Aus dem Department für Diagnostische Labormedizin der
Universität Tübingen

Institut für Medizinische Mikrobiologie und Hygiene

The Influence of Symbiont Bacteroides vulgatus on Th17 Differentiation via Dendritic Cells

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Klees, Johanna

2021

Dekan:	Professor Dr. B. Pichler
1. Berichterstatterin:	Professorin Dr. J.- S. Frick
2. Berichterstatter:	Professor A. Weber
Tag der Disputation:	25.03.2021

to my love Philip and my family

Contents

Index of Figures

Index of Tables

Index of Abbreviations

Actb ..... *gene of β-actin*
APC ..... *antigen-presenting cell*
B.vulgatus ..... *Bacteroides vulgatus*
B.vulgatus mpk ..... *Bacteroides vulgatus strain mpk*
BHI ..... *brain heart infusion*
BMDC ..... *bone marrow-derived dendritic cell*
CD ..... *cluster of differentiation*
DC ..... *dendritic cell*
DPBS ..... *Dulbecco's phosphate buffered saline*
E.coli ..... *Escherichia coli*
E.coli mpk ..... *Escherichia coli strain mpk*
EAE ..... *experimental autoimmune encephalomyelitis*
ELISA ..... *enzyme-linked immunosorbent assay*
FMO ..... *fluorescence minus one*
G-CSF ..... *granulocyte colony-stimulating factor*
HIES ..... *hyper IgE syndrome*
IBD ..... *inflammatory bowel disease*
IFNγ ..... *interferon gamma*
IL ..... *interleukin*
iTreg ..... *inducible regulatory T cell*
IκBζ ..... *inhibitor of nuclear factor kappa B zeta*
LAC ..... *leukocyte activation cocktail*
LAP ..... *latency associated peptide*
LB ..... *Luria-Bertani*
MACS ..... *magnetic activated cell sorting*
MHC II ..... *major histocompatibility complex class II*
MOI ..... *multiplicity of infection*
mpk ..... *Max von Pettenkofer-Institute*
MS ..... *multiple sclerosis*
Mst1 ..... *gene of MST1*
MST1 ..... *mammalian sterile 20-like kinase 1*

Nfkbiz ..... *nuclear factor kappa B inhibitor zeta-gene*
NF-κB ..... *nuclear factor kappa B*
OMV ..... *outer membrane vesicle*
OVA..... *ovalbumin*
PFA ..... *paraformaldehyde*
PMA ..... *phorbol 12-myristate 13-acetat*
PRR..... *pattern recognition receptor*
qRT-PCR..... *quantitative reverse transcription polymerase chain reaction*
RA ..... *rheumatoid arthritis*
Rag1..... *recombination activating gene 1*
Rag2..... *recombination activating gene 2*
rIL-17 ..... *recombinant IL-17*
rIL-22 ..... *recombinant IL-22*
RORγt ..... *RAR-related orphan receptor gamma t*
ROS ..... *reactive oxygen species*
RT ..... *room temperature*
SD ..... *standard deviation*
SFB ..... *segmented filamentous bacteria*
SPF ..... *specific-pathogen-free*
STAT3 ..... *signal transducer and activator of transcription 3*
Th cell..... *T helper cell*
Th1 cell..... *T helper 1 cell*
Th17 cell..... *T helper 17 cell*
Th2 cell..... *T helper 2 cell*
TLR ..... *Toll-like receptor*
TNFα ..... *tumor necrosis factor alpha*
Treg cell..... *regulatory T cell*
Vβ5.1 ..... *T cell receptor Vβ5.1*
WT..... *wild-type*

1 Introduction

1.1 Intestinal Microbiota

The human body is a complex system: Diverse tissues are made up of various cell types, that all cooperate to fulfil the tasks necessary for life. But this is not enough. A healthy living individual is dependent on myriads of bacteria, archaea, fungi and protozoa that cover all its outer and inner contact surfaces like the skin and the intestinal mucosa. Bacteria form the majority. They outnumber human nucleated cells 10:1 (Luckey, 1972, Savage, 1977, Sender et al., 2016). The biggest fraction of bacteria, about 10 billion, reside in the colon (Luckey, 1972, Sender et al., 2016).

