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Mechanisms of the tolerance and homeostasis of immune cells

Doctoral Thesis

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Prohlášení:

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

V Praze Oksana Tsyklauri

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Contents

List of Abbreviations

AIRE	Autoimmune regulator
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS I	Autoimmune polyglandular syndrome type I
ATP	Adenosine triphosphate
BACH2	Broad complex-tramtrack-bric a brac and Cap'n'collar homology 2
BBS	Bardet-Biedl Syndrome
BBSome	BBS protein complex
BCL	B-cell lymphoma
BMI	Body mass index
CAR	CXCL12- abundant reticular cell
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DEF6	Differentially expressed in FDCP 6 homolog (DEF6)
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EV	Extracellular vesicles
FDA	U.S. Food and Drug Administration
FOXP3	Forkhead box P3
GITR	Glucocorticoid-induced TNFR family related gene
GVHD	Graft-versus-host disease
Gzm	Granzyme
HFD	High-fat diet
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell costimulator
IFITM	Interferon-induced transmembrane protein
IL	Interleukin
IL-2-ic	Interleukin-2 immunocomplexes

IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
JAK	Janus kinase
KLR	Killer cell lectin-like receptor subfamily
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific Src-family tyrosine kinase
LE	Lupus erythematosus
LRBA	Lipopolysaccharide-responsive and beige-like anchor protein A
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MS	Multiple sclerosis
mTEC	Medullar thymic epithelial cell
MyD88	Myeloid differentiation primary response gene (88)
MZ	Marginal zone
NOD	Non-obese diabetes
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand
PEG	Polyethylene glycol
pMHC	Complex of a peptide and major histocompatibility complex molecules
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
Sirpα	Signal-regulatory protein alpha
SLE	Systemic lupus erythematosus
SP	Single-positive
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
TCR	T-cell receptor
TLR	Toll-like receptor
TNFR	Tumor-necrosis factor receptor
TNFRSF	Tumor-necrosis factor receptor superfamily
TRA	Tissue-restricted antigens
WNT	Wingless and Int-1 signaling pathway
Xcr1	X-C motif chemokine receptor 1

Abstract

The ability of the immune system to tolerate self-antigens while mounting appropriate responses to pathogens is indispensable for the survival of the organism. Despite years of research, many details of the mechanisms of self-tolerance are still not well understood.

The objective of this thesis is to extend our knowledge of the mechanisms of immune tolerance. The core of the PhD thesis consists of five publications related to two main research directions. The first one addresses the mechanisms of peripheral immune tolerance established by regulatory T cells (Tregs). We showed that Tregs increase the quorum of self-reactive CD8+ T cells required for the induction of autoimmunity. In addition, we identified a novel subset of antigen-stimulated $CD8^+$ T cells, which expand in the absence of Tregs. We called them super-effector T cells. We revealed that the administration of IL-2 phenocopies the absence of Tregs, i.e., it induces super-effector T cells, and enhances $CD8^+$ T cell response in autoimmunity and cancer. Our results provide strong evidence that the major suppressive mechanism of Tregs is limiting IL-2 availability for $CD8^+$ T cells. Furthermore, in a collaborative project, we have shown that MyD88 signaling in thymic epithelial cells contributes to the development of Tregs and thus to the establishment of tolerance.

The second project deals with Bardet-Biedl Syndrome (BBS) ciliopathy. Despite multiple indications that ciliary disorder might influence the hematopoietic compartment, immune system of patients with BBS has not been studied before. We observed that BBS patients have higher incidence of certain autoimmune diseases, and altered blood components. Moreover, we revealed that BBSome deficiency alters the B-cell development in mice. Interestingly, some of the hematopoietic system alterations were caused by the BBS-induced obesity whereas others were caused by intrinsic defects in the bone marrow stromal cells. Because the molecular functions of particular BBS proteins were not clear, we performed a meta-analysis of published clinical data of BBS patients. This revealed that the identity of the causative gene partially predicts the clinical outcome of the BBS disease.

Overall, we provide new insights into the mechanisms of the tolerance and homeostasis of immune cells in health and disease.

Abstract (Czech Version)

Schopnost imunitního systému tolerovat vlastní antigeny a současně vytvářet vhodné reakce na patogeny je nezbytná pro přežití organismu. Navzdory dlouhodobému výzkumu mnoho detailů mechanismů autotolerance není stále dobře pochopeno.

Cílem této práce je rozšířit naše znalosti o mechanismech imunitní. Jádro disertační práce tvoří pět publikací, které se týkají dvou hlavních výzkumných směrů. První se zabývá mechanismy periferní imunitní tolerance, které zprostředkovávají regulační T-lymfocyty. Ukázali jsme, že Tregy zvyšují kvorum autoreaktivních $CD8^+$ T-lymfocytů nutné pro indukci autoimunity. Kromě toho jsme identifikovali novou podskupinu antigen-stimulovaných $CD8^+$ T-lymfocytů, které expandují v nepřítomnosti Tregů. Nazvali jsme je super-efektorové T-lymfocyty. Zjistili jsme, že podávání IL-2 mimikuje nepřítomnost Tregů, tedy indukuje super-efektorové T-lymfocyty a zvyšuje imunitní odpověď $CD8^+$ T-lymfocytů v modelu autoimmunity a rakoviny. Naše výsledky ukazují, že hlavní supresní mechanismus Tregů omezuje dostupnost IL-2 pro $CD8^+$ T-lymfocyty. Kromě toho jsme v rámci spolupráce ukázali, že signalizace přes MyD88 v thymických buňkách je přispívá k vývoji Tregů a následnému pro nastolení imunitní tolerance.

Druhý směr se zabývá ciliopatií Bardet-Biedlova syndromu (BBS). I přes četné náznaky, že ciliární poruchy mohou ovlivnit hematopoetický kompartment, nebyl imunitní systém pacientů s BBS dosud podrobně studován. Pozorovali jsme, že pacienti s BBS mají vyšší výskyt určitých autoimunitních onemocnění a změněné některé krevní parametry. Navíc jsme odhalili, že nedostatek BBS4 mění vývoj B-lymfocytů u myší. Je zajímavé, že některé změny hematopoetického systému byly způsobeny obezitou vyvolanou BBS, zatímco jiné byli vyvolané defekty ve stromálních buňkách kostní dřeně. Protože detaily funkce konkrétních proteinů BBS nebyly známé, provedli jsme meta-analýzu publikovaných klinických dat pacientů s BBS, která odhalila, že identita kauzativního genu částečně předpovídá klinický výsledek onemocnění BBS.

Celkově naše práce přinesla nové poznatky o mechanismech tolerance a homeostázy imunitních buněk ve zdraví a nemoci.

Preface

During my graduate studies in the doctoral program 'Immunology' at the Faculty of Sciences, Charles University, I worked on my PhD project at the Laboratory of Adaptive Immunity at the Institute of Molecular Genetics of the Czech Academy of Sciences in Prague under the supervision of Dr. Ondřej Štěpánek. My research was focused on immune tolerance in health and disease.

My PhD thesis consists of two main projects. The first is dedicated to Treg-mediated suppression of $CD8^+$ T lymphocytes. We have shown that limiting IL-2 is the major mechanism of Treg-mediated tolerance of $CD8^+$ T cells in the context of autoimmunity and cancer. In addition, we have identified a novel population of IL-2-induced $CD8^+$ super-effector T cells that appear in the absence of Tregs.

My second project focuses on immune system homeostasis in a ciliopathy called Bardet-Biedl Syndrome (BBS). By analyzing clinical data from BBS patients and from mouse models, we have found a previously uncharacterized link between ciliopathy and a dysregulated immune system.

Finally, I have been involved in several projects that are closely related to my research topics. First, I contributed to the project devoted to genotype/phenotype correlation in BBS, where I co-created a database containing all reported BBS patients. Second, I participated in a collaborative project investigating the role of TLR signaling in mTECs in Treg development. Specifically, I developed a $CD4^+/CD8^+$ T cell-mediated diabetes model for functional characterization of Tregs from mice with MyD88-deficient mTECs. Last but not least, I contributed to a review article summarizing the phenotypic and functional heterogeneity of $CD8^+$ Tregs.

My dissertation includes the following chapters: introduction summarizing the current state of knowledge on the subject, aims of the research projects, brief description of the main methods, results and discussion of each project, conclusion, and reprints of manuscripts either published or submitted for publication.

1. Introduction

Physiological immune response protects the organism from invading pathogens and transformed cells. However, if aimed at self-antigens, immune response can lead to the development of autoimmune diseases. In this way, tolerance to self is one of the essential features of the physiology of the immune system. On the other hand, mechanisms of self-tolerance represent a major barrier to the induction of robust anti-tumor immune responses.

Both protective and self-reactive immune responses are mediated by the adaptive immune system, in particular T and B lymphocytes. The potency of T cells to cause autoimmunity is a result of enormous diversity of antigen recognizing T-cell receptors (TCRs), characterized by a variable level of self-reactivity. Furthermore, T lymphocytes play important roles in the self-tolerance establishment. There are two major branches of controlling self-reactive lymphocytes: (i) the clonal deletion, i.e., the elimination of highly self-reactive cells during T-cell development in thymus (so-called central tolerance), and (ii) the suppression of potentially self-reactive clones which escaped central tolerance in the periphery (peripheral tolerance). Regulatory T cells (Tregs), represent a distinct subset of T lymphocytes that play an important role in both branches of immune tolerance.

1.1 Loss of self-tolerance

Self-tolerance is the ability of the immune system to recognize what is 'self' and prevent an immune response against it. Disruption of immune tolerance can lead to autoimmune diseases. To date, more than 80 autoimmune diseases have been described [1]. Pathogenesis of autoimmune diseases is a complex process in which various genetic factors (such as polymorphisms of HLA and other genes associated with the immune response, selective X chromosome inactivation, microchimerism etc.) and environmental triggers (including tissue damage, infections, stress, smoking, diet, body mass index (BMI), gastrointestinal microbiome etc) contribute to the failed Treg-mediated suppression of self-reactive cells.

1.1.1 Environmental factors of self-tolerance

Despite years of research, the impact of extrinsic factors on the development of autoimmunity is still debated. Even when a link between a particular environmental factor and autoimmune disease is indicated, it is often unclear whether it is a trigger, a pathological mechanism, or a

consequence of autoimmune disease. Nevertheless, risk of several autoimmune diseases is strongly associated with several factors:

1.1.1.1 Tissue damage

One of the oldest hypotheses to explain the origin of autoimmunity is based on the idea that tissue damage, especially severe or repetitive, might be a trigger for an immune response to self. The concept, known as the hidden (or cryptic) antigens theory, states that tissue damage can lead to the release of antigens that are normally hidden from the immune system and can activate autoreactive immune cells [2]. The cryptic antigen hypothesis has its limitations. First, most hidden self-antigens would originate from immune-privileged tissues such as testes, eyes, and brain, however there are autoimmune diseases that affect unprivileged sites such as joints, heart, and intestine. Second, damaged autologous tissues administered to model animals are not capable of inducing autoimmune disease; an immunogenic adjuvant is always required [3, 4].

Nevertheless, there are examples of autoimmune diseases in which the contribution of damage-induced release of autoantigens has been demonstrated. Damage caused by UV irradiation in people prone to lupus erythematosus (LE) leads to the release of autoantigens such as dsDNA, that trigger the proliferation of self-reactive T and B cell clones [5, 6]. In more details, UV irradiation induces massive apoptosis of keratinocytes that cannot be efficiently eliminated by phagocytes. This is due to increasingly evident deregulation of apoptosis in LE patients [6-8]. Consequently, apoptotic cells undergo secondary necrosis and release nuclear antigens as well as proinflammatory compounds.

The release of damage-associated molecular patterns (DAMP) also appears to play an important role in the development of other autoimmune diseases. In particular, the role of DAMPs-induced events such as activation of TLRs and the inflammasome has been suggested in rheumatoid arthritis (RA), LE, Sjogren's syndrome, systemic sclerosis, and ankylosing spondylitis [9, 10].

