cell-mediated antigen uptake in the colon (Knoop et al., 2017). Weaning reaction also seems to have an upper time limit, i.e. it cannot be shifted to older age, however the molecular determinants of this border are yet unknown. Although all the complexity of immune responses mounted at weaning is only beginning to be uncovered, Tregs induced at this timepoint seem crucial for long-term intestine homeostasis. Similar to the neonatal wave of thymic Tregs, weaning-derived Rorγt^{+} Tregs generated in the intestine, are crucial transducers of the long-lasting memory of immune response that took place during weaning (Al Nabhani et al., 2019). However, while the transient upregulation of IFNγ and TNFα at weaning was reported (Al Nabhani et al., 2019), the functional significance of this observation for the intestinal homeostasis is currently unknown. Nevertheless, the availability of complex microbiota and dietary antigens, present at weaning, together

with high efficiency of GAPs-mediated antigen uptake at this timepoint poses the weaning period as the “sweet spot” for the induction of tolerance to intestinal antigens.

Regionalization

The physical barrier is largely different in the small intestine and colon. In the colon, luminal microbes are largely segregated from the epithelium by a thick layer of mucus. In the small intestine, microbes may often come to contact with epithelial cells, since it contains only a thin layer of mucus, and the barrier function is largely ensured by the production of antimicrobial compounds (Clevers and Bevins, 2013; Johansson et al., 2011; Mowat and Agace, 2014). This difference might be the driving force of divergent immune responses that are mounted in response to microbes present in these tissues. While colonic reactions to microbiota largely drive the generation of Tregs, in the small intestine, homeostatic proinflammatory immune responses are kept in equilibrium with immune suppression (Mowat and Agace, 2014).

Since the small intestine is the primary side of nutrients absorption, it also contains the largest concentration of food antigens. In contrast, colon contains the greatest diversity of commensal microbes. Conversely, the tolerance to food antigens is primarily induced in the small intestine, while the tolerance to commensal microbiota is mostly induced in the colon (Kim et al., 2016). Interestingly, the presence of some commensal microbes is generally limited to the small intestine. One of these microbes is SFB, which is also a potent inducer of homeostatic proinflammatory immune responses (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009) (see section *Tolerate or respond?*). Thus, it is possible that tropism specific for the small intestine, together with the ability to attach to the intestinal epithelium is a defining factor for the microbes to induce homeostatic proinflammatory immune responses.

Interestingly, the capacity of goblet cells to deliver luminal antigens to intestinal APCs is differentially regulated in the small intestine and colon. While in the colon GAPs open shortly after birth, but close soon after the weaning, small intestine GAPs show an opposing trend, gradually increasing their activity from the weaning (Knoop et al., 2017).

Collectively, recent studies discussed in this section highlight the importance of localization and timing of intestinal immune responses, suggesting that neonatal and weaning time windows are crucial for the establishment of proper intestinal immune equilibrium. In addition, while the small intestine combines tolerance to food antigens with homeostatic immunogenic responses and tolerance to intestinal microbiota, the colon seems to be rather specialized for the induction of tolerance to microbiota. However, it is only recently that we started to uncover the immense dynamic of intestinal immune responses through time and space and thus many further studies are needed to really understand these processes.

Breakdown of intestinal homeostasis

In previous sections I have summarized mechanisms which enable establishment of intestinal immune homeostasis. In this section I will illustrate the crucial importance of these processes and describe deleterious outcomes which occur if intestinal homeostasis fails.

Failure of thymic tolerance

The importance of functional central tolerance can be demonstrated by the existence of severe human disorders, such as Autoimmune polyglandular syndrome type-1 (APS-1) or Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX). In the case of APS-1, patients carry mutations of *AIRE* gene, which leads to complete or partial dysfunction of the AIRE protein (Consortium, 1997; Nagamine et al., 1997), leading to disruption of mTECs' ability to produce TRAs and thus to incomplete thymic selection of self-reactive T-cell (Anderson et al., 2002; Liston et al., 2003). As a result, patients develop a number of autoimmune symptoms typically including destruction of thyroid glands, adrenal glands and gonads (Perheentupa, 2006). Notably, the majority of APS-1 patients also develop chronic mucocutaneous candidiasis (CMC) (Perheentupa, 2006). This phenomenon was long a mystery, since it is not a typical complication in other autoimmune disorders, but rather it develops in immunosuppressed or immunodeficient patients (Huppler et al., 2012). As explained above, it was recently shown that susceptibility of APS-1 patients to CMC is rather linked to extrathymic Aire function (Dobeš et al., 2022) (for more details please see section *Antigen presentation* in *Tolerance to food and microbiota antigens* section).

On top of canonic symptoms described above, some APS-1 patients also develop intestinal pathology represented either by malabsorption, chronic constipation or diarrhea (Perheentupa, 2006). While the pathogenesis of these APS-1 symptoms is unknown, it is worthy of note that Aire plays a role in the expression of PC-specific α-defensins in mTECs and APS-1 patients show lowered numbers of PCs (Dobeš et al., 2015). Thus, we can speculate that PC destruction, due to incomplete elimination of T-cell which recognize PC-specific autoantigens in the state of AIRE disfunction could be a driving force for a portion of intestinal symptoms observed in APS-1 patients.