1.1.1 Symbionts and pathobionts – *Bacteroides vulgatus* and *Escherichia coli*

Organisms of the intestinal microbiota can reside transiently or permanently in the digestive tract. Their effects on their host organism range from beneficial to harmful, which may change, depending on the circumstances. Simplified, considering their effect on the host organism, they can be subdivided into pathogens and commensals, that comprise symbionts and pathobionts (Figure 1.1.1).

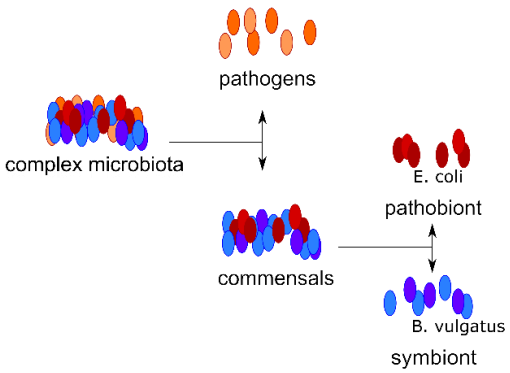

Figure 1.1.1: Composition of bacterial microbiota

Commensals are microbes that permanently reside in our bodies. Symbionts are defined as advantageous commensals, while pathobionts are mostly harmless commensals in healthy individuals. However, they are potentially harmful in predisposed hosts, where they can promote diseases or autoimmune reactions.

Pathogens are damaging and lead to diseases, following Koch's third postulate (Hornef, 2015). In a healthy host organism, symbionts and pathobionts outbalance pathogens by growth competition. Disturbances of this balance arise through defects in immunity or iatrogenic alterations of microbiota composition. This can, e.g., result from antibiotic therapy. It may lead to infectious diseases or autoimmune disorders like inflammatory bowel disease (IBD), an umbrella term for Crohn's disease and ulcerative colitis (Hornef, 2015).

One symbiotic representative of the human and the murine gut microbiome is *Bacteroides vulgatus*. *B. vulgatus* has been reported to have pathobiont features in literature before (Bamba et al., 1995). However, this does not seem to be true for every strain. The strain *B. vulgatus* mpk has even been shown to act anti-inflammatorily and thus aid equilibrium of the immune system in several studies (Waidmann et al., 2003, Frick et al., 2010, Muller et al., 2008, Steimle et al., 2016b). This illustrates that the complex effects of bacteria might differ even within species level. Another representative of *Bacteroidetes*, the symbiont *Bacteroides fragilis*, has been demonstrated to suppress intestinal inflammation in animals with *Helicobacter hepaticus*-induced colitis (Mazmanian et al., 2008). Besides, patients with IBD show lower proportions of *Bacteroidetes* in their intestines than healthy controls (Frank et al., 2007, Ott et al., 2004). All of this indicates a role of some *Bacteroidetes* in intestinal homeostasis. On the pathobiont side, there are strains of the widely known *Escherichia coli*, e.g. the strain *E. coli* mpk.

1.1.2 Influences on composition and function of intestinal microbiota

Though most organisms host microbes in their gastrointestinal system, the composition of microbiota seems to differ considerately between species (Ley et al., 2008). Even within a species, each individuum harbours its own unique set of microbiota (Human Microbiome Project, 2012). The distribution of microbes in the gut seems to depend on the genetics of the host organism and external influences like long-term nutrition or use of antibiotics. (Goodrich et al., 2014, Wu et al., 2011, De Filippo et al., 2010, Ley et al., 2008, Blekhman et al., 2015, Jernberg et al., 2007). In humans, two bacterial divisions predominate this microbial microenvironment: *Firmicutes* and *Bacteroidetes* (Hold et al., 2002). The murine

microbiota shows this abundance of *Firmicutes* or *Bacteroidetes,* as well, while differing on the level of genera (Ley et al., 2005). Mice are therefore considered a useful animal model in the study of host-microbiota interactions (Ley et al., 2005, Low et al., 2013).