1.1.1.2 Infections

The possible role of infections in the development of autoimmune diseases has long been debated. Several hypotheses have been proposed, including the adjuvant or bystander effect, excessive activation of the innate immune system, molecular mimicry, and antigenic

complementarity (reviewed in [2]). In particular, persistent infections, such as Epstein-Barr virus (EBV) and hepatitis C virus, are frequently associated with autoimmune diseases [11]. It appears that they not only act as adjuvants, but also directly affect the host immune system via infection of B cells. HCV has been reported to increase the number of depleted B cells and marginal zone-like B cells in the blood of chronically infected patients [12], induce the proliferation of autoreactive IgM^+ B cells [13], and induce the generation of effector $CD8^+$ T cells specific for apoptotic T cell-associated self-epitopes [14]. EBV employs a number of so-called immune evasion mechanisms, which include inhibition of apoptotic cell elimination [15], regulation of host MHC-I and MHC-II expression [16], and production of soluble mediators that interfere with host cytokines [17, 18]. These effects contribute to immune system dysregulation, potentially leading to autoimmunity.

Molecular mimicry hypothesis is based on the idea that similarities between self-antigen and foreign antigen favor activation of autoreactive T or B cells, which might result in autoimmunity [19]. The concept of molecular mimicry led to the antigenic complementarity hypothesis, which states that autoimmune diseases are caused by specific combinations of multiple microbial antigens, at least one of which mimics a self-antigen [20, 21]. Both these hypothesis are essentially based on structural similarities between self- and pathogen antigens, cross-reactivity between pathogens and self-antigens, and anti-idiotic activity of antibodies against some microorganisms, but unfortunately they lack a solid experimental evidence base. Although cross-reactivity between pathogens and self-antigens is relatively common [22], this is not strong evidence for mimicry as a pathogenic mechanism per se.

The best-known example of an autoimmune disease presumably driven by molecular mimicry is the streptococcal antigen M protein, whose α-helical coiled-coil structure is similar to intramyocellular proteins (such as myosin and tropomyosin), proposed to be responsible for rheumatic fever, particularly rheumatic heart disease in the second half of the 20th century [23]. This concept was challenged by the Chhatwal group, who observed that protein M type 3 and type 18 form a complex with human collagen type IV that triggers the formation of autoantibodies against collagen [24, 25]. In this case, the pathogenesis of rheumatic fever does not require molecular mimicry with streptococcal antigens.

Interestingly, there are studies that claim that infections have a protective effect against some autoimmune diseases. For example, experiments with mouse models of autoimmune diseases show a positive effect of bacterial/viral/helminthic infections [26-28]. In addition, widely discussed hygiene hypothesis is based on the observation that a lower number of infections in Western countries correlates with a higher incidence of certain autoimmune diseases and allergies [29, 30]. This hypothesis is strongly supported by epidemiological data. An interesting example is a study comparing the populations of Karelia (Russia) and Finland that demonstrated significantly lower allergy incidence in Karelian children, despite the fact that two groups belong to the same ethnic group and live in the same climate [31]. Moreover, while there are no differences in the occurrence of anti-islet β-cell autoantibodies [32] or the frequency of HLA DQ genotypes predisposing to T1D, the incidence of T1D is six times higher in the Finnish population than in the Karelian population [33]. It should be noted, however, that while a lower socioeconomic level is often associated with poorer sanitary conditions, the observed differences might be a result of different nutritional patterns [34-36].

1.1.1.3 Microbiome

Although the gut and other mucosal surfaces of the body are a source of microbial antigens, host-microbiome contact has little in common with infection because of the complex ecological associations formed between them. The link between the microbiome composition and autoimmune diseases is becoming increasingly evident, however, the mechanism behind the observed changes is often unclear [37, 38]. One of the proposed mechanisms is the induction of pTreg by microbial metabolites [39]. Data from SPF and germ-free mouse models help to elucidate the effects of the microbiome. In non-obese diabetic (NOD) mice, certain imbalances involving the microbiota can both reduce and accelerate the onset of T1D [40]. NOD mice, housed in SPF conditions have a higher incidence of diabetes than mice raised under conventional conditions [41]. In contrast, germ-free mice are protected from various autoimmune diseases such as experimental arthritis [42] and experimental autoimmune uveoretinitis [43], and do not develop food allergy [44]. It should be noted however that germ-free mice are known to have an underdeveloped immune system, so the results of the aforementioned studies should be interpreted with caution [45].

1.1.1.4 Vitamin D deficiency

Vitamin D has numerous immunosuppressive effects, including promotion of pTregs [46], inhibition of Th1 and Th17 cell differentiation [47, 48], suppression of B cell function [49], and reduction monocyte activation [50]. Furthermore, vitamin D may influence the gut microbiome (reviewed in [38]).

Vitamin D3 supplementation in mouse models of autoimmune disease improves symptoms and survival in the EAE model of MS [51-53], suppresses the incidence and/or severity of arthritis in CIA (model of RA) [54, 55], and reduces colon damage in TNBS-induced colitis (model for Crohn's disease) [56]. Data on the effect of vitamin D in humans are less conclusive. Several cross-sectional studies reported an association between vitamin D deficiency and a higher incidence of autoimmune thyroid disease (summarized in [57]), ankylosing spondylitis [58], and RA [59], whereas other studies contradict these associations [58, 60-62]. This discrepancy is partly due to limited number and heterogeneity of participants. A meta-analysis on the therapeutic effect of vitamin D in MS found no benefit on relapse recurrence or patient quality of life [63].

1.1.1.5 Obesity

White adipose tissue is not only involved in the energy storage, but also plays the role of an endocrine organ that releases proinflammatory cytokines and mediators, such as TNF-α, IL-6, and leptin [64]. Inadequate accumulation of white adipose tissue leads to obesity.

Obesity is often associated with a metabolic syndrome characterized by insulin resistance, high triglyceride and low high-density lipoprotein levels, hypertension, and low grade systemic inflammation [65]. Accordingly, obesity is a risk factor for autoinflammatory diseases in humans [66]. High BMI is associated with an increased risk of MS [67, 68], RA [69], and autoimmune thyroiditis [70].

Studies with mice fed a high-fat diet (HFD) aim to investigate the mechanism behind this association. Tregs are decreased in visceral adipose tissue of obese HFD mice [71], as well as in peripheral blood of obese humans [72], consistent with the role of leptin in inhibiting Treg proliferation [73]. Recent study also demonstrated fewer IgA^+ B cells and less secretory IgA in diet-induced obese mice [74]. It has been reported that obese HFD mice have a larger Th17 cell population, produce more IL-17 and are more susceptible to EAE and colitis than lean

littermates [75]. It should be noted that HFD alters the gut microbiota and thus indirectly affects the immune system, even in the absence of obesity [76, 77].

Usually, obesity is caused by excessive caloric intake relative to caloric expenditure, which may be a consequence of lifestyle (lack of physical activity, high-fat and high-sugar diet). It can also be a result of a genetic predisposition, as in the case of BBS (ciliopathy caused by a malfunction of the ciliary transport complex BBSome). Obesity in BBS is associated with leptin resistance and abnormal adipogenesis [78], but the molecular mechanism is not completely clear.

Obesity is one of the typical BBS symptoms seen in almost all patients with homozygous mutation s [79]. The extent of morbid obesity (BMI > 40) is significantly higher in BBS than in the general population. In addition, BBS patients have higher leptin and triglyceride levels and abdominal visceral fat mass compared to BMI-matched controls [78, 79]. Moreover, since BBSome regulates leptin receptor trafficking [28], BBS might affect immune cells, the vast majority of which are leptin-responsive [80]. Altogether, there are some indications that BBS patients might have higher risk of autoimmune disorders. However, the immune system in BBS or any other ciliopathy has not been studied.

In summary, a variety of environmental factors may be associated with the development of autoimmune diseases. However, the available data are often controversial. Further research is needed to better understand the potential contribution of external factors to the breakdown of immune tolerance.

1.1.2 Genetic factors of self-tolerance

The development of autoimmune diseases cannot be explained by a single genetic factor, considering that concordance rates in identical twins are below 50% for most autoimmune diseases [81]. However, relatively high concordance has been found in HLA-identical siblings [82]. HLA, or human leukocyte antigen, is a group of genes localized in the major histocompatibility complex (MHC) region. The function of HLA molecules is the antigen presentation, so they are involved in several steps of immune cell development and immune response. The risk for certain autoimmune diseases associated with HLA haplotypes is likely due to specific amino acid sequence variations within the antigen binding grooves, resulting in altered repertoire of presented peptides [83]. To date, specific HLA variants have been

described to significantly influence the risk for various autoimmune diseases. The risk amino acid positions in HLA have been described for RA, systemic lupus erythematosus (SLE), Sjogren's syndrome, ankylosing spondylitis, psoriasis, T1D, Graves' disease, IBD, multiple sclerosis, and other autoimmune diseases [84].

At the same time, there are genes whose mutations are associated with a systematic loss of tolerance leading to excessive inflammation and life-threatening multi-organ autoimmunity. The most important genes whose mutations can lead to the loss of immune tolerance are *AIRE*, *FOXP3*, *CTLA4* and *FAS* (reviewed in [85]). The first of them is ensuring the central tolerance, while the other three are required for the proper functioning of peripheral immune tolerance, particularly Treg function.

1.1.2.1 Tregs and Tregopathy

Canonical T regulatory cells are $CD4^+$ T lymphocytes characterized by expression of the transcription factor FOXP3 and various surface markers such as CD25 (α-chain of IL-2R) and CTLA-4. FOXP3 is essential for the development and stability of the phenotype of Tregs. Together with other transcription factors, it shapes a unique gene expression signature by activating anti-inflammatory genes and repressing proinflammatory genes [86]. *FOXP3* loss of function mutations results in Treg deficiency, and leads the IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans, or scurfy phenotype in mice, which are analogous in their nature [87, 88]. IPEX patients and scurfy mice suffer from excessive lymphocyte activation, lymphocytic infiltration into peripheral organs and lethal autoimmunity [88, 89].

IPEX syndrome is classified as an example of tregopathies – autoimmune diseases caused by monogenic mutations that lead to defects in Treg development, survival, and/or function. Tregopathies share many common symptoms. Heterozygous germline mutations of *CTLA4* result in an IPEX-like disease that manifests as lymphocytic organ infiltration, increased numbers of effector T cells, and multiple types of autoimmunity [90-92]. Loss of function mutations of genes *LBRA* and *DEF6* phenocopies *CTLA4* insufficiency, since LBRA and DEF6 proteins are required for CTLA-4 mobilization on the cell surface [93-95].

Mutations affecting the α-chain of IL-2R also cause an IPEX-like disease, with autoimmunity and lymphoproliferation, but also severe immunodeficiency [96]. STAT5b defects overlap with IL-2Rα deficiency, as STAT5 associates with the β-chain of IL-2R [97, 98].

Although the immunosuppressive properties of $CD4^+$ $FOXP3^+$ Tregs were initially controversial [99], they are now widely recognized. In addition, their successful use in clinical trials (reviewed in [100-102]) is inspiring researchers to investigate tolerogenic effects of other immune cell types, including proposed regulatory $CD8^+$ T cells [103, 104], regulatory B cells [105-107], and tolerogenic DC [108-110].

Regulatory $CD8^+$ T cells, or $CD8^+$ Tregs, were initially described even earlier than $CD4^+$ $FOXP3^+$ Tregs, however, their properties and even existence are still controversial [103, 111]. This may be due to the lack of solid *in vivo* experiments with $CD8^+$ Tregs. Moreover, it seems that the phenotypes of the proposed $CD8^+$ Tregs are different. A more detailed study of regulatory $CD8^+$ T lymphocytes could contribute to a better understanding of the suppressive properties of the immune system.

1.2 Tregs as one of the key mechanisms of self-tolerance

Most $CD4^+$ $FOXP3^+$ Tregs develop in the thymus (tTregs), however there are also Tregs that develop peripherally (pTregs) from conventional ($FOXP3^-$) $CD4^+$ T cells. The overall role of tTregs and pTregs remains controversial despite years of research. It is generally believed that tTregs are mainly involved in tolerance to autoantigens, whereas pTregs prevent overt inflammation in the presence of exogenous antigens.

1.2.1 Modern view on Treg development in thymus

Tregs, as well as other T lymphocytes, are derived from hematopoietic stem cells that migrate from the bone marrow to the thymus where they undergo further developmental stages. One of the most important steps of the T-cell development in the thymus is the establishment of a mature T-cell repertoire with diverse TCRs that recognize peptide antigens presented by APCs. During this process, $CD4^+$ and $CD8^+$ T cells with the potential to recognize peptides displayed by MHC class II and MHC-I molecules, respectively, are selected. This step, called positive selection, creates an incentive for the formation of a pool of self-reactive cells that, if released from the thymus, can cause autoimmunity. To prevent this, developing T cells go thought another round of selection (negative selection), in which the cells that exhibit overt reactivity

to self-pMHC ligands are eliminated by clonal deletion [112]. It is well recognized now that the process of negative selection is not absolute, and some self-reactive clones escape the deletion [113, 114]. A notable example is Tregs, which appear to arise from T-cell clones with increased TCR affinity for self-peptide MHC-II complexes. Diversification of self-reactive thymocytes into Tregs is thought to be a mechanism of central tolerance in addition to clonal deletion.