In contrast to APS-1, mTECs' ability to produce TRAs is unperturbed in IPEX patients, however these patients carry mutations of *FOXP3* gene (Bennett et al., 2001; Wildin et al., 2001), the master regulator of Treg differentiation program (Fontenot et al., 2003; Fontenot et al., 2005; Hori et al., 2003). The abrogation of FOXP3 function leads to inability of T-cells to undergo Treg development, which, similar to APS-1, results in multiorgan autoimmune manifestations. However, unlike in APS-1, in IPEX patients the intestine is one of the primary sites of pathology (Wildin et al., 2002). As explained above, Tregs are generated both in the thymus and in the immune periphery and in particular at the mucosal barriers in response to food and commensal microbiota antigens (Idoyaga et al., 2013;

Josefowicz et al., 2012; Weiss et al., 2012). Since Tregs play a crucial role in the intestinal homeostasis (Liu et al., 2003; Mottet et al., 2003) it is likely that intestinal pathology in IPEX is linked to abrogation of both thymic and intestinal Tregs development in contrast to APS-1, where intestinal Treg differentiation is likely unperturbed.

Failure of tolerance to food and microbiota antigens

In contrast to thymic tolerance, failure of tolerance to antigens from intestinal lumen results either in food allergies or IBD. As described above, tolerance to food and microbiota antigens is mediated by distinct APCs and thus the disruption of DC tolerogenic properties is the likely the cause of food allergies (Coombes et al., 2007; Esterházy et al., 2016; Sun et al., 2007), while the perturbation of antigens presentation in ILC3s is associated with IBD (Hepworth et al., 2015).

Food allergy. Food allergy is a pathologic reaction to harmless food antigens included in the diet. While the precise mechanism that drives the pathogenesis of food allergy is not clear, it is generally accepted that these conditions arise from the failure of oral tolerance (Wambre and Jeong, 2018).

One of the possible explanations why tolerance to food antigens fails in food allergy arose from the investigation of the nature of typical food antigens. Interestingly, food allergy is targeted only to a small fraction from a vast variety of antigens that are typically included in diet. It was found that some typical food allergens, such as Ara h 1, major allergen of peanuts, acts as an adjuvant, driving pro-Th2 polarization of intestinal DCs (Shreffler et al., 2006). A common mechanism behind adjuvant-like properties of food antigens might be their glycosylation pattern, since specific protein glycosylation is able to skew immune response to proallergic Th2 (Hilmenyuk et al., 2010; Hsu et al., 2007).

Importantly, microbiota dysbiosis was implicated in the pathogenesis of food allergy (Zhao et al., 2019). As described above, intestinal microbes are able to actively promote development of intestinal tolerance. While these microbes likely developed this ability primarily to be tolerated by the immune system, it also enables them to shape immune responses to other targets, such as food antigens. Thus, one can imagine that perturbation of intestinal microbiota composition by external factors (such as antibiotics treatment) or genetic predisposition (such as mutations in antimicrobial proteins or molecules that regulate them) can predispose immune system to develop food allergy due to the loss of microbiota-driven tolerogenic environment.

Inflammatory bowel diseases. IBD are a group of disorders that are characterized by chronic inflammation in the small intestine or colon (or even upper gastro-intestinal tract in some cases). Two major components of IBD are Crohn's disease (CD) and ulcerative colitis (UC). While UC is limited to the colon, CD can affect any part of the gastro-intestinal tract, however it manifests typically in the colon or terminal ileum. Although these diseases were historically viewed as idiopathic, i.e. without

any obvious causative factor, in recent decades a large number of studies have aimed to understand mechanisms that drive the development of IBD. Thus, it is the current view that the three major components: genetic predisposition, pathologic immune reaction to intestinal microbiota and dysbiosis (disruption of microbiota composition and/or functionality), in combination, are the driving force of IBD development (Ramos and Papadakis, 2019).

Genome-wide association studies (GWAS) found that mutations associated with IBD mostly target genes, which play a role in the intestinal homeostasis establishment. Just to name a few, these genes include *NOD2*, *IL10*, *RORC* and *IL23R*, signifying that genetic basis of IBD is tightly linked to the functionality of intestinal homeostasis (Jostins et al., 2012). While cases of IBD with strong genetic association mostly manifest in early childhood, the majority of IBD patients are diagnosed much later in their lives, suggesting that although genetic background plays a role in IBD development, other factors must contribute to IBD development (Ramos and Papadakis, 2019).

One of the principal causes of IBD development is the uncontrolled inflammatory response to commensal microbiota. As described above (see *Tolerance to food and commensal antigens* section), $MHCII^+$ ILC3s are responsible for the establishment of tolerance to commensal microbiota (Hepworth et al., 2013). Importantly, it was found that the IBD patients show lowered $MHCII^+$ expression in ILC3s as well as low frequencies of $ROR\gamma t^+$ Tregs in their intestines and associated lymphoid organs (Hepworth et al., 2015; Lyu et al., 2022), suggesting that perturbation of $MHCII^+$ ILC3-mediated T-cell selection might be the causative factor for IBD development. However, the underlying mechanism that might explain why MHCII on ILC3s is lowered in IBD patients is yet unknown. Also, it is unclear, what is the mechanistic difference between homeostatic inflammatory immune responses that are mounted to some intestinal microbes (see section *Tolerate or respond?*) and deleterious chronic inflammation observed in IBD.