The microbiota is linked to many systemic functions of the host: It supports digestion (Almquist et al., 1938, Bentley and Meganathan, 1982), drug metabolism (Backhed et al., 2005) and influences body weight (Ley et al., 2005, Ridaura et al., 2013, Turnbaugh et al., 2009). Importantly, commensals also shape the immune system, e.g., by training immune cells (Backhed et al., 2005, Mazmanian et al., 2005), by inducing defensive (Macpherson et al., 2000) or regulatory mechanisms (Zaph et al., 2008, Steimle et al., 2016a). Exposition to a wide range of microbiota during the neonatal period is beneficial. In mice, animals without such contact in early life showed signs of dysregulation of the immune system and were more likely to develop an inflammation (Olszak et al., 2012).

1.2 Mucosal Immunity

The intestines are hollow organs with the obvious task of facilitating digestion and nutrient uptake. They resemble a muscular tube with a luminal mucosa consisting of an epithelial lining and underlying lamina propria. As ingested materials might be potentially harmful and as especially the colon is inhabited by many microbes, this mucosa is also in charge of upholding an immunological barrier that keeps a balance between warding off pathogens and tolerating symbiotic commensals. Several mechanisms contribute to this task (synopsis in Figure 1.2.1).

The first barrier is a physical one: A single layer of mucus lines the small intestines and the colonic epithelium is coated by a loose outer and a dense inner layer of mucus. It seems that bacteria usually do not penetrate this inner layer in bigger numbers (Johansson et al., 2008), though a mechanism of crossing of outer membrane vesicles (OMVs) has been described by our group and might be important for immune modulation by commensal bacteria (Maerz et al., 2017).

Beside this and other, e.g. B cell-mediated mechanisms, T cell-mediated processes are crucial: Cells of high importance in this context are dendritic cells (DCs), T helper17 (Th17) cells and regulatory T (Treg) cells. They are described in the next sections in more detail.

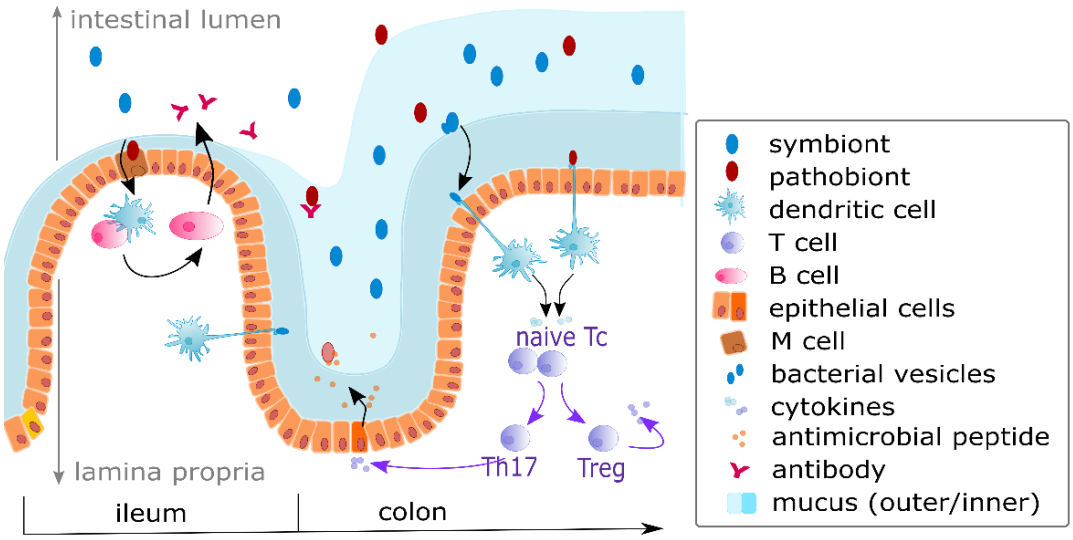

Figure 1.2.1: The mucosal barrier under homeostatic conditions
Depicted are the intestinal lumen, the outer and inner mucus layer, with differential distribution of commensal symbiotic and pathobiontic bacteria and the epithelium and lamina propria of the mucosa. Host contact with bacterial antigen is mediated by dendritic cells or M cells. M cells transcytose antigens into the lamina propria, where B cells are triggered to produce IgA antibodies that are transported into the mucus layer. Dendritic cells probe the mucosal surface by extending their long dendrites and induce Treg or Th17 cells, dependent of stimulus and surrounding microenvironment. Treg cells release ameliorating cytokines, dampening immune response, while Th17 cells release IL-22 and aid production of antimicrobial peptides by epithelial cells.