The hypothesis that Tregs are self-reactive was born in early 2000s, even before FOXP3 was discovered to be a Treg marker. The Caton research group showed that interactions with a self-peptide can induce thymocytes that bear an autoreactive TCR to undergo selection to become $CD4^+$ $CD25^+$ regulatory T cells in TCR transgenic mice [115]. Later, this observation was confirmed by several studies which emphasized the role of strong TCR signaling in Treg cell fate [116-119]. Nevertheless, the field stayed controversial, and while some studies claimed that the Treg TCR repertoire is distinct and more self-reactive than its conventional counterpart [120, 121], the others did not observe this trend [122, 123]. A potential source of this discrepancies is Treg heterogeneity. It seems rational that TCR repertoire of tTregs must be biased toward self-recognition, while pTregs might express TCRs similar to those found on $CD4^+$ T helpers, as they develop originally from the same T-cell pool. Indeed, it has been shown that colonic Tregs utilize different TCRs from those used by Treg cells in other locations, indicating the process of peripheral Treg development driven by colonic microbiota [124].

Collectively, current evidence suggests that the strength of TCR signaling acts as an instructive cue for thymic Treg cell development [125-127], yet particular TCR specificity alone is not sufficient to induce Treg cell differentiation [128]. According to the so-called two-step model, strong TCR signaling is just the first step in this process, leading to the upregulation of IL-2Rα expression and providing the advantage of high-affinity IL-2 binding (second step), which in turn promotes the upregulation of FOXP3, the master regulator of Tregs [121, 128, 129].

According to the two-step model, thymic $CD25^+$ $FOXP3^-$ $CD4^+$ SP cells are Treg-precursors. Indeed, adoptively transferred thymic $CD25^+$ $FOXP3^-$ $CD4^+$ SP cells developed into mature $CD25^+$ $FOXP3^+$ Tregs in MHC class II-deficient mice [121]. Along this line, it has been shown that thymocytes unable to express IL-2Rα were less efficient, but still able, to develop $FOXP3^+$

Treg cells, in contrast to IL-2Rγ$^{-/-}$ [130] and IL-2Rβ$^{-/-}$ mice [131] which had a major block in Treg development. These observations, on the one hand, supported the key role of IL-2 signaling in Treg development, but on the other hand indicated that IL-15 (that also utilizes IL-2Rβ) might be involved in the process. Recent studies support this possibility [131-134]. Nevertheless, the role of IL-15 seems to be limited to induction of Treg development in IL-2 absence [135]. Moreover, IL-2 is required not only for the development, but also for proliferation, survival and suppressive function of Tregs [136, 137]. Given the fact that FOXP3 blocks *Il2* gene transcription [138], Tregs depend solely on extrinsic sources of IL-2. Interestingly, FOXP3 has been proposed as a pro-apoptotic signal, which must be counterbalanced by survival signals mediated by γc family cytokines [139, 140].

The CD28-CD80/86 costimulatory axis was another addition to the classical two-step model, as it appeared to be essential for the formation of Treg precursors [141] and the induction of FOXP3 expression [142]. Moreover, TCR/CD28 stimulation drives the upregulation of TNFRSF members such as GITR, OX40, and TNFR2, promoting Treg fate in thymocytes [143].

Although the two-step model remains the predominant paradigm for Treg development, it should be noted that it is rather simplistic and does not describe all possible pathways of Treg generation. Recent studies have proposed an alternative Treg progenitor population, namely thymic IL -15 dependent CD25^{-} FOXP3$^{+/low}$ cells [144, 145]. Similar to CD25^{+} Treg precursors, a substantial proportion of CD25^{-}FOXP3$^{+/low}$ cells differentiates into mature Tregs upon adoptive transfer in mice. Importantly, single-cell transcriptomic analysis confirm the presence of both CD25^{+} FOXP3^{-} and CD25^{-} FOXP3$^{+/low}$ Treg precursors in the human thymus [146]. Taken together, these observations suggest that both types of Treg precursors contribute to the formation of the mature Treg pool.

1.2.2 Role of thymic APC in tolerance establishment

Thymic T-cell development is coordinated by diverse stromal populations, consisting of epithelial cells, fibroblasts, endothelial cells, as well as cells of hematopoietic origin such as DC and B cells. Thymic epithelial cells, further divided into cortical (cTEC) and medullary (mTEC) cells, account for the majority of thymic stromal components and play the most important role in thymocyte development by driving both positive and negative selection.

mTECs express tissue-restricted antigens (TRAs) [147] under the control of transcription factors AIRE (in addition, the role of FEZF2 has been proposed) [148, 149]. TRAs serve as a projection of the self-antigen repertoire at the local site of negative selection – the thymus – allowing deletion of self-reactive T cells before they reach the periphery. Humans expressing a defective form of AIRE have impaired clonal deletion and consequently develop the severe multi-organ autoimmune disease APECED, also known as APS I [150]. Several rodent models of APECED have been established, but they do not fully recapitulate the phenotype of human patients and do not develop lethal autoimmunity (reviewed in [151]). Nevertheless, *Aire*-deficient mice do have impaired negative selection of developing self-reactive thymocytes and show age-dependent lymphocytic infiltration of organs [152, 153]. Disruption of *Aire* in rats may better recapitulate the clinical features of APECED patients, but this has only been investigated in a limited number of studies [151, 154].

The role of AIRE in establishing self-tolerance relies not only on clonal deletion but also on the generation of Tregs. There are several lines of evidence for this. First, mice and humans with mutated AIRE have decreased frequency of Treg precursors in the thymus [155, 156]. Second, Tregs isolated from APECED patients show decreased suppressive capacity *in vitro* [156]. Third, expression of *Aire* has been reported to promote the formation of a specific subset of Tregs that develops early in life and has a specific transcriptome and activation profile [157]. In addition, AIRE has been shown to influence the TCR repertoire of Tregs. TCR sequencing of mouse $Aire^{-/-}$ convential $CD4^+$ T cells revealed the presence of self-reactive TCRs typically associated with Tregs [158]. A similar phenomenon was described in AIRE-deficient humans, who had conventional $CD4^+$ T cells with self-reactive TCRs normally found in the Treg compartment [159]. Overall, the available data suggest that the autoimmunity associated with AIRE deficiency is due to two failed tolerance mechanisms: the first is clonal deletion, and the second is the development of Tregs.

Recently, a distinct type of mTECs has been identified by single-cell mapping of the thymic stroma. So-called tuft cells or mTEC IV are remarkably similar to intestinal tuft cells expressing broad diversity of taste receptors that are thought to influence cell polarization and chemotaxis [160, 161]. Thymic tuft cells have also been found to produce high levels of IL-25 [160, 161].

Other thymic APCs (mainly DC) also appear to play an important role in T cell development, but the details of their function are controversial despite years of research. Thymic DCs present TRAs to developing thymocytes same as mTECs, although they do not express *AIRE*. This phenomenon was clarified when several studies described antigen transfer from *AIRE*-expressing mTECs to DCs (so-called indirect antigen presentation or cross-presentation) [162, 163]. However, it appears that the role of DCs in establishing tolerance is not limited to cross-presentation. A study by Perry et al. proposed that the role of mTECs and DCs in Treg cell differentiation is redundant due to cross-presentation only for some antigens, whereas it is not redundant for the other antigens [164]. In addition, migratory DC subsets, in particular SIRPα^+ DC and plasmacytoid DC, might contribute by transporting peripheral antigens to the thymus and presenting them to developing T cells [165, 166]. Some studies also suggest that DCs provide IL-2 to thymic Tregs [167]. Altogether, our understanding of overall role of thymic DC compared with mTECs is incomplete. Further research is needed to fill the gaps in our knowledge regarding their contribution to T cell development, particularly Treg development.

1.2.3 Treg development in the periphery

Besides their development in the thymus, regulatory $CD4^+$ $FOXP3^+$ Tregs can also be induced peripherally. To date, most researchers agree that pTregs build tolerance to non-self antigens, including those derived from the microbiota of mucosal surfaces [168] and fetal antigens during pregnancy [169]. As mentioned previously, the TCR repertoire of Tregs in the colon differs from other locations [124] and is strongly influenced by the composition of the microbiota [170], which seems to be a result of peripheral transformation of conventional $CD4^+$ T cells into Tregs. A recent study confirmed that specific commensal epitopes reprogram naïve $CD4^+$ T cells into the Treg lineage [171]. In sharp contrast, experiments with germ-free mice found that pTregs can develop in the colon even in the absence of microbiota. Moreover, they show no visible phenotypic or functional defects [172, 173]. At the same time, other studies claim that immune tolerance to the microbiota is established by tTregs [170], calling into question the overall role of pTregs. It has been suggested that special populations of tTregs are required for the formation of pTregs, which may explains this controversy [174].

1.3 Mechanisms of Treg-mediated suppression

Tregs have the extraordinary ability to suppress immune responses of both the innate and adaptive immune systems. Tregs have been reported to affect various cell types, including T lymphocytes ($CD4^+$, $CD8^+$, and γδ T cells [175, 176]), B lymphocytes [177, 178], NK cells [179, 180], NKT cells [181], DCs [182], macrophages [183-185], and neutrophils [186].

A variety of Treg-mediated suppressive mechanisms have been proposed in the literature, which can be classified into several categories, such as (a) contact-dependent modulation of target cell phenotype, (b) secretion of immunosuppressive cytokines, (c) release of extracellular vesicles, (d) IL-2 deprivation, (e) metabolic perturbations. The overall contribution of these mechanisms to immune tolerance is under debates.

Although it seems plausible that Tregs use specific suppression mechanisms to inhibit a particular cell type, most of our knowledge of the mechanism of Treg action comes from studies characterizing Treg suppression of $CD4^+$ T lymphocytes. The mechanisms for suppressing the immune response of $CD8^+$ T cells are less studied, despite the fact that effector $CD8^+$ T cells play a critical role in the initiation and progression of autoimmune diseases [187]. In particular, $CD8^+$ T cells are thought to contribute to the pathogenesis of T1D [188], MS [189], autoimmune arthritis [190], vitiligo [191], and severe aplastic anemia [192]. In addition, a better understanding of Treg-mediated suppression of $CD8^+$ T cell responses against tumors may open new approaches for cancer immunotherapy [193].

1.3.1 Contact-dependent suppression

Tregs express numerous cell membrane receptors that mediate contact-dependent inhibition of immune responses. Immune checkpoint regulators such as CTLA-4, PD-1, and LAG3 are the best characterized inhibitory receptors of Tregs. Using CTLA-4, Tregs prevent costimulation of conventional $CD28^+$ T cells by CD80/CD86 ligands expressed on DCs [194]. Moreover, the interaction of CTLA-4 with CD80/CD86 leads to their trans-endocytosis and lysosomal degradation, preventing DCs from promoting effector T-cell activation and function [195]. Low surface expression of CTLA-4 ultimately leads to Treg dysfunction and prolonged T cell activation and migration, causing lymphocytic infiltration of multiple organs and a breakdown in peripheral immune tolerance [90]. Mice lacking *Ctla-4* suffer from early-onset multi-organ autoimmunity, whereas heterozygous mice show no visible immune system defects [196].

PD-1 is a receptor that belongs to the immunoglobulin superfamily. It is expressed on activated lymphocytes and monocytes [197]. PD-1 ligands (PD-L1 and PD-L2) are members of the B7 co-stimulatory molecule family and are expressed on APCs, endothelial and epithelial cells and also on activated lymphocytes [198]. PD-1–PD-L interactions regulate T and B cells at multiple checkpoints, inhibiting co-stimulation mediated effects such as cell activation, expansion, and cytokine secretion [199]. Mice deficient in PD-1 exhibit spontaneous late-onset lupus-like autoimmunity [197], and the loss of PD-1 expression on Tregs has been associated with Treg phenotype instability and function [198]. In contrast, it has been shown recently that monoclonal antibody blocking PD-1 enhances immunosuppressive function of PD-1^+ Treg via activation of the TCR and CD28 signals, thus PD-1^+ Treg cells in the tumor microenvironment are involved in the resistance to PD-1 blockade therapies [200]. In addition, another recent study utilizing mice selectively lacking PD-1 in Tregs demonstrated enhanced immunosuppressive function of PD-1 deficient Tregs [201]. Therefore, the role of PD-1 in Tregs remains to be elucidated.