Failure of microbiota regulation

While in the previous subsection I have focused on the failure of immune system to tolerate harmless antigens in the intestine that results in intestinal pathology, here I will focus on the perturbation of complementary mechanism, i.e. the propensity of dysbiotic gut microbiota to cause intestinal pathology. The idea that pathogenesis of IBD and other conditions may be driven by changes in the intestinal microbiota came from microbiome profiling studies, which found that composition of intestinal microbiota is altered in patients suffering from these conditions. While this observation was reproduced multiple times, it became evident that no universal microbiota signature can be assigned as IBD-predisposing or even IBD-associated, since changes in microbiota in IBD patients are largely specific for each individual or a group of individuals. However, what seems to be universally applicable is that the diversity of microbial community in the intestine shrinks in dysbiotic state (Franzosa et al., 2019; Gevers et al., 2014; Morgan et al., 2012; Pascal et al., 2017).

Despite these observations it is not clear if changes in microbiota composition are the results of intestinal inflammation in IBD state, or if microbiota alteration operates upstream of inflammation and may serve as one of the inflammation triggers. Importantly, in mice it was functionally proven that dysbiotic microbiota from mice with IBD-like disease can induce similar pathology in genetically susceptible recipients (Couturier-Maillard et al., 2013; Elinav et al., 2011; Schaubeck et al., 2016). Furthermore, we have shown that microbiota from mice which show subclinical intestinal inflammation in the intestine, along with a robust drop in their microbial diversity, is capable to induce severe intestinal pathology after its transfer to IL-10$^{-/-}$ recipients, which are prone to microbiota-dependent intestinal inflammation (Brabec et al., 2023). This suggests that microbiota dysbiosis can indeed occur upstream of intestinal pathology rather than as its result and it can be one of the primary drivers of IBD.

Summary

In this literary overview I have aimed to provide a mechanistic insight into the processes which govern intestinal homeostasis establishment and maintenance. I have shown that intestinal homeostasis is established in a multilayered system, starting from thymic T-cell selection, which ensures tolerance to intestinal self-antigens, followed by intestine-specific mechanisms that drive tolerance to food and microbiota antigens. As I highlighted, compared to other tissues, intestinal immune system is faced with additional challenge, the necessity to keep intestinal microbes in check through homeostatic proinflammatory immune responses, which drive microbe-limiting effector mechanisms. Thus, intestinal homeostasis is defined by the tightly controlled equilibrium between pro- and anti-inflammatory responses rather than indiscriminative tolerance. Furthermore, intestinal microbes actively modulate immune responses that target them, adding another layer of complexity to the already complex system controlling intestinal homeostasis. Finally, I have described typical cases, in which intestinal homeostasis fails, leading to often severe intestinal pathology. For schematic summary of multilayered model of intestinal homeostasis please see Fig. 4.

Throughout this review I have demonstrated the crucial role of immune-epithelial interactions, which orchestrate indispensable processes such as thymic T-cells selection done by mTECs, intestinal lumen antigens acquisition by M-cells and goblet cells, and effector mechanisms executed through secretion of antimicrobial compounds from PCs and other cells of in the intestinal epithelium. As can be seen from the presented publications, immune-epithelial interactions and their role in intestinal homeostasis were the primary focus of my Ph.D. studies. Now I will briefly summarize the original aims of my Ph.D. studies, which are addressed in respective publications shown below.