1.2.1 Dendritic cells

DCs subsume a heterogenous group of short-lived antigen-presenting cells (APCs) with branch-like protrusions, called dendrites (Steinman and Cohn, 1973). Precursors can be both lymphoid and myeloid progenitor cells. They occur in most body tissues, prominently in the epidermis and mucosa and in lymphatic organs like lymph nodes or the spleen, travelling from one to another via the blood or lymph. Differences in phenotype, function and predominant localization are used to define distinct subsets of DCs (Steimle and Frick, 2016, Johansson and Kelsall, 2005). Bone marrow-derived DCs (BMDCs) are well-established cultivated dendritic-like cells for *in vitro* studies (Lutz et al., 1999).

Function and maturation states of dendritic cells

Traditionally, DCs are regarded the most efficient APCs (Inaba et al., 1983). They carry pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) on their surface that bind antigens. Stimulation of these receptors triggers maturation

of DCs. Upon antigen contact, DCs incorporate, process and, when activated, display fragments of these antigens on MHC II molecules on their surface, together with co-stimulatory proteins such as CD40 and CD80/86 (Fujii et al., 2004). The encounter with naïve T cells is thought to take place mainly in lymph nodes, where stimulated DCs actively migrate to. Antigen presentation, co-stimulation and secretion of stimulatory cytokines together activate T cells to polarize into different subtypes and proliferate (Mellman and Steinman, 2001, Rescigno and Di Sabatino, 2009). This initiates an adaptive immune response.

Correspondingly to these functions, DCs isolated from body tissue show distinct stages of maturation (Pierre et al., 1997). In immature DCs, phagocytic processes are highly active, while the cells feature only small amounts of antigen-displaying MHC II on their surface. In mature DCs, MHC II and co-stimulatory protein display is intensified, while phagocytic abilities decrease immensely. So, translated into function, immature DCs are antigen-uptakers that then change into mature DCs that are antigen-presenters (Mellman and Steinman, 2001).

But, lack of DCs also results in a decrease in self-tolerance and emergence of autoimmune processes (Ohnmacht et al., 2009). These and other findings suggest that DCs are also involved in establishment of self-tolerance. Correspondingly, a third stage of maturation has been described, that is called semimaturation. A global definition of semimaturity is difficult. Functionally, semimature DCs do not induce proinflammatory T cells like Th1 or Th17 cells, but rather are tolerogenic (Steimle and Frick, 2016). Furthermore, DCs in a semimature state can be subjected to maturation stimuli like contact to pathobionts without changing their phenotype (Frick et al., 2006, Steimle and Frick, 2016).

Mediation of immune tolerance by dendritic cells

Induction of a tolerant semimature DC phenotype, resulting in less T cell activation, has been shown by our group, e.g. in *Rag1*$^{-/-}$ mice. These mice are a model of a mixed Th1/Th17-mediated chronic colitis. The *Rag1* gene is essential for V(D)J recombination (Mombaerts et al., 1992). Thus, *Rag1*$^{-/-}$ mice do not have functional B and T cell receptors. Colitis in the *Rag1*$^{-/-}$ mouse model is induced by administration of wild-type (WT) naïve T cells to *Rag1*$^{-/-}$ mice. Inflammation in

this model goes along with IFNγ and IL-17 production in the absence of Treg cells (Ostanin et al., 2009). An *in vivo* mono-colonization of *Rag1*$^{-/-}$ mice with *B. vulgatus* mpk showed more DCs with a semimature phenotype and less T cell proliferation in colonic lamina propria isolates than mono-colonization with *E. coli* mpk (Steimle et al., 2016b). Thus, semimature DCs can support homeostasis and prevent uncontrolled inflammatory responses towards commensal bacteria (Steimle and Frick, 2016). This is of crucial importance, as body parts like the skin, oral cavity and the gut are habitats of useful microbial communities that need to be treated friendly by our immune system. Failure of auto-tolerance and tolerance of commensal microbes has been linked to severe pathologic states such as IBD or psoriasis (Ananthakrishnan, 2015, Sakaguchi et al., 2006).