LAG-3 is a Treg cell surface protein, a homolog of the CD4 protein, which can bind MHC-II on APCs with high affinity. Binding of LAG-3 to MHC blocks DC ability to activate $CD4^+$ T cells [202]. It also inhibits the maturation of DC [203]. Indeed, *in vitro* suppression of conventional $CD4^+$ T cells by Tregs was inhibited by the LAG-3 antibody [204]. In contrast, another study showed that LAG-3 deficient Tregs were able to suppress the proliferation of $CD4^+$ T cells *in vivo* [205].

Another proposed contact-dependent mechanism of immunosuppression is FASL-induced triggering of apoptosis of FAS-expressing DC [206] and $CD8^+$ effector T cells [207]. Human *FAS* gene defects leading to impaired apoptosis cause autoimmune lymphoproliferative syndrome (ALPS) [208]. It should be noted, that mutations of *FAS* are the most common but not the only cause of ALPS – the disorder can also be caused by mutations in *FASLG*, *FADD*, *CASP8,* and *CASP10* [209]. The disease is characterized by chronic lymphoproliferation, increased risk of lymphoma, and autoimmunity, particularly autoimmune cytopenias. FAS-induced apoptosis has been previously described as one of the mechanisms of Treg-mediated suppression. Some studies have suggested that Treg dysfunction may contribute to the pathogenesis of ALPS [210]. However, another study has shown that Tregs isolated from FAS-

ALPS donors have normal suppressive capacity [211]. Moreover, conventional T cells from *FAS*-mutated patients could be effectively suppressed by Tregs, suggesting that ALPS is not a result of Tregs dysfunction [211].

Recently, Akkaya et al. proposed a novel contact-dependent mechanism of Treg-mediated suppression, which targets antigen presentation by DC [212]. Using advanced microscopy techniques, they demonstrated strong binding of a Treg's antigen receptor to cognate pMHC-II expressed on DC, resulting in its removal from the DC surface. As a result, DC loses the ability to present only this specific pMHC-II antigen to $CD4^+$ T cell [212]. However, this mechanism cannot explain Treg-mediated suppression of $CD8^+$ T cell, which are MHC-I restricted.

1.3.2 Release of immunomodulatory agents

The release of immunosuppressive cytokines, particularly IL-10, TGF-β, and IL-35, is another proposed mechanism of Treg-mediated suppression [213]. In studies from the late 20th century, IL-10 was found to affect the phenotype of DC by decreasing their production of IL-12, IFN-γ or TNF-α [214], and downregulating the expression of B7 family molecules [215]. Secretion of IL -10 by Treg cells appears to be particularly important at mucosal surfaces, such as the intestine and airways [216]. Mice lacking IL-10 or IL-10R in Tregs are highly susceptible to colitis [217, 218]. Similarly, mutations in IL-10 and/or IL-10R are associated with Crohn's disease and ulcerative colitis in humans [219]. TGF-β belongs to transforming growth factor superfamily and plays multiple roles in immune system. It has been shown to modulate the immune response during colitis [220], cancer [221-223], allergy [224], and experimental autoimmunity [225], acting mainly as an immunosuppressant. The immunosuppressive effect is partly due to the ability of TGF-β to block cell cycle progression through the G1 phase, thus inhibiting cell proliferation [226]. It also regulates cell polarity, cytoskeleton [227], and apoptosis [228]. Interestingly, TGF-β promotes generation of pTregs from conventional $CD4^+$ T cells [229], but at the same time, it induces formation of potent effectors, T helper 17 (Th17) [230].

IL-35 is an anti-inflammatory cytokine mainly produced by Tregs and its expression is induced by proinflammatory stimuli [231]. After binding to its receptor (IL-35R), it activates the JAK-STAT signaling pathway [232]. The therapeutic effect of IL-35 has been demonstrated in

mouse models of IBD and psoriasis [233], EAE [234], CIA [235], and acute graft-versus-host disease [236]. The immunosuppressive effect of IL-35 is mainly achieved by promoting Tregs differentiation from naïve $CD4^+$T lymphocytes and M2 macrophage polarization [235-239].

Secretion of extracellular vesicles (EVs) by Tregs is a relatively recent observation. EVs are cell-derived membranous structures that may contain various proteins, lipids, and small RNAs that are transferred from one cell to another [240]. EVs of Treg origin exhibit immunological modulatory abilities *in vitro*, such as suppression of $CD4^+$ T cell proliferation, reduction of IFNγ and IL-2 levels, and increase of IL-10 secretion by DCs [241]. VS also ameliorated the development and severity of arthritis in mouse model [242]. Interestingly, IL-35-expressing EVs have been described, that act by coating of bystander lymphocytes with IL-35, thus promoting their exhaustion [243, 244].

1.3.3 Metabolic perturbations

Tregs can affect the metabolism of target cells through the action of CD39 and CD73 [245]. These are surface ectonucleotidases that mediate the conversion of extracellular ATP (which can promote inflammation) into adenosine [246]. Adenosine receptors (A1, A2a, A2b, and A3) are expressed on various immune and non-immune cells and promote immunosuppression. In particular, binding of A2aR on effector T cells has been shown to suppress TCR signaling and inhibit proliferation and cytokine secretion [247].

IL-2 deprivation is another mechanism of metabolic perturbations, which will be discussed in the next chapter.

1.3.4. IL-2 deprivation

IL -2 deprivation, or competition for IL-2 is one of the mechanisms of Treg-mediated immunosuppression that has been repeatedly described in the literature. IL-2 promotes the survival and proliferation of T cells [248], including Tregs. Moreover, Tregs require IL-2 for their proper functioning [249, 250]. At the same time, unlike conventional T cells, Tregs do not express IL-2, which makes them dependent on external sources of this cytokine [249]. Tregs express high affinity IL-2R, which consists of the α (CD25), β (CD122), and γ (CD132) subunits, whereas naive conventional T lymphocytes express dimeric IL-2R, which lacks the α-chain. In this way, Tregs have an advantage over naive T cells in accessing IL-2, and by consuming it, they can limit the immune response. However, IL-2Rα expression on T cells can

be induced by antigen stimulation or by IL-2 itself [251]; thus, IL-2 signaling may be self-enhancing. Based on the above, IL-2 competition must be naturally limited to an early time window before the autocrine IL-2 loop causes IL-2Rα expression.

Mathematical modeling confirms that IL-2 limiting mechanism of suppression is time and distance dependent [252]. In addition, it predicts that Tregs can limit IL-2 only for moderately stimulated but not strongly stimulated T helpers, because of rapid and abundant IL-2 secretion in the latter case [252].

Interestingly, IL-2Rα-deficient mice have elevated serum levels of IL-2 that can be dramatically reduced by adoptive transfer of $FOXP3^+$ $CD25^+$ Tregs [253], emphasizing the important role of IL-2 consumption by Tregs in immune homeostasis.

Despite a strong theoretical base, the IL-2 deprivation hypothesis still lacks sufficient experimental evidence. Early studies from 2004 reported that the addition of IL-2 abrogated $CD4^+$ $CD25^+$ T cell-mediated suppression of conventional T cell proliferation *in vitro* [137, 254], suggesting a the role for IL-2R signaling in Treg-mediated suppression. However, it should be noted that these studies were performed before specific Treg markers were established and only CD25 was used as a Treg marker. A 2007 study, also based on *in vitro* assays, suggested that Treg-mediated IL-2 deprivation triggers apoptosis of conventional $CD4^+$ T cells [255]. However, IL-2Rα deficient Tregs turn out to be fully competent as *in vitro* suppressors of $CD4^+$ T cell activation, suggesting that CD25-mediated IL-2 signaling plays little or no role in the suppressive activity of Tregs [256]. This conclusion was supported by experiments with mouse conventional $CD4^+$ T cells and human Tregs in co-culture [257]. Interestingly, a recent study, which used Treg-specific IL-2Rα deficient mouse model, claimed that IL-2 is dispensable for the control of $CD4^+$ T cells, but is important for the suppression of the immune response of $CD8^+$ T cells [249]. Overall, there is still a gap in our knowledge regarding the role of IL-2 consumption in Treg-mediated suppression.

2. Aims of the study

The study is focused on the mechanisms of immune tolerance. Our aim was to extend the existing knowledge on the role of Tregs in the establishment of immune tolerance. In addition, we aimed to investigate the factors for the break of tolerance in BBS ciliopathy.

In the first part of this work, we studied the immune system of BBS patients and *Bbs4*-deficient mice (mouse model of BBS). Ciliary protein dysfunction could affect the hematopoietic compartment, including the immune system [258], and possibly disrupt the immune response and self-tolerance. As an initial step, we analyzed clinical data from BBS patients to identify potential immune disorders. Next, we obtained a *Bbs4*-deficient mouse model of BBS. We analyzed the immune cells of BBS mice in steady state. We also tested the functional capabilities of *Bbs4*-deficient lymphocytes in various *in vitro* and *in vivo* assays.

We wanted to elucidate the mechanism by which BBS deficiency affects the hematopoietic system. We hypothesized that *Bbs4* deficiency might affect ciliated stromal cells and cause dysregulation of the stromal cell niche in the bone marrow. To test this hypothesis, we measured the expression of several key cytokines required for B-cell development in the bone marrow. In a final step, we generated embryonic fibroblasts from *Bbs4*-deficient and tested whether the expression of cytokines depends on BBS4.

In addition, BBS is often associated with obesity, which might contribute to the break in tolerance [259]. To investigate how obesity affects the immune cells of *Bbs4*-deficient mice, we analyzed the immune organs of lean and obese *Bbs4*-deficient mice. We also measured leptin levels in the blood serum of obese and lean *Bbs4*-null mice.

In the second part of the study, we were particularly interested in the mechanisms of Treg-mediated suppression of $CD8^+$ T lymphocytes. We used a mouse model of $CD8^+$ T cell-driven experimental diabetes to test the hypothesis that Tregs prevent autoimmunity caused by self-reactive $CD8^+$ T cells.

It has been proposed that Tregs inhibit the $CD8^+$ T-cell response to low affinity but not high affinity antigens [260]. To test whether Tregs preferentially suppress high- or low- affinity $CD8^+$ T-cell responses, we used variable affinity antigens to induce experimental diabetes in Treg-depleted and Treg-replete mice.

Next, we examined the immune system of Treg-depleted mice in the course of experimental diabetes, with particular attention to the phenotype of self-reactive $CD8^+$ T cells activated in the presence or absence of Tregs.

We also investigated the interplay between Tregs, self-reactive $CD8^+$ T cells, and conventional $FOXP3^-$ $CD4^+$ T cells by reconstituting the T cell compartment in $CD3\varepsilon^{-/-}$ RIP.OVA mice. We analyzed how the presence or absence of conventional and regulatory T cells affected the onset of experimental diabetes. Next, we examined the phenotype of self-reactive $CD8^+$ T cells activated in the absence of conventional $CD4^+$ T cells and/or Tregs.

Several studies have proposed IL-2 limiting as a mechanism of lymphocyte suppression [130, 249, 254, 255], but experimental evidence is limited and results are controversial. First, we performed an *in vitro* suppression assay with the addition of recombinant IL-2 to test if excessive IL-2 interferes with Treg-mediated suppression. Next, we tested whether IL-2 signaling is upregulated in activated $CD8^+$ T cells in Treg-depleted mice by pSTAT5 staining. To test whether IL-2 abrogates Treg-mediated suppression *in vivo*, we used IL-2 immunocomplexes in an experimental diabetes assay. Finally, we tested whether IL-2 immunocomplexes interfere with Treg-mediated suppression of anti-tumor responses in leukemia and melanoma mouse models.

2.1 Specific aims of the study

1. Clarify the genotype-phenotype association in Bardet-Biedl Syndrome.

2. Characterize the effects of BBSome deficiency on immune cell development and homeostasis.

3. Investigate the effects of ciliopathy-associated obesity on the hematopoietic system and self-tolerance.

4. Investigate the ability of Tregs to suppress high- and low- affinity self-reactive $CD8^+$ T cells.

5. Reveal the mechanism of suppression of $CD8^+$ T cells by Tregs.

6. Investigate the contribution of thymic DCs to Treg development.

7. Clarify the heterogeneity and suppressive properties of the proposed $CD8^+$ Tregs.

3. Materials and methods

The prevalent models in the whole study were murine models. We used next murine strains: DEREG [261], RIP.OVA [262], CD3ε$^{-/-}$ [263], Ly5.1 and OT-I RAG2$^{-/-}$ [264]. Mouse *Bbs4*$^{tm1a(EUCOMM)Hmgu}$ allele with a gene trap cassette was obtained from KOMP (UC Davis, CA, USA) as frozen sperm and used for *in vitro* fertilization. Males and females aged from 6 to 25 weeks were used for the experiments. If possible, age and sex-matched pairs of animals were used in the experimental groups. All animals were bred in the animal facility of Institute of Molecular Genetics in accordance with laws of the Czech Republic. All animals were kept in individually ventilated cages under specific pathogen free condition. Animal protocols were approved by the Czech Academy of Sciences, Czech Republic.