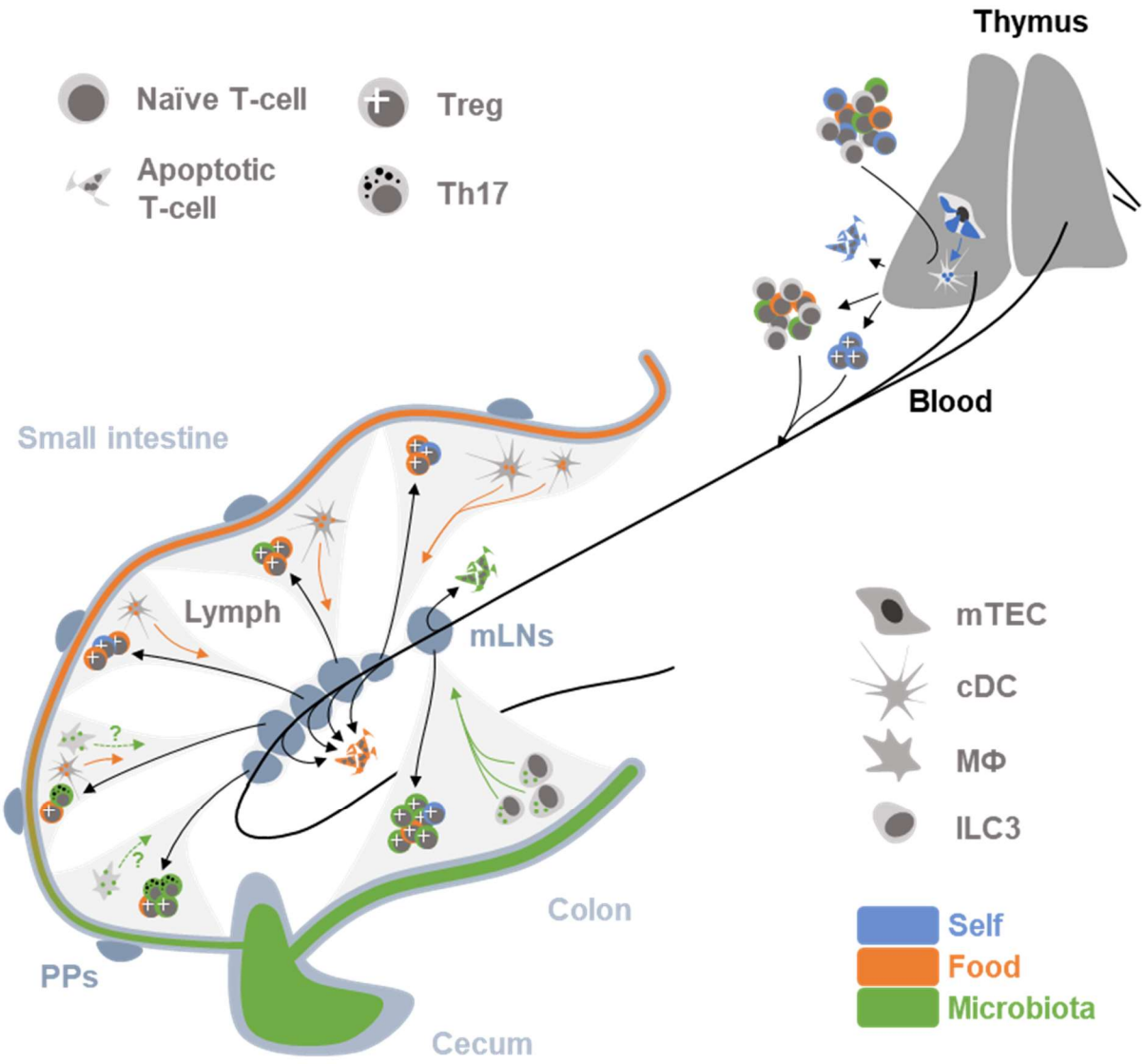

Figure 4. Multilayered model of intestinal immune homeostasis. In the thymus mTEC-cDC cooperation ensures deletion and Treg diversion of self-specific T-cell clones, releasing self-specific Tregs, together with naïve T-cells with all the other specificities to the system. In the small intestine, cDCs uptake food antigens and migrate to mLNs, where they mediate deletion and Treg diversion of food-specific clones T-cell clones. Generation of homeostatic proinflammatory T-cell responses (Th17) to microbiota is driven by intestinal macrophages (MΦ), but it is currently unclear if they migrate to mLNs. In the colon, ILC3 uptake microbiota antigens and migrate to mLNs, where they mediate deletion and Treg diversion of microbiota-specific clones T-cell clones. Self-, food- and microbiota-specific Tregs together with microbiota specific Th17 migrate to the intestines, where they enforce immune homeostasis by immune suppression and microbiota regulation.

Thesis aims

Originally, my Ph.D. was focused mainly on the immune regulation of PCs antimicrobial functions and their role in intestinal homeostasis. However, during the course of my Ph.D. studies, my scientific interests broadened to the study of intestinal homeostasis as a whole. My position in the laboratory of Dominik Filipp and many fruitful discussions with my colleagues allowed me to extend the view of intestinal immunity and take into account many other aspects, such as thymic T-cells selection. Thus, my personal scientific growth resulted in several studies presented below, which contributed to the view of intestinal homeostasis as a complex system of interdependent processes. Specifically, we aimed to address the following questions:

1) Are Paneth cells directly regulated by IL-17 and what is the physiologic importance of this regulatory mechanism?

Rationale: IL-17 is the major driver of antimicrobial molecules expression, while PCs are the major producers of antimicrobial compounds in the intestine. However, the direct link between IL-17 and PCs was previously unknown.

2) How does TLR stimulation affect mTEC biology and what is the impact of this process on thymic dendritic cells, cooperative antigen transfer and T-cell selection?

Rationale: While mTECs were known to express TLRs, the function of TLR-mediated stimulation of mTECs, as well as its impact on thymic T-cell selection were unknown.

3) Are mTEC-derived antigens delivered to specific thymic APC subsets?

Rationale: Several different thymic APC subsets were known to acquire antigens from mTECs, and this process was known to contribute to thymic T-cell selection. Since recent studies have shown large heterogeneity of mTECs, we speculated that different mTEC subsets might deliver antigens to distinct populations of thymic APCs.

4) What is the identity and function of Aire$^+$ cells in the secondary lymphoid organs?

Rationale: Previous studies showed that Aire$^+$ APCs reside outside of the thymus, in lymph nodes, but their cellular identity as well as function(s) was not known.

5) What is the impact of Nod2-mediated stimulation of intestinal epithelium on the chronic malnutrition?

Rationale: Certain microbes are able to alleviate some deleterious effects of chronic malnutrition, however the mechanism of their action was not known.