1.2.2 T cells - Th17 cells and regulatory T cells

For decades it had been scientific consensus that T helper cells (Th cells) comprise two distinct lineages: Th1 and Th2 cells (Mosmann et al., 1986). But as additional lineages have been discovered, the picture has broadened. In context with autoimmune diseases, Th17 and Treg cells are of high importance (Noack and Miossec, 2014).

Th17 cells – differentiation, function and regulation

In mice, early differentiation towards Th17 cells is directed by the transcription factor STAT3 (Yang et al., 2007). STAT3 induces the expression of another transcription factor, the orphan nuclear receptor RORγt, that acts as main regulator of Th17 differentiation (Ivanov et al., 2006). Early induction of Th17 cells depends on the cytokines IL-6 and TGFβ1 (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006), while stabilization and activity are mediated by IL-23 (Langrish et al., 2005, Aggarwal et al., 2003, McGeachy et al., 2009) and expansion is aided by IL-1β (Gulen et al., 2010) and autocrine effects of IL-21 (Nurieva et al., 2007). Studies on human Th17 cells show high similarities to murine differentiation: TGFβ proved to be indispensable for induction of Th17 cells, in combination with either IL-6 and IL-23 or IL-21. ROR-c, a human homologue of the murine RORγt is the main regulator (Noack and Miossec, 2014, Yang et al., 2008a, Manel et al., 2008).

IL-6 is a pleiotropic acute phase protein and cytokine that is essential in infection control and inflammation. It has been observed to act pro- and anti-inflammatorily, depending on the context (Hunter and Jones, 2015). TGFβ is similarly pleiotropic in its functions, being a notable regulator of immune cells, e.g. by inducing Th17 cells and regulatory T cells, while repressing Th1, Th2 and cytotoxic T cells (Li et al., 2006). TGFβ exists in several subtypes, with TGFβ1 as the most abundant one. It is synthesized as a longer precursor together with the latency associated peptide (LAP). To become functional, this dimer needs to be disassembled (Annes et al., 2003). IL-23 is comprised of a subunit it shares with IL-12, IL-12p40, and a unique subunit, IL-23p19. It has mainly been associated with stabilization of Th17 cells and IL-17 secretion (Langrish et al., 2005).

Characteristic cytokines produced by Th17 cells are IL-17, IL-22 and GM-CSF (Zheng et al., 2007, Langrish et al., 2005). Cells that produce IL-17A constituently have been shown to reside mainly in the lamina propria of the small intestine and, in smaller amounts, in the colon (Ivanov et al., 2006). IL-17, more precisely IL-17A, as a representative of the IL-17 cytokine family, has a broad range of effects. Its influence is exerted on immune cells like neutrophils, but also on non-hematopoietic cells like epithelial cells and fibroblasts. E.g., the cytokine upregulates gene expression of cytokines like IL-6, TNFα or G-CSF (Onishi and Gaffen, 2010), thus regulating neutrophilic response. IL-22, together with IL-17, induces the expression of antimicrobial peptides such as defensins, e.g. in keratinocytes (Wolk et al., 2004). Correspondingly, Th17 cells seem to be involved in defence against a range of pathogens that are inadequately addressed by mere Th1 reaction, especially fungi. Examples are *Candida albicans (Huang et al., 2004, Eyerich et al., 2008)*, that have been studied in mice and humans, and *Pneumocystis jirovecii* and its murine equivalent *Pneumocystis carinii (Rudner et al., 2007)*. Defects in the IL-23/IL-17 axis have been described for patients with an autosomal dominant form of a disorder called the hyper IgE syndrome (HIES), in which among other symptoms mucocutaneous candidiasis and severe pulmonary infections occur repeatedly (Milner et al., 2008). This is further indirect evidence for the relevance of Th17 in pathogen clearance.

However, effective pathogen clearance capability can turn into susceptibility to autoimmune disorders. Th17 cell involvement has been implied in human IBD (Duerr et al., 2006, Liu et al., 2015, Fujino et al., 2003), asthma (Molet et al., 2001), psoriasis (Blauvelt, 2008), rheumatoid arthritis (RA) (Kirkham et al., 2006) and multiple sclerosis (MS) (Matusevicius et al., 1999, Tzartos et al., 2008).