Data were collected using various experimental protocols. The key approach of the study was execution of *in vivo* or *ex vivo* experiments, followed by survival monitoring, Western blot, flow cytometry, RT-qPCR or scRNAseq analysis.

Predominant *in vivo* assay which was used in this project is experimental diabetes. Briefly, OT-I cells were adoptively transferred into a RIP.OVA host. On the following day, the host mice were immunized. Three approaches of immunization were applied in different experiments: intravenous injection of 5000 CFU of OVA expressing *Listeria monocytogenes*, intravenous injection of DC loaded with OVA peptide, or intraperitoneal injection of OVA peptide in combination with LPS. Level of glucose in the urine of RIP.OVA mice was monitored on a daily basis. On day 7 post-infection, blood glucose was measured.

Other *in vivo* assays used in the study include treatment of mice with IL-2 immunocomplexes (IL-2/JES-6 and IL-2/S4B6), CD8$^+$ T-cell mediated killing assay, *Listeria monocytogenes* infection, and generation of bone-marrow chimeric mice.

In vitro and *ex vivo* assays performed in the study include generation of bone-marrow-derived DCs, Treg suppression of the proliferation of anti-CD3ε-antibody stimulated CD8$^+$ T cell, APC-T-cell conjugation assay, B-cell activation with 4-hydroxy-3-nitrophenylacetic acid succinimide ester (NP-Osu)-loaded T2-Kb cells, or with F(ab')2-goat anti-mouse IgM antibody.

Detailed methods are described in the corresponding publications.

4. Results and discussion

4.1 Phenotype of BBS patients varies depending on causative gene mutation.

Bardet-Biedl Syndrome (BBS) is a pleiotropic genetic disease caused by dysfunction of the primary cilia, the main symptoms of which include rod-cone dystrophy, polydactyly, obesity, learning difficulties, hypogonadism, and renal abnormalities. BBS can be caused by loss-of-function mutations in any of the 24 BBS genes known to date. Eight of the BBS genes encode subunits of a protein complex called the BBSome (BBS1, 2, 4, 5, 7, 8, 9, 18), other genes encode chaperonins (BBS6, 10, 12) assisting the BBSome assembly, or a GTPase (BBS3) assisting BBSome function. The biology and function of each BBS protein is not yet fully understood. BBS is characterized by substantial phenotypic variability between patients. We hypothesized that individual BBS proteins may have different functions, resulting in different symptoms in patients. However, BBS is a rare disease, making it challenging to study genotype-phenotype correlation in human patients. To overcome this obstacle, we adopted a meta-analytic approach (Ad. 1).

We collected all records of BBS patients for whom the genotype (homozygous mutation in a specific BBS gene) and the phenotype (presence or absence of specific BBS symptoms) were reported, and created the largest database of BBS cases published to date (the database was created in 2018). This database contains clinical records of 916 BBS patients, extracted from 85 original studies.

Using this database, we revealed partial correlation between the patient's genotype and the severity of the disease. We demonstrated that mutations in *BBS3* (encoding the BBSome-associated GTPase) typically result in milder disease compared with mutations in other BBS genes. A recent study of twelve BBS3 patients from eight families originating from La Réunion Island reported a typical BBS phenotype including retinal pigmentosa, obesity, polydactyly in most patients, but only two patients had renal insufficiency and none of the patients had hypogonadism [265], which is consistent with our findings. However, eight of twelve patients from La Réunion Island were found to have learning difficulties [265], which is in contrast to our finding that cognitive impairment is a rare symptom in BBS3 patients.

Within the BBSome, mutations in *BBS1* and *BBS8* cause the lowest mean severity, whereas mutations in *BBS2* and *BBS7* are responsible for the highest mean disease severity. Mutations

in genes encoding BBSome core (*BBS2*, *BBS7*, and *BBS9*) were associated with higher frequency of renal anomalies than mutations in *BBS1*, *BBS4*, and *BBS8.*

Retinal dystrophy is the most common symptom of BBS. The incidence of retinal dystrophy in our cohort was 94.4%. Studies published after the creation of our database confirmed this observation and reported a retinis pigmentosa phenotype in up to 100% of patients, regardless of the gene affected [265-268]. This makes retinal dystrophy a strong diagnostic feature of BBS.

Renal anomalies were the most common among patients with mutations in BBS chaperonins. This observation was later supported by a cohort study of twenty Italian BBS patients, where they observed higher frequency of renal abnormalities in patients with variations in BBSome chaperonin than in patients with mutations in BBSome proteins [269].

Obesity was another frequent symptom (penetrance >70%) but had a comparable incidence in patients with a mutated BBSome or BBS chaperonin genes. Interestingly, a recent study investigating the body mass patterns of 552 BBS patients found that BBS1 patients had a lower BMI in childhood compared to the BBS10 cohort, however, this difference attenuated in adolescence [270]. This study also found a significantly higher BMI in female BBS patients than in male patients.

We found that *BBS1* mutations are associated with a milder phenotype. *BBS1* had a relatively low penetrance for reproductive system abnormalities and polydactyly compared with the other patients. This finding was later confirmed by a recent cohort study involving 27 BBS patients from Puerto Rico [267]. They found that BBS1 patients had a milder ocular phenotype and less frequent renal and genital anomalies than patients with *BBS7* mutations. Interestingly, they described a mutation of *BBS1* (c.1645G > T) that leads to a more severe phenotype with early-onset visual symptoms and a higher frequency of polydactyly [267].

Overall, our results suggest the association between the genotype and phenotype of BBS patients. In this way, the affected gene partially predicts the clinical outcome of BBS. Moreover, our data provided new insights for a better understanding of the role of individual BBS proteins.

4.2 BBS ciliopathy is linked to altered hematopoiesis and dysregulated self-tolerance

There is some evidence that ciliary dysfunction may affect the hematopoietic system, such as the homology between the immunological synapse and the primary cilium [271, 272], requirement of BBSome for Sonic Hedgehog, leptin, and WNT signaling [273], and the frequent obesity in BBS patients. However, the immune system of patients with ciliopathy has not yet been studied. We decided to elucidate the mechanism by which BBS deficiency affects the hematopoietic system (Ad. 2).

As a first step, we analyzed clinical data of BBS patients. We found that BBS patients had a higher prevalence of certain autoinflammatory diseases, such as T1D, Hashimoto's thyroiditis, RA, and IBD. BBS has not been previously recognized as a risk factor for autoimmune diseases. A single cohort study reported multiple autoimmune diseases (Crohn's disease, RA, T1D, Hashimoto's thyroiditis, primary sclerosing cholangitis, and psoriasis) in three of 15 BBS patients [274]. However, most cohort studies do not report the presence or absence of immune system disorders in BBS patients.

Next, we examined the blood test results of BBS patients. We found that BBS patients had higher white blood cell counts, particularly neutrophils and eosinophils, than the control group. However, when we used a BMI-matched control group, these differences were no longer significant, suggesting that the increased white blood cell count in BBS patients is caused by obesity. Interestingly, BBS patients had increased levels of C-reactive protein, a slight decrease in hemoglobin, and mild thrombocytopenia compared with the BMI-matched control group, suggesting that these changes are obesity-independent. It should be noted that although these changes could be primary effects of BBS, they may also reflect BBS-associated pathologies such as renal insufficiency [275].

In a next step, we examined the immune system of BBS mouse models carrying mutations in *Bbs4* or *Bbs18*. Based on the homology between the cilium and the immunological synapse [258], we hypothesized that BBSome dysfunction might affect T-cell responses. We tested the functionality of *Bbs4*-deficient T cells in several assays. First, we examined the ability of T cells to form an immune synapse with APC *ex vivo*. Contrary to our hypothesis, conjugation of *Bbs4*-deficient T cells with APC was not altered. Next, we tested the ability of *Bbs4*-

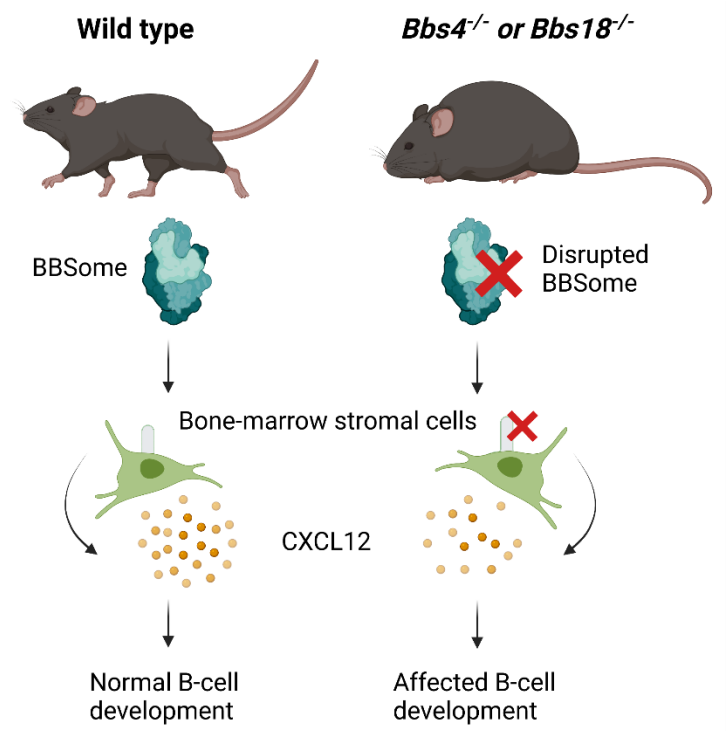
deficient OT-I T cells to induce autoimmune diabetes in RIP.OVA mice expressing ovalbumin under the rat insulin promoter. The autoimmune diabetes assay examines several aspects of T-cell functionality, including their ability to interact with APC, proliferate, infiltrate the pancreas, and destroy β-cells. Remarkably, *Bbs4*-deficient OT-I T cells showed the same ability to induce diabetes as *Bbs4*$^{+/+}$ OT-I T cells. Based on these observations, we concluded that BBS4 does not play an important role in the T-cell-mediated immune response.

A recent study by the Baldari group investigated the role of BBS1 in the formation of the T cell-APC immune synapse [276]. In apparent contrast with our findings, they observed that *BBS1* depletion in human T cells resulted in defective centrosome polarization and impaired accumulation of endosomal TCR–CD3 complexes at immune synapses. Moreover, they proposed that BBS1 promotes centrosome repositioning by assisting the dynein-dependent recruitment of 19S regulatory subunit of the proteasome to allow for the clearance of centrosome-associated F-actin during immune synapse assembly [276]. This discrepancy may be due to several factors. First, BBS1 and BBS4 functions in BBSome assembly are not redundant [277]. In addition, our meta-analysis of BBS patients' data suggests that individual BBS proteins may have BBSome-independent role. In this way, the lack of BBS1 or BBS4 proteins might lead to different T-cell phenotype. Another possible factor is a difference between mouse and human T cell biology. Last but not least, the observed defects in the formation of immune synapses do not exclude the possibility of a normal immune response of *BBS1*-deficient T cells, which was not investigated in the Baldari group study.

While the T-cell compartment was not altered in our mouse model of BBS, B-cell development and homeostasis were disrupted. Particularly, *Bbs4*- and *Bbs18*-deficient mice had significantly increased numbers of pre-B cells in the bone marrow, whereas other B-cell populations in the bone marrow were unaffected. The fact that these alterations were present in both *Bbs4*- and *Bbs18*-deficient mice suggests that the observed phenotype is caused by BBSome dysfunction (both BBS4 and BBS18 proteins are subunits of BBSome complex). At the same time, mice with a specific deletion of *Bbs4* in hematopoietic cells showed no signs of altered B-cell development, suggesting B-cell extrinsic mechanism of development block seen in *Bbs4*-null mice. However, this phenotype was not dependent on obesity, as *Bbs4*-gene-trap mice used in our study showed B-cell developmental alterations, but they were not obese.