Results

Here I provide a list of primary publication in which I have participated throughout my Ph.D. studies, that constitute the core body of my scientific work related to the topic of this thesis:

Brabec T, Voboril M, Schierova D, Valter E, Splichalova I, Dobes J, Brezina J, Dobesova M, Aidarova A, Jakubec M, Blumberg R, Waissman A, Kolar M, Kubovciak J, Srutkova D, Hudcovic T, Schwarzer M, Fronkova E, Pinkasova T, Jabandziev P and Filipp D. **IL-17 driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum.** *In press, Mucosal Immunology, 10.1016/j.mucimm.2023.01.005*

Vobořil M, **Brabec T**, Dobeš J, Šplíchalová I, Březina J, Čepková A, Dobešová M, Aidarova A, Kubovčiak J, Tsyklauri O, Štěpánek O, Beneš V, Sedláček R, Klein L, Kolář M, Filipp D. **Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation.** *Nat Commun. 2020 May 12;11(1):2361. doi: 10.1038/s41467-020-16081-3.*

Vobořil M, Březina J, **Brabec T**, Dobeš J, Ballek O, Dobešová M, Manning J, Blumberg RS, Filipp D. **A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer.** *Elife. 2022 Jan 31;11:e71578. doi: 10.7554/eLife.71578.*

Dobeš J, Edenhofer F, Vobořil M, **Brabec T**, Dobešová M, Čepková A, Klein L, Rajewsky K, Filipp D. **A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire.** *Eur J Immunol. 2018 Mar;48(3):546-548. doi: 10.1002/eji.201747267. Epub 2017 Dec 27.*

Yamano T, Dobeš J, Vobořil M, Steinert M, **Brabec T**, Ziętara N, Dobešová M, Ohnmacht C, Laan M, Peterson P, Benes V, Sedláček R, Hanayama R, Kolář M, Klein L, Filipp D. **Aire-expressing ILC3-like cells in the lymph node display potent APC features.** *J Exp Med. 2019 Mar 27. pii: jem.20181430. doi: 10.1084/jem.20181430.*

Schwarzer M, Gautam UK, Makki K, Lambert A, **Brabec T**, Joly A, Šrůtková D, Poinsot P, Novotná T, Geoffroy S, Courtin P, Hermanová PP, Matos RC, Landry JJM, Gérard C, Bulteau AL, Hudcovic T, Kozáková H, Filipp D, Chapot-Chartier MP, Šinkora M, Peretti N, Boneca IG, Chamaillard M, Vidal H, De Vadder F, Leulier F. **Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice.** *Science. 2023 Feb 24;379(6634):826-833. doi: 10.1126/science.ade9767.*

I have also participated in one review article and one study related to embryonic hematopoiesis:

Filipp D, **Brabec T**, Vobořil M, Dobeš J. **Enteric α-defensins on the verge of intestinal immune tolerance and inflammation.** *Semin Cell Dev Biol. 2018 Jan 29. pii: S1084-9521(17)30407-X. doi: 10.1016/j.semcdb.2018.01.007.*

Splichalova I, Balounová J, Vobořil M, **Brabec T**, Sedlacek R, Filipp D. **Deletion of TLR2 + erythro-myeloid progenitors leads to embryonic lethality in mice.** *Eur J Immunol. 2021 Sep;51(9):2237-2250. doi: 10.1002/eji.202049142. Epub 2021 Jun 21.*

IL-17 driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum

In this study we investigated one of the effector mechanisms of homeostatic proinflammatory immune responses in the intestine (see *Tolerate or respond?* section in *Current state of knowledge*), namely the impact of direct IL-17 stimulation on PC antimicrobial functions. Through PC-specific genetic depletion of IL-17 receptor, we have found that IL-17 increases the expression of specific set of enteric α-defensins in ileal PCs. In turn, the abrogation of PCs' ability to sense IL-17 leads to subclinical inflammation in the ileum and drop in ileal microbiota diversity. Interestingly fecal microbiota remained largely unaffected, arguing for the region-specific function of enteric α-defensins that are affected by IL-17 mediated stimulation of PCs. Importantly, ileal microbiota from mice lacking IL-17 receptor on PCs is able to induce severe intestinal inflammation in IL-10$^{-/-}$ mice, suggesting that regulation of microbiota through IL-17 driven stimulation of PCs is crucial to maintain intestinal homeostasis. Finally, we analyzed a small cohort of pediatric patients suffering from Crohn's disease and found that sub-cohort of these patients show similar phenotype to mice lacking IL-17 receptor on PCs. Interestingly, these patients showed the most severe inflammation in their ileum among all the patients in our cohort, suggesting that mechanism, similar to the one we discovered in mice, might also operate in humans.

This is the central study of this thesis in which I have participated as the first author. I have formed the hypotheses, designed the experiments and wrote the manuscript, often with the help facilitated by the discussion with my supervisor Dominik Filipp as well as Jan Dobeš, Matouš Vobořil, Evgeny Valter, Jiří Březina, Marin Schwarzer, Dagmar Schierova and other coauthors. I have also performed the majority of the experimental work presented in this study, but I want to stress that I am really grateful for all the help with particular experiments rendered by all the coauthors. Namely, I want to acknowledge the help of Marin Schwarzer for performing all the microbiota transfer experiments, Dagmar Schierova for performing all the microbiota sequencing, Evgeny Valter for all the histopathology scoring and Peter Jabandziev for organizing the collection of patient samples.