T cell differentiation is regulated by DCs. As DCs are in contact not only with pathogenic microbes but also with commensals, this interaction is likely to influence Th17 induction in the intestines. On the one hand, commensal bacteria such as segmented filamentous bacteria (SFB) induce Th17 cells in the small intestines indirectly through DCs, thus possibly contributing to mucosal clearance of pathogens (Goto et al., 2014). On the other hand, exposure to commensal LPS of low endotoxicity aids to maintain intestinal homeostasis (Gronbach et al., 2014, Steimle et al., 2019).

A key cytokine for Th17 induction released by DCs is the acute phase protein IL-6. Its production is influenced by many regulatory processes. E.g., in some cells it is increased by inhibitor of NF-κB zeta (IκBζ) (Kitamura et al., 2000), and in DCs decreased by MST1 via a MST1-p38MAPK pathway (see below) (Li et al., 2017). IκBζ is an atypical IκB protein, a protein family first described as inhibitors of the cell regulatory protein nuclear factor kappa B (NF-κB) (Kitamura et al., 2000, Yamazaki et al., 2001). NF-κB is a protein dimer that as a transcription factor has numerous effects on cell cycle, apoptosis and inflammation (Oeckinghaus and Ghosh, 2009). In contrast to its name, IκBζ, in association with the NF-κB subunit p50, is no inhibitor but an activator and acts as a coregulatory transcription factor. It is involved in upregulating the transcription of proinflammatory molecules like IL-6 in many cell types, e.g. macrophages and T cells (Kitamura et al., 2000, Libermann and Baltimore, 1990, Yamamoto et al., 2004, Okamoto et al., 2010). Additionally, it has been shown that transfer of *Nfkbiz*$^{-/-}$ T cells, cells that lack the gene of IκBζ, into *Rag2*$^{-/-}$ mice, does not lead to inflammation (Okamoto et al., 2010). In this mouse model transferral of functional Th1 and Th17 cells induces experimental autoimmune encephalomyelitis (EAE), a model for MS. IκBζ seems to regulate Th17 differentiation indirectly through positive effects on the transcription factor RORγt,

its absence thus preventing from Th17-induced EAE (Okamoto et al., 2010). In this context, it is noteworthy that *Nfkbiz* has also been shown to be a susceptibility locus for the autoimmune diseases IBD (Liu et al., 2015) and psoriasis (Tsoi et al., 2015).

Mammalian sterile 20-like kinase 1 (MST1), also known as kinase responsive to stress 2 or serine/threonine kinase 4 (STK4), is a ubiquitous mammalian kinase *(Creasy and Chernoff, 1995)*. It contributes to such different processes as regulation of cell cycle and apoptosis in different tissues (Song et al., 2010, Graves et al., 1998) and reactive oxygen species (ROS) production in phagocytes (Katagiri et al., 2009, Geng et al., 2015). Recently, it has been shown to play a crucial role in Th17 differentiation as negative regulator of IL-6 production in DCs via a MST1-p38MAPK signaling pathway (Li et al., 2017).

Treg cells - differentiation and function

Treg cells are a line of T cells that have suppressive function on the immune system. Their phenotype is $FOXP3^+CD3^+CD4^+CD25^+$ and they produce IL-10 and TGFβ (Noack and Miossec, 2014, Fontenot et al., 2005). Inducible regulatory T cells (iTregs) derive from $CD4^+$ naïve T cells in the periphery of the body, e.g. following stimulation by mesenteric lymph node DCs in the intestines (Coombes et al., 2007). Induction of iTreg cells is orchestrated by the transcription factor FOXP3 (Fontenot et al., 2005). FOXP3 is induced by the cytokine TGFβ (Chen et al., 2003) and by retinoic acid (Mucida et al., 2007, Coombes et al., 2007). Beneficial for Treg cell expansion is IL-10 (Hsu et al., 2015).

These iTreg cells mediate tolerance to autoantigens and non-pathogenic antigens (Harrison and Powrie, 2013). They control other T cells, e.g. by secreting inhibitory cytokines, such as IL-10 and TGFβ. This is of special importance once a pathogen has been successfully fought and immune homeostasis needs to be re-established to prevent chronic inflammation (Sakaguchi et al., 2006).