Based on this, we hypothesized that the observed differences were caused by the disruption of the bone marrow stromal cell niche. Of note, bone marrow stromal cell are ciliated [278], whereas hematopoietic cells do not form cilia [279], which further supports our hypothesis. We analyzed the expression of the key cytokines regulating B-cell development in the bone marrow of *Bbs4*-deficient mice and found a decreased expression of *Cxcl12*. The same effect was observed in *Bbs4*$^{-/-}$ mouse embryonic fibroblasts.

We hypothesized that the decrease in Cxcl12 expression was caused by enhanced WNT signaling as a consequence of BBSome dysfunction [280, 281]. We were able to suppress *Cxcl12* expression in ST2 cells by triggering canonical WNT signaling. Collectively, these results provide evidence that BBSome deficiency restricts *CXCL12* expression in bone marrow stromal cells, leading to a partial blockage of B-cell development (Scheme 1).



Scheme 1. *Loss of Bbs4 or Bbs18 disrupts BBSome and causes cilia impairment in bone marrow stromal cells, leading to limited expression of CXCL12 and subsequent disruption of B cell development. See Ad. 2 for details.*

Interestingly, a recent study reported that *CXCL12* deletion in bone marrow CXCL12-abundant reticular cells (so-called CARs) reduced stromal-hematopoietic coupling, suggesting that physical contact of *CXCL12*-expressing stromal cells and hematopoietic cells is CXCL12-dependent. They also observed that *CXCL12* deficiency resulted in enhanced conversion of CARs to marrow adipocytes [282]. Enhanced bone marrow adiposity impairs hematopoiesis and has been associated with aging, osteoporosis, T1D, and growth hormone deficiency [283]. We have not examined bone marrow adiposity in BBS mouse model, but this may be an interesting direction for future research.

Another B-cell phenotype which we observed in *Bbs4*- and *Bbs18*-deficient mice was an increased percentage of late mature B cells in the spleen and lymph nodes, and a decreased percentage of marginal zone (MZ) B cells. The molecular mechanism underlying these alterations is less clear. However, we can speculate that it might be dependent on CXCL12 too. A study by Wang et al. found that receptor for CXCL12, CXCR7, is expressed on MZ but not on follicular B cells [284]. Furthermore, they demonstrated that inhibition of CXCR7 signaling led to disrupted MZ architecture and reduced numbers of MZ B cells [284]. Accordingly, *Cxcr7*-null mice have decreased number of MZ B cells [285]. Although we do not have experimental proofs, we suppose that BBSome deficiency affects *CXCL12* expression in the spleen in the same way as it does in the bone marrow. Therefore, these findings support the possibility that reduced MZ B population in BBSome-deficient mice might be due to restricted CXCL12 expression.

Altogether, our study revealed a link between ciliopathy BBS, homeostasis of hematopoietic system, and autoimmunity. Some of the hematopoietic system alterations in BBS are caused by the BBS-induced obesity, whereas others are caused by intrinsic defects in the bone marrow stromal cells. Further investigations are needed to better understand the molecular mechanisms underlying the observed manifestations of BBS.

4.3 Tregs suppress the formation of super-effector CD8$^+$ T cells by limiting IL-2

To investigate the role of CD4$^+$ FOXP3$^+$ Tregs in the autoimmune response of self-reactive T cells (Ad. 3), we took advantage of mice expressing the diphtheria toxin receptor in FOXP3$^+$ Tregs, enabling their selective depletion by administration of diphtheria toxin. We employed a model of experimental autoimmune diabetes based on the transfer of ovalbumin-specific

$CD8^+$ OT-I T cells into Treg-depleted or Treg-replete mice expressing ovalbumin in pancreatic β-cells. Subsequent priming of OT-I T cells by administration of OVA induces activation of OT-I T cells and leads to destruction of pancreatic β-cells.

Using this model, we observed that the induction of diabetes in the majority of Treg-replete mice required 10^6 OT-I T cells, whereas in Treg-depleted mice this number dropped to 10^4. Accordingly, the expansion of OT-I T cells was enhanced in Treg-deficient mice in comparison to Treg-replete mice. These results demonstrate the crucial role of Tregs in autoimmunity prevention.

To investigate how Tregs suppress priming of self-reactive T cells by antigens with various affinities in the context of experimental diabetes, we used bone marrow-derived DCs loaded with OVA peptide (KD ~ 50 μM) or its variants Q4R7 (KD ~ 300 μM) or Q4H7 (KD ~ 850 μM). Tregs decreased the susceptibility to diabetes upon priming with each of these peptides, although a higher number of OT-I T cells was required in case of low affinity antigens to break the tolerance. These results demonstrate that Tregs increase the quorum of self-reactive $CD8^+$ T cells required for inducing autoimmune diabetes irrespective of the priming antigen affinity, while preserving the hierarchy of the biological potencies of these antigens. In contrast, a study by Pace et al. suggested that Tregs exclusively suppress low-affinity T-cell responses [286]. This discrepancy is most likely explained by the fact that the study by Pace et al. examined the role of Tregs in the $CD8^+$ T cell response to *Listeria monocytogenes* infection, whereas we used pathogen-free priming of $CD8^+$ T cells (DC loaded with antigen).

To test if Tregs inhibit self-reactive $CD8^+$ T cells directly or via suppressing by-stander $CD4^+$ T helpers, we adoptively transferred conventional $CD4^+$ T cells or Treg to *Cd3ε*$^{-/-}$ RIP.OVA hosts and induced diabetes via OT-I T cells and DC-OVA transfer. We observed that Tregs reduced, whereas conventional $CD4^+$ T cells increased the incidence and accelerated the onset of diabetes, in comparison to mice receiving no $CD4^+$ T cells. We also revealed that conventional $CD4^+$ T cells enhanced the expansion of $Klrg1^+$ OT-I T cells in spleens, while Tregs had the opposite effect. Overall, we demonstrated that Tregs suppress $CD8^+$ T cells independently of conventional $CD4^+$ T cells.

Next, we investigated the mechanism of Treg-mediated suppression. We hypothesized that Tregs suppress $CD8^+$ T cells by limiting IL-2 availability. We observed that OT-I T cells from

Treg-deficient mice had upregulated IL-2 signaling (IL-2Rα and pSTAT5) early after priming. We also showed that Tregs suppressed proliferation and IL-2Rα expression in $CD8^+$ T cells *ex vivo*, and that the addition of IL-2 abrogated this effect. To test if IL-2 limits the effect of Tregs *in vivo*, we provided IL-2/anti–IL-2 antibody immunocomplexes (IL-2-ic), namely IL-2/S4B6 and IL-2/JES6 to RIP.OVA mice during the OT-I priming in the course of diabetic assay. Both types IL-2-ic induced the autoimmune diabetes similarly to the Treg-depletion.

We hypothesized that enhanced IL-2 signaling may overcome Treg-mediated suppression of anti-tumor $CD8^+$ T response. We test the effect of IL-2/JES6 in two mouse models of cancer: BCL1 leukemia and B16F10 melanoma. In both models, IL-2ic combined with a chemotherapeutic drug doxorubicin slowed down disease progression. Antibody-mediated depletions of $CD8^+$ T cells abrogated anti-tumor effect, whereas $CD4^+$ T cell depletion improved it. Overall, these results showed that Tregs are unable to suppress $CD8^+$ T cells in the excess of IL-2. This conclusion goes in line with some previous studies suggesting IL-2 restriction as a Treg mechanism for suppression of effector $CD8^+$ T cells [287, 288].

Interestingly, IL-2/JES6 selectively stimulates IL-$2R\alpha^+$ cells and thus has been considered as an immunosuppressive agent acting via Treg stimulation [289]. Indeed, strong exogenous IL-2 signals stimulate the expansion of Tregs and the expression of their effector molecules, which should enhance their suppressive capacity. In apparent contrast, our study demonstrates that IL-2/JES6 administered during the OT-I priming dramatically increases diabetes incidence, although it also induces Treg population expansion in treated mice. Accordingly, IL-2/JES6 in combination with chemotherapy significantly prolonged the survival of mice in two different tumor models in a $CD8^+$ T-cell-dependent manner. Collectively, these results reveal that strong IL-2 signals potentiate $CD8^+$ T-cell immune response, promoting anti-tumor immunity and autoimmunity despite its stimulatory effects on Tregs.

Nevertheless, IL-2 therapy aimed at Treg expansion is currently being investigated in many autoimmune diseases. Preclinical studies generally demonstrate a positive effect. Administration of IL-2/JES6 suppressed the induction of arthritis in the CIA model [290], prevented diabetes in NOD mice [291] and graft-versus-host disease [292]. A recently described NKTR-358, a PEGylated IL-2 designed to preferentially bind the trimeric IL-2R, ameliorated disease progression in a mouse model (MRL/MpJ-Faslpr) of SLE [293]. However,

clinical trials of IL-2 therapy look less promising. A phase I clinical trial combining polyclonal Tregs and low-dose IL-2 therapy in patients with T1D showed that while IL-2 expands exogenously administered Tregs it also expand cytotoxic cells [294], which goes in a line with our conclusions. Accordingly, in another phase I/II clinical trial no significant change in glycaemic control was observed in patients receiving low dose IL-2 therapy compared with the placebo group [295].

On the other hand, the IL-2 cancer therapy shows a great potential. The FDA approved a recombinant IL-2 called aldesleukin for metastatic renal cancer in 1992 and for metastatic melanoma in 1998 [296, 297]. However, due to serious side effects, such as potentially fatal vascular leak syndrome, investigation of IL-2 therapy was abandoned for some time. There has been a resurgence of interest in this topic, stimulated by recent advances in understanding the structure of IL-2 and its receptors and the role it plays in the immune system. The recently described bempegaldesleukin is a βγ-chain oriented IL-2 agonist [298]. It consists of recombinant human IL-2 fused with an average of six releasable PEG groups. PEG groups were added to IL-2 to increase the half-life of the cytokine and allow gradual release of the active IL-2 to avoid side effects. In addition, the placement of the PEG chains results in bempegaldesleukin preferentially binding IL-2Rβ, which is normally expressed on effector T cells, rather than the trimeric IL-2Rαβγ, which is typically expressed on Tregs [298]. A clinical trial of bempegaldesleukin in combination with an anti-PD-1 mAb showed encouraging antitumor activity in first-line treatment of metastatic melanoma, with an overall response rate of 59.5%, while 47.4% of patients showed complete clearance of target lesions [299]. These results demonstrate that IL-2 therapy can be successfully combined with a checkpoint inhibitor. Importantly, efficacy was observed regardless of baseline PD-L1 expression, suggesting therapeutic potential for patients with poor prognosis for response to PD-1/PD-L1 blockade. The efficacy of bempegaldesleukin in other cancers is currently being investigated in several clinical trials.

Another IL-2R agonist called nemvaleukin consists of an IL-2 variant fused to the α-chain, blocking its ability to interact with endogenous α-chain of cells expressing trimeric IL-2R. Nemvaleukin showed the ability to selectively expand natural killer cells and effector and memory $CD8^+$ T cells [300]. A phase II trial of nemvaleukin in combination with

pembrolizumab in patients with ovarian, fallopian tube, and primary peritoneal cancer is now ongoing.

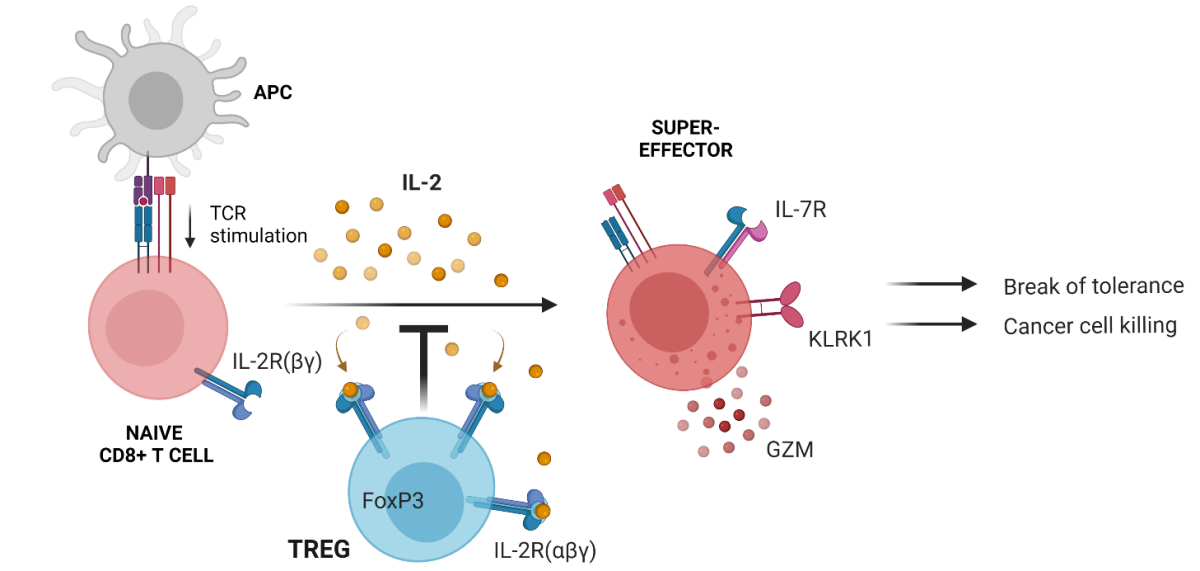
The recent focus on approaches aimed at avoiding the interaction of IL-2 with the IL-Rα is potentially interesting. However, we should keep in mind that while naïve effector T cells express the dimeric IL-2R, they upregulate expression of the α-chain shortly after activation. Thus, it is likely that tumor-reactive T cells express trimeric IL-2R in cancer patients. Therefore, blocking the interaction of IL-2 with IL-2Rα may not only prevent Treg stimulation, but also reduce its stimulatory effect on cytotoxic T cells.