Brabec T, Voboril M, Schierova D, Valter E, Splichalova I, Dobes J, Brezina J, Dobesova M, Aidarova A, Jakubec M, Blumberg R, Waissman A, Kolar M, Kubovciak J, Srutkova D, Hudcovic T, Schwarzer M, Fronkova E, Pinkasova T, Jabandziev P and Filipp D. **IL-17 driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum.** *In press, Mucosal Immunology, 10.1016/j.mucimm.2023.01.005*

Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation.

In this publication we have investigated the role of TLR signaling in mTECs. We have found that upon TLR stimulation mTECs upregulate a set of chemokines in TLR transducer MyD88-dependent manner. Next, we analyzed the heterogeneity of thymic APC, along with their capacity to sense these chemokines. Through this analysis we identified a subpopulation of $CD14^+$ cDCs, which accumulated in response to TLR mediated stimulation of mTECs. Furthermore, these cells increased their capacity to acquire mTEC-produced antigens after TLR mediated stimulation of mTECs. Thus, we have identified a novel mechanism which regulates CAT to a specific population of DCs.

Next, we focused on the physiological role of this mechanism. Although mice with mTEC-specific MyD88 genetic depletion did not show changes in the general thymic T-cell development, we found that these mice have decreased frequency of thymically generated Tregs. Importantly, this effect was dependent on the MHCII expression in DCs, arguing for the role of DC mediated presentation of mTEC derived antigens. We also discovered that Tregs found in mice with mTEC-specific MyD88 depletion show weaker capacity to suppress T-cell activation. Finally, we have used T-cell transfer approach and showed that T-cells from mice with mTEC-specific MyD88 depletion induce intestinal inflammation, likely due to the disruption of their Treg functionality. Thus, we have identified a mechanism, which orchestrates T-cell selection through the cooperation of mTECs with a specific set of cDC. Strikingly, this mechanism, which operates in the thymus, ultimately contributes to intestinal homeostasis.

In this study I have participated as a coauthor. I have contributed mostly intellectually, through discussion, experimental design, and critical reading of the manuscript. I have also performed experiments, which tested the functionality of Tregs.

Vobořil M, **Brabec T**, Dobeš J, Šplíchalová I, Březina J, Čepková A, Dobešová M, Aidarova A, Kubovčiak J, Tsyklauri O, Štěpánek O, Beneš V, Sedláček R, Klein L, Kolář M, Filipp D. **Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation.** *Nat Commun. 2020 May 12;11(1):2361. doi: 10.1038/s41467-020-16081-3.*

A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer.

This is a follow up study of the one, presented just above. In this study we have tested the hypothesis that specific subsets of mTECs deliver antigens to specific types of thymic APCs. We used a set of mouse models, which express fluorescent proteins in different populations of mTECs. By correlating the expression pattern of these fluorescent proteins in different mTEC populations and the positivity for these markers in thymic APCs, which acquired them through CAT we identified several possible mTEC-APC pairs. While this approach has many limitations, it was the first study to analyze this complex system. On top of these findings, we identified thymic aDCs as the most efficient population participating in CAT. Furthermore, we have found that unlike other thymic APCs, single $CD14^+$ cDC is able to acquire antigens from multiple mTECs as well as from other thymic APCs with strikingly high efficiency.

Due to the descriptive nature of this study, there is no direct relation to intestinal homeostasis. However, as I have discussed above, the process of CAT is important for the generation of thymic Tregs, which ultimately play a crucial role in the intestinal homeostasis.

In this study I have participated as a coauthor. I have mainly contributed by the bioinformatic analysis of large datasets. I have also contributed intellectually, through scientific discussion, experimental design, and critical reading of the manuscript.

Vobořil M, Březina J, **Brabec T**, Dobeš J, Ballek O, Dobešová M, Manning J, Blumberg RS, Filipp D. **A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer.** *Elife. 2022 Jan 31;11:e71578. doi: 10.7554/eLife.71578.*

A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire

In this short study, we have generated a novel mouse model, which enables cre mediated cell type-specific depletion of *Aire* gene. We have also performed a rigorous validation of this model using several different cre drivers and comparison to whole-body $Aire^{-/-}$ mice. Interestingly, we have also found that thymic Aire expression does not affect fertility, which is severely diminished in whole-body $Aire^{-/-}$ mice.

Due to the technical nature of this publication, it has no direct link to intestinal homeostasis. However, the generation of this mouse model enabled the identification of $Aire^{+}$ ILC3 as the central regulators of immune response to *Candida albicans* (Dobeš et al., 2022), as described below.

In this study I have participated as a coauthor. I have mainly contributed by flow cytometry and gene expression validation of Aire deletion in several different mouse models. I have also critically read the manuscript.