Relationship of Th17 and iTreg cells

Th17 and iTreg cells are reciprocally regulated: Both Th17 and iTreg cells are induced by TGFβ. In combination with IL-6, FOXP3 induction by TGFβ is inhibited and instead STAT3 and RORγt are upregulated, favouring Th17 cell differentiation (Bettelli et al., 2006, Yang et al., 2007). IL-6 deficiency or high concentrations of TGFβ lead to domination of Treg cells (Bettelli et al., 2006, Zhou et al., 2008). Besides, retinoic acid and IL-2 suppress Th17 cell differentiation as well, supporting instead iTreg cell differentiation (Coombes et al., 2007, Mucida et al., 2007).

It is now known that Th cells are cells of high plasticity (Figure 1.2.2). Th17 and iTreg cells convert into the other cell type under the right stimuli (Yang et al., 2008b). E.g., human iTreg cells can be reprogramed into Th17 cells under influence of IL-1β (Koenen et al., 2008). In other context, continuing stimulation of Th17 cells with IL-6 and TGFβ leads to production of IL-10 in addition to IL-17, forming a subset of “protective Th17” (McGeachy et al., 2007). In inflammatory tissue Th17 cells that are continually stimulated with IL-12 or IL-23 may also turn into Th1-like Th17 cells that release IFNγ (Lee et al., 2009, Annunziato et al., 2007, Boniface et al., 2010). Most surprising, perhaps, are findings concerning a group of Treg cells that can inhibit Th17 cells or produce IL-17 themselves, depending on the nature of their surrounding milieu (Beriou et al., 2009). These findings of additional complexity in the commitment of T cells show how crucial microenvironmental stimuli like cytokines are for T cell differentiation.

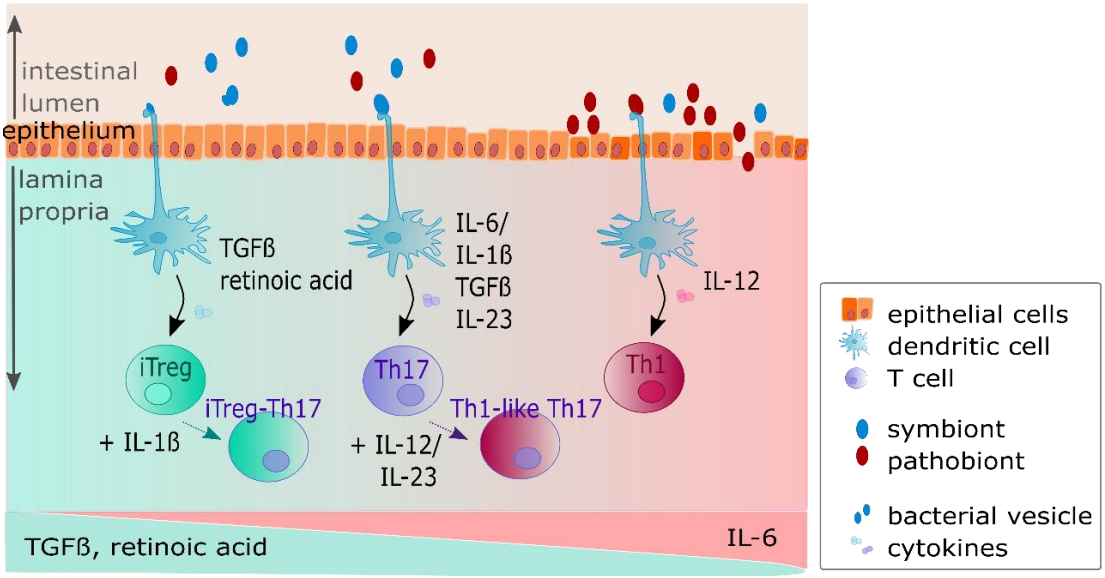

Figure 1.2.2: Plasticity of Th cells and Treg cells of the intestinal mucosa
adaptation after (Bhaumik and Basu, 2017). Continuum of anti- to pro-inflammatory conditions from left to right represented by barrier functionality of the epithelium and predominance of TGFβ vs. IL-6. Activated DCs release different cytokines that drive iTreg, Th17 or Th1 differentiation. Additional microenvironmental conditions can lead to conversion to other cell types.

1.3 T cell-mediated Autoimmunity

To maintain a physiological state, sorting out auto-reactive immune cells and preserving the balance of pro- and anti-inflammatory