To further investigate how Tregs influence the phenotype of $CD8^+$ T cells, we analyzed the transcriptomes of OT-I T cells primed in the presence or absence of Tregs. We revealed that Tregs reduce the expression of IL-2 responsive genes, such as *Il2rα* and *Gzmb*. Furthermore, we identified previously unknown $CD8^+$ T cell subset, characterized by the expression of *Gzmb*, *Ilr7a*, *Cd103* and natural killer cell markers *Klrk1*, *Ifitm1-3* and *Cd7*, but not expressing *Cd49d*, which appeared almost exclusively in the absence of Tregs. We called these cells super-effector T cells. We hypothesized that super-effector T cells might be induced by excessive IL-2 signals. Indeed, we were able to induce super-effector T cells in mice via IL-2 administration and antigenic stimulation. Later we tested cytotoxic properties of these cells in *in vivo* killing assay based on adoptive transfer OT-I T cells together with OVA-loaded target cells to host mice. $Klrk1^+$ super-effector induced by the combination of the antigen and IL-2ic showed the most potent cytotoxic activity.

Of note, antigenic stimulation in combination with OX40 and 4-1BB activating antibodies induces IL -$7R\alpha^+$ effector $CD8^+$ T cells, as shown in a study by Lee et al [301]. 4-1BB signaling has been shown to increase IL-2 production and IL-2Rα expression in T cells [302]. Accordingly, OX40/4-1BB-induced effector $CD8^+$ T cells expressed high levels of IL-2Rα [301]. Taken together, this suggests that the described IL-$7R\alpha^+$ effector $CD8^+$ T cells not only share phenotypic features but also develop via a mechanism similar to that of super effector T cells. Interestingly, the authors of this previous study also refer to these cells as 'super-effectors'.

Next, using a human CD8$^+$ T cells atlas, which we generated by integrating publicly available single cell transcriptomic data sets, we identified potential human counterparts of super effector CD8$^+$ T cells, expressing *IL7R*, *KLRD1*, *IFITM3*, and *CD7*. However, these cells express GZMA and GZMB genes on a lower level than the conventional effector cells, probably reflecting the absence of recent antigenic stimulation. Obtaining single-cell transcriptomic data from patients with autoimmune diseases will improve our understanding of the T-cell phenotypes that trigger autoimmunity and allow identification of potential human super-effector T cells.

Overall, we demonstrated that antigenic stimulation of CD8$^+$ T cells in the presence of excessive IL-2 leads to the generation of super-effector T cells, which are characterized by a specific phenotype and superior cytotoxic properties (Scheme 2). This cell subset may play an important role in the tolerance breakage. Our results support the hypothesis that the main mechanism of Treg-mediated suppression of self-reactive CD8$^+$ T cells is the reduction of IL-2 availability.



Scheme 2. *Tregs prevent the expansion of super-effector CD8$^+$ T cell via limiting available IL-2. See Ad. 3 for details.*

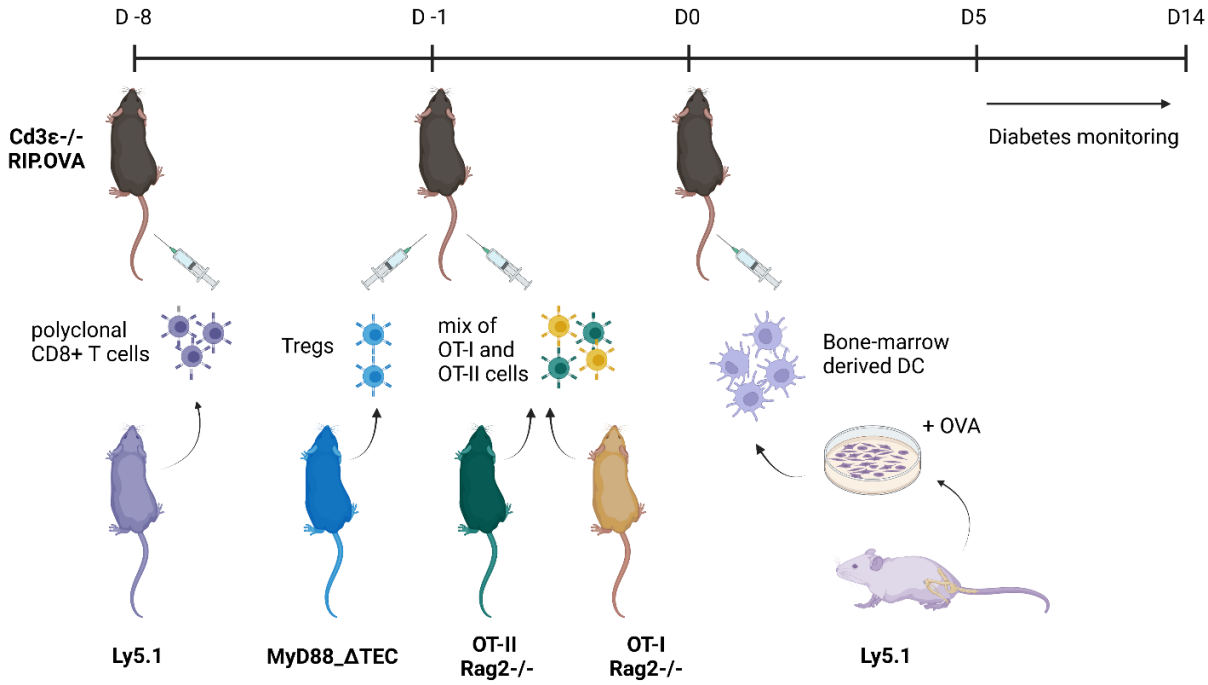
4.4 Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation

The development of tTreg is mediated by mTECs and DCs which present tissue-restricted self-antigens. However, the overall contribution of mTECs and DCs is still poorly understood. In this project (Ad. 4) we identified a population of thymic Sirpα^{+} monocyte-derived CD14^{+} DCs that are enriched in the thymic medulla. We observed that TLR/MyD88 signaling increases cooperative antigen transfer between TECs and the thymic CD14^{+} monocyte-derived DCs. In more details, in response to TLR stimulation, mTECs upregulate the expression of cytokine genes, including *Il1f6*, *Csf2*, *Ccl25*, *Ccl4*, and *Ccl24*. These cytokines promote the migration of CD14^{+} DCs to the proximity of mTECs. As a result, CD14^{+} DCs are able to acquire mTEC-derived antigens. We also observed that the number of monocyte-derived DCs is diminished in mice with MyD88-deficient TECs. Furthermore, the frequency of thymic CD25^{+} FOXP3^{+} Tregs was decreased in mice with MyD88-deficient TECs (MyD88$^{\Delta TECs}$).

To test the functionality of Tregs from mice with MyD88-deficient TECs, we developed the mouse model of autoimmune diabetes based on adoptive transfer of ovalbumin-specific CD8^{+} OT-I T cells and CD4^{+} OT-II T cells to T-cell deficient *Cd3ε*$^{-/-}$ RIP.OVA mice expressing ovalbumin in pancreatic β-cells. Immunization of host mice with OVA-loaded DCs leads to OT- II and OT-I T cell activation and subsequent destruction of pancreatic β-cells (Scheme 3).

Using this model, we demonstrated that Tregs from MyD88$^{\Delta TECs}$ mice have a significantly lower ability to prevent the early onset of diabetes. Accordingly, Tregs from MyD88$^{\Delta TECs}$ mice could not efficiently suppress the expansion of effector Klrg1^{+} OT-I T cells.

Our results are consistent with previous studies. The importance of TLR /MyD88 signaling in AIRE-deficiency-induced autoimmunity was demonstrated in experiments with *Myd88*/*Aire* double knockout mice. These mice develop more severe symptoms of autoimmunity than *Aire*-deficient mice, suggesting that MyD88 signaling may play a role in tolerance induction [303]. It seems plausible that the more severe phenotype of *Myd88*/*Aire* double knockout mice could be caused by the absence of MyD88 signaling in mTECs, leading to downregulation of the chemokines required to recruit CD14^{+} thymic DCs and consequently a deficiency of Tregs.



Scheme 2. *Mouse model of autoimmune diabetes. $CD8^+$ OT-I T cells and $CD4^+$ OT-II T cells are adoptively transferred to $Cd3\varepsilon^{-/-}$ RIP.OVA that have previously received polyclonal $CD8^+$ T cells. Next, T cells are primed with adoptively transferred OVA-loaded DCs. Diabetes monitoring is performed by measuring glucose in blood and urine. See Ad. 4 for details.*

It should be noted that MyD88 mediates signals not only from TLR but also from the receptors for IL-1 family cytokines (IL-1β, IL-18, IL-33). We tested whether IL-1β, IL-18, or IL-33 could trigger chemokine response in mTECs. We found that IL-1β stimulation leads to a cytokine response similar to that observed with TLR9 stimulation. This suggests that IL-1β may act as a co-regulator of chemokine and cytokine expression in mTECs.

Developing thymocytes encounter self-antigens presented by various types of thymic APC, including mTECs, B cells, and DCs. It is believed that thymic Treg development requires antigen presentation by both mTECs and DCs [304]. Some studies emphasize the indispensable contribution of DC for this process, particularly $Sirp\alpha^+$ DCs [305, 306]. Accordingly, we found that mTEC-intrinsic TLR9/MyD88 signaling increased the cell ratio of $Sirp\alpha^+$DCs to $Xcr1^+$cDC1, which correlated with an increased production of thymic Tregs.

Altogether, these data describe a MyD88-dependent function of mTECs and thymic $CD14^+$ DCs that is required for the generation of Tregs, and consequently for the establishment of immune tolerance.

4.5 $CD8^+$ Tregs represent a heterogeneous population with different phenotypes and properties

Tregs play a key role in peripheral self-tolerance and prevention of autoimmunity. In contrast to the well-established $CD4^+$ $FOXP3^+$ Tregs, $CD8^+$ Tregs are still controversial. There are several reasons for this. First, only a limited number of studies to date have demonstrated the suppressive function of $CD8^+$ Tregs *in vivo*, and even fewer have described the mechanism underlying this suppression. Second, the phenotype and often the properties of $CD8^+$ Tregs differ considerably across publications. We decided to write a literature review to clarify the phenotype and functional properties of the $CD8^+$ Tregs proposed in the literature (Ad. 5). We critically evaluated the published data to determine whether one or more of these subsets could be established as true $CD8^+$ Tregs or whether additional experimental evidence is required.

Because the phenotypes of $CD8^+$ Tregs proposed in different studies did not match, we revisited the concept of $CD8^+$ Tregs by characterizing individual $CD8^+$ T-cell subsets with the proposed regulatory capacity separately. The proposed $CD8^+$ Treg subsets include $CD8^+$ $FOXP3^+$ T cells, $CD8^+$ $CD122^+$ T cells, $CD8^+$ $CD28^{low/-}$ T cells, $CD8^+$ $CD45RC^{low}$ T cells, T cells expressing CD8αα homodimer and Qa-1-restricted $CD8^+$ T cells (Table 1).