Dobeš J, Edenhofer F, Vobořil M, **Brabec T**, Dobešová M, Čepková A, Klein L, Rajewsky K, Filipp D. **A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire.** *Eur J Immunol. 2018 Mar;48(3):546-548. doi: 10.1002/eji.201747267. Epub 2017 Dec 27.*

Aire-expressing ILC3-like cells in the lymph node display potent APC features

In this study we have investigated the cellular identity of extrathymic Aire$^+$ cells found in LNs. We discovered that these cells show both transcriptomic and protein signature of ILC3, marked by the expression of Rorγt and the absence of other hematopoietic lineages markers. Since these cells express high levels of MCHII, it poses them close to previously identified MHCII$^+$ ILC3s (Hepworth et al., 2013), which play a major role in the tolerance to antigens form intestinal microbiota. Although recent studies found that Aire$^+$ ILC3-like cells do not select microbiota-specific Tregs (Kedmi et al., 2022; Lyu et al., 2022), the knowledge obtained in the study presented here was utilized by Jan Dobeš in a follow-up study, which identified Aire$^+$ ILC3-like cells as the crucial regulators of the immune response to *Candida albicans* (Dobeš et al., 2022). While *Candida albicans* is normally well tolerated and behaves as a commensal, in immunosuppressed individuals and in APS-1 patients, *Candida albicans* can overgrow or even disseminate from gastrointestinal tract and cause severe complications.

Thus, the identification of Aire$^+$ ILC3 fostered the discovery of an important mechanism that mediates homeostatic immune reactions in the intestine, which is needed to keep *Candida albicans* under immune control.

In this study I have participated as a coauthor. I have mainly contributed by flow cytometry phenotyping of Aire$^+$ ILC3-like cells. I have also critically read the manuscript.

Yamano T, Dobeš J, Vobořil M, Steinert M, **Brabec T**, Ziętara N, Dobešová M, Ohnmacht C, Laan M, Peterson P, Benes V, Sedláček R, Hanayama R, Kolář M, Klein L, Filipp D. **Aire-expressing ILC3-like cells in the lymph node display potent APC features.** *J Exp Med. 2019 Mar 27. pii: jem.20181430. doi: 10.1084/jem.20181430.*

Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice

In this study we have shown that a specific strain of lactobacillus, *Lactiplantibacillus plantarum* WJL, is able to alleviate detrimental effects of chronic undernutrition in infant mice through the activation of Nod2, intracellular PRR, in intestinal epithelium. *L. plantarum* WJL colonization increases the absorption surface of the intestinal epithelium by Nod2-dependent induction of epithelial proliferation. This ultimately results in better growth of infant mice facing chronic undernutrition on hypoprotidic and hypolipidic diet.

While this study goes beyond the concept of intestinal immune homeostasis, which I have introduced in the *Current state of knowledge* section, it illustrates how are immune mechanisms, such as the PRR-mediated recognition of commensal microbes, utilized in other physiologic processes. Also participating in this study allowed me to gain insight into cross-disciplinary research, which is currently one of the main sources of scientific advancement.

In this study I have participated as a coauthor. I have mainly contributed by the flow cytometry validation of mouse models through the analysis of cre-driven expression of fluorescent proteins. I have also contributed by discussion, bioinformatic analysis of previously obtained datasets and critical reading of the manuscript.

Schwarzer M, Gautam UK, Makki K, Lambert A, **Brabec T**, Joly A, Šrůtková D, Poinsot P, Novotná T, Geoffroy S, Courtin P, Hermanová PP, Matos RC, Landry JJM, Gérard C, Bulteau AL, Hudcovic T, Kozáková H, Filipp D, Chapot-Chartier MP, Šinkora M, Peretti N, Boneca IG, Chamaillard M, Vidal H, De Vadder F, Leulier F. **Microbe-mediated intestinal NOD2 stimulation improves linear growth of undernourished infant mice.** *Science. 2023 Feb 24;379(6634):826-833. doi: 10.1126/science.ade9767.*

Discussion and conclusions

In the previous sections I have introduced a model of multilayered establishment of intestinal immune homeostasis, that is based on many previous studies integrated with novel findings, which I have presented here. The baseline of intestinal homeostasis is set in the thymus, where T-cells reactive to self-antigens are either eliminated or converted to Tregs, based on their affinity to peptide-MHC complexes and the abundance of self-antigens in the thymus. This is largely done by mTECs and their cooperation with thymic DCs.

In the original studies presented here, we have demonstrated the complexity of mTEC-DC cooperation network and we identified one of the novel mechanisms, which regulates CAT to $CD14^+$ cDCs and ultimately drives the generation of thymic Tregs that are crucial for intestinal homeostasis. While we found that this mechanism is dependent on TLR-mediated stimulation of mTECs, the source of TLR ligands inside the thymus is currently unknown. Since we showed that this mechanism is independent of intestinal microbiota, it is likely that TLR ligands are derived from endogenous sources. Possible source of these ligands could be apoptotic cells, as large amount of developing thymocytes undergo apoptosis due to their positive and negative selection in the thymus (Surh and Sprent, 1994). Dying cells can release many molecules, which can be recognized as endogenous PRR ligands, serving the purpose of so-called danger signals. One such example could be Hmgb1 protein, which is released from dying cells (Bell et al., 2006) and it is also recognized by some TLRs (Yu et al., 2006). In support of this hypothesis, it was found that increase in thymic apoptotic rate induces thymic Treg generation (Konkel et al., 2014), similar to TLR stimulation of mTECs (Vobořil et al., 2020).