It is noteworthy that some of these cell phenotypes potentially overlap. For example, $CD8^+$ $FOXP3^+$ cells may overlap with $CD8^+$ $FOXP3^+$ $CD45RC^{low}$, however $CD8^+$ $FOXP3^-$ $CD45RC^{low}$ Tregs have been proposed as well. Similarly, as $CD8^+$ $CD122^+$ T cells might include $CD8^+$ $CD122^+$ $PD\text{-}1^+$ T cells, $CD8^+$ $CD122^+$ $CD44^+$ $CD49d^{low}$ T cells, and $CD8^+$ $CD122^+$ $Ly49^+$ T cells, the possible overlap between these subsets is not clear. Moreover, it remains to be resolved, whether the regulatory function is typical for the subset as a whole or whether only a subpopulation within a subset is immunoregulatory. Clarification of the $CD8^+$ Treg phenotype is complicated by the limited cell markers used in the original studies. Emerging approaches of single cell transcriptomics might help to overcome these problems by uncovering specific and shared markers of $CD8^+$ Tregs.

Subset name	Additional markers	*In vivo* animal models	Credibility level
$CD8^+$ $FOXP3^+$ Tregs (mouse, human)	CD25, GITR, CTLA-4, CD103, ICOS, CD73 TNFR-2, CD39, CD26	graft survival, GVHD, CIA in humanized mice	**High**: described repeatedly, mechanism of suppression proposed
$CD8^+$ $CD122^+$ Tregs (mouse, human)	CD44, $CD49d^{low}$, CD62L, Ly49, CD38, PD-1, CXCR3	experimental colitis, EAE, diabetes, allograft survival	**Intermediate**: described repeatedly, mechanism of suppression proposed, but may overlap with multiple $CD8^+$ T subsets
$CD8^+$ $CD28^{low/-}$ Tregs (mouse, human)	CD62L, CD103, Tim-3, CD45RB, CD57, KLRG-1, CD27, CD62L, FOXP3, IL-10RA, perforin, granzyme B	experimental colitis, EAE	**Low**: conflicting evidence, phenotypic overlap with exhausted T cells
$CD8^+$ $CD45RC^{low}$ Tregs (rat, human)	CD45RO, CD122, GITR, FOXP3	GVHD, allograft survival, experimental uveitis	**Low**: conflicting evidence, substantial variation in population size
$CD8\alpha\alpha^+$ Tregs (mouse, human)	$CD4^+$ or $CD4^-$, PLZF, granzymes, perforin	EAE, experimental colitis	**Intermediate**: mechanism of suppression proposed, but phenotype is unclear

Table 1. *$CD8^+$ Treg subsets proposed in literature. NA – not available. See Ad. 5 for details.*

It seems plausible that different $CD8^+$ Treg populations employ distinct mechanisms of suppression. Side-by-side comparison of the proposed $CD8^+$ Treg phenotypes would dramatically improve our understanding of $CD8^+$ Treg heterogeneity In addition, it would be beneficial to study the behavior of $CD8^+$ Tregs under different conditions, as it appears that some of the subsets may have both regulatory and proinflammatory properties depending on the context (particularly perforin and granzyme-expressing $CD8^+$ Tregs).

The *in vitro* suppressive activity has been reported for each of the aforementioned subsets. Moreover, several preclinical *in vivo* models indicated the importance of several putative $CD8^+$ Treg subsets in immune tolerance. These results encourage the search for the future use of

$CD8^+$ Tregs in clinical applications. Our review was followed by several reports that have contributed to the $CD8^+$ Treg field.

The study by Giang et al. reported expansion of $CD4^+$ $FOXP3^+$ and $CD8^+$ $FOXP3^+$ Tregs in mice treated with nanoparticles encapsulating IL-2 and TGF-β [307]. However, the suppressive capacity of the proposed $CD8^+$ Tregs was not demonstrated *in vitro* or *in vivo*. The authors demonstrated that the mice treated with nanoparticles have reduced GVHD disease manifestations [307], but this effect could be potentially achieved by $CD4^+$ Tregs alone. It should be noted that FOXP3 is transiently expressed by activated $CD8^+$ T cells and the expression of FOXP3 alone does not account for the suppressive properties of the cell.

Another study focused on $CD8^+$ $CD122^+$ $PD1^+$ Tregs. This study investigated the effect of a combined therapy of anti-OX40L, Rapamicin and a low dose of IL-2 in a mouse graft survival model (heart transplant) [308]. They observed that the therapy prolonged graft survival and increased the percentage of $CD4^+$ $FOXP3^+$ Tregs, but also expanded $CD8^+$ $CD122^+$ $PD1^+$ T cell population. To confirm their suppressor abilities, they sorted $CD8^+$ $CD122^+$ $PD1^+$ and $CD8^+$ $CD122^+$ $PD1^-$ T cells from the long-term survival grafts and adoptively transferred them into the transplant model. Interestingly, both $CD8^+$ $CD122^+$ $PD1^+$ T cells and $CD8^+$ $CD122^+$ $PD1^-$ T cells were able to suppress graft rejection. These observations further support our conclusion that several subsets of $CD8^+$ $CD122^+$ Tregs may have regulatory properties.

$CD8^+$ $CD28^-$ Tregs have been proposed as markers for invasive nonfunctioning pituitary adenomas, as a high percentage of cells with this phenotype have been detected in blood of the patients [309]. In parallel, another study observed an increased number of $CD8^+$ $CD28^-$ Tregs in patients with renal transplant [310]. Although the authors of these studies referred to these cells as $CD8^+$ Tregs, they did not address their suppressive properties. Importantly, loss of surface CD28 is a hallmark of senescent $CD8^+$ T cells, which are known to expand during aging but also in some health disorders including cancer [311, 312]. In this way, the authors may have misidentified the cells they observed.

In summary, the field of $CD8^+$ Tregs remains controversial. However, despite the controversy and conflicting evidence accumulating in this field, the immunosuppressive properties of $CD8^+$ Tregs have been repeatedly demonstrated. We believe that our critical review made an important step toward evidence-based classification of the potential $CD8^+$ Tregs.

5. Conclusion

Self-tolerance is one of the essential features of the immune system indispensable for the organism homeostasis. This PhD thesis is focused on the mechanisms of immune tolerance. Our aim was to extend the existing knowledge on the role of Tregs in the establishment of immune tolerance. In addition, we aimed to investigate the factors for the break of tolerance in BBS ciliopathy.

BBS can be caused by dysfunction of numerous genes, but the role of specific BBS proteins was not clear. We performed a meta-analysis of published clinical data of BBS patients, which revealed that the identity of the causative gene partially predicts the clinical outcome of the BBS disease. Importantly, we found that BBS patients had a higher incidence of autoimmunity than the general population. We examined the blood tests of BBS patients and identified several immune-related alterations, some of which were caused by BBS-associated obesity. To further investigate the effects of BBS on the immune system, we took advantage of BBS mouse model. Similar to human patients, BBS-null mice were obese, but we were able to generate non-obese mouse model of BBS, *Bbs4* gene-trap. We found that both obese and non-obese BBS mice had impaired B-cell development, caused by intrinsic defects in bone marrow stromal cells.

In the second part of the study, our focused on the mechanisms of Treg-mediated peripheral tolerance. In particular, we were interested in Treg-mediated suppression of CD8+ T lymphocytes, which has not been studied intensively. We have shown that Tregs suppress both high- and low-affinity self-reactive $CD8^+$ T cell responses. Therefore, Tregs establish peripheral immune tolerance by suppressing self-reactive $CD8^+$ that escaped elimination via central tolerance mechanisms. Furthermore, we have shown that Tregs do not require $CD4^+$ T helpers to suppress $CD8^+$ T cell responses, indicating that Tregs directly suppress $CD8^+$ T cells.

Using several *in vitro* and *in vivo* approaches, we demonstrated that the major immunoregulatory mechanism used by Tregs to suppress $CD8^+$ T cells is the limitation of available IL-2. In addition, we found a novel cell population, which we called super-effector T cells, that occurs only in Treg-depleted or IL-2 treated mice, further supporting our conclusion that the absence of Tregs leads to the excess of available IL-2. We have demonstrated the role of super-effector T cells in breaking tolerance to self and tumors. In

addition, our collaborative study contributed to our understanding of the role of the thymic DC in Treg development. Altogether, our study brings new concepts to the field of immune tolerance and cancer immunology.

In addition, we have made a significant contribution to the field of $CD8^+$ Tregs by classifying the $CD8^+$ Treg subsets proposed in the literature and elucidating their immunosuppressive properties.

5.1 Summary of major findings

1. We demonstrated that the identity of the causative gene partially predicts the clinical outcome of the BBS disease.

2. We revealed that BBSome deficiency causes ciliary defects in bone marrow stromal niche, leading to altered B-cell development.

3. We have shown that obesity in BBS is associated with altered blood test parameters, indicating that obesity is an additional factor predisposing to loss of tolerance in BBS.

4. We have shown that Tregs suppress both high- and low- affinity $CD8^+$ T cell responses in a $CD4^+$ T cell-independent manner.

5. We found that Tregs suppress $CD8^+$ T cell responses and prevent the formation of highly cytotoxic super-effector $CD8^+$ T cells by limiting IL-2.

6. We demonstrated that MyD88-dependent cooperation of mTECs and thymic $CD14^+$ DCs is required for sufficient generation of Tregs, and thus for the establishment of immune tolerance.

7. We demonstrated that $CD8^+$ Tregs represent heterogeneous group of $CD8^+$ T cell that includes several cell subsets characterized by different functional properties and varying levels of suppressive capacity.

6. Publications

The texts of the publications can be found at the end of this thesis.

6.1. List of publications

1. Niederlova V, Modrak M, **Tsyklauri O**, Huranova M, Stepanek O. Meta-analysis of genotype-phenotype associations in Bardet-Biedl syndrome uncovers differences among causative genes. Hum Mutat. 2019 Nov;40(11):2068-2087. PMID: 31283077. (IF_{2021}= 4.87).
2. **Tsyklauri O***, Niederlova V*, Forsythe E, Prasai A, Drobek A, Kasparek P, Sparks K, Trachtulec Z, Prochazka J, Sedlacek R, Beales P, Huranova M, Stepanek O. Bardet-Biedl Syndrome ciliopathy is linked to altered hematopoiesis and dysregulated self-tolerance. EMBO Rep. 2021 Feb 3;22(2):e50785. PMID: 33426789. (IF_{2021}= 8.8).
3. **Tsyklauri O**, Chadimova T, Niederlova V, Kovarova J, Michalik J, Malatova I, Janusova S, Rossez H, Drobek A, Vecerova H, Galati V, Kovar M, Stepanek O. Regulatory T cells suppress the formation of super-effector CD8 T cells by limiting IL-2. bioRxiv 2021.11.10.467495.
4. 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. PMID: 32398640. (IF_{2021}= 14.92).
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6.2 Contribution

Ad 1. I contributed to the creation of the database of BBS patients' available records. In more detail, I contributed to screening of the data from the primary sources (PubMed, Google Scholar and Euro-Wabb databases), reading of the full texts of potentially relevant articles and their evaluation, and extraction of the data concerning individual BBS patients phenotype and genotype. Extracted data were further analyzed via other researchers.

Ad 2. I joined the project when BBS patients' data were already collected and analyzed. I contributed to characterization of the phenotype, in particular immune system of *Bbs4*- and *Bbs18*-deficient mice. I performed data collection and analyzes for majority of mouse model experiments (Figure 1D-K, Figure 2A-F, Figure 3A-F, Figure 4A-F, Figure 5B, E, G). I also co-wrote the manuscript with Dr. Ondřej Štěpánek.

Ad 3. I joined the project when we already showed that Tregs can prevent $CD8^+$ T-cell mediated diabetes in RIP.OVA model, and that Tregs are able to suppress $CD8^+$ T cells in the absence of $CD4^+$ T helpers. I contributed to further development of the project. I performed data collection (Figure 2A-H, Figure 3D-G, Figure 4A-H, Figure 5A-I, Figure 6A-G) and data analysis (Figure 2A-H, Figure 3D-G, Figure 4A-H, Figure 5I, Figure 6A-G) for substantial part of experiments and co-wrote the manuscript with Dr. Ondřej Štěpánek.

Ad 4. I joined the project in its final phase. Nevertheless, I made a contribution to the project via developing and optimizing the mouse model of $CD4^+/CD8^+$ T-cell-induced diabetes, which was used for the characterization of functional properties of Tregs isolated from $MyD88^{\Delta TECs}$ mice. I performed and analyzed diabetes experiments, including survival monitoring, glucose measurements in blood and urine, and flow cytometry analysis of spleens as a final step of the experiment (Figure S8).

Ad 5. I contributed to the screening of relevant articles and co-wrote the literature review.

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8. Reprints of publications