We have also identified that thymic aDCs are the most efficient population in the antigen acquisition from mTECs. Outside of the thymus, DC activation occurs in the peripheral tissues after the recognition of antigen. This leads to the upregulation of antigen presentation machinery, costimulatory molecules and cytokines, which together enable efficient activation of cognate T-cells. At the same time activated DCs upregulate Ccr7, which enables them to migrate to draining lymph nodes, where cognate T-cell traffic (Liu et al., 2021). However, the situation in the thymus is very much different. First, it is unclear which signals lead to intrathymic DC activation. Similar to TLR stimulation of mTECs, cells dying inside the thymus can also serve the purpose of thymic cDC activation, since the uptake of apoptotic cells inside the tumor was shown to drive activation of local DCs, mounting a phenotype intriguingly similar to thymic aDCs (Maier et al., 2020). In the thymus, it is tempting to speculate that the uptake of dying mTECs could be the driving force of thymic aDC generation, since it would ensure the uptake of mTEC-produced TRAs. Second, it is unclear what is the function of Ccr7 expression in thymic aDCs. In this regard it is interesting to mention that Ccl21, the ligand for Ccr7, is expressed by a population of mTECs distinct from those expressing Aire. These mTECs use Ccl21 to attract developing thymocytes to the medulla once their positive selection is finished (Ueno et al., 2004; Witt et al., 2005). Thus, it is possible that aDCs use Ccr7 to migrate to the vicinity of $Ccl21^+$ mTECs. Since

these mTECs do not express Aire and thus nor TRAs, it is possible that TRA-fed aDCs might use this mechanism to extend TRA presentation to this niche of the thymus medulla. Importantly, the identification of mechanisms that drive aDC generation would enable the determination of physiological role of thymic aDCs, which is currently unknown.

In the intestine, the thymic T-cell selection is complemented by the process of oral tolerance, which ensures the selection of T-cells reactive to food and microbiota antigens and their conversion to Tregs. Through the review of previous studies, I have provided a mechanistic insight into the processes, which ensure acquisition of these antigens, their presentation and finally effector mechanisms that enforce oral tolerance. However, microbial antigens cannot be simply ignored, as their donors, intestinal microbes, might overgrow and perturb intestinal homeostasis. Thus, the disruption of the tightly regulated equilibrium between the tolerance to these antigens and homeostatic immune responses may result in severe intestinal pathology.

In a central study of this thesis, we have identified IL-17 mediated stimulation of PCs as one of the mechanisms used by the intestinal immune system to regulate intestinal microbiota and thus prevent intestinal pathology. We have shown that IL-17 preferentially stimulated the expression of a specific set of PC-produced enteric α-defensins, Defa20, Defa21, Defa22 being the most dominant. Previous findings that these antimicrobial peptides are mostly limited to the ileum (Castillo et al., 2019; Haber et al., 2017) suggest a specific microbiota regulatory mechanism in this region. This notion would fit with our observation that mice with PC specific deletion of IL-17 receptor show microbiota alteration as well as inflammation susceptibility specifically in the ileum. However, it is currently unclear if ileum-specific defensins bear a different antimicrobial function than the rest of defensins produced by PCs, different specificity for some microbes or just increase the overall microbicidal capacity of ileal PCs. Furthermore, it is noteworthy that a sub cohort of CD patients develop ileum restricted pathology, that is associated with the drop in the production of PCs defensins (Wehkamp et al., 2005). While we did provide an analysis of CD patients and shown that even in humans, IL-17 might contribute to intestinal homeostasis through the induction of PC antimicrobial functions, we analyzed only a small cohort of patients. Thus, we were unable to correlate IL-17 function with ileum-specific pathology. Since mutations in Th17-associated genes are often found in patients with CD (Jostins et al., 2012), future research should clarify, if IL-17 mediated stimulation of PC antimicrobial functions plays a role in the pathogenesis of ileal form of CD.

We have also shown that the susceptibility to ileum pathology is conveyed by the disruption of microbial community in this tissue. However, the only reproducible microbial change we were able to detect in mice with PC specific deletion of IL-17 receptor was the drop in microbial diversity and we did not observe overgrowth of any specific bacterial strains. Thus, it is unclear what change in the microbiota quality drives the intestinal pathology. To address this question, future research should move from general quantification of microbiota composition to the analysis of microbiota functionality. One

of the possible avenues of this research could be the analysis of functional genetics, such as scRNA-seq, of the intestinal microbiota (Kuchina et al., 2021). As the application of this approach on mammalian cells and tissues recently lead to the rapid development of many biological fields, we are yet to see wide-spread usage of this method in microbiota research. I believe that the combination of multiparametric analysis of intestinal immune system and epithelial compartment together with functional analysis of local microbiota might facilitate dramatic advances in the understanding of host-microbe interactions, which ensure their homeostatic co-habitation. However, although the application of this method in human patients might be the most straightforward way to take, I think that usage of mouse models, which allow a precise analysis of specific mechanisms used in the intestinal homeostasis will still be indispensable.

In summary, we have brought several new findings identifying new mechanisms of thymic T-cell selection and effector mechanisms used by the intestinal immune system to control intestinal microbiota. I have integrated our findings with previous studies, to deliver a bigger picture of how these processes collaborate to ensure intestinal homeostasis.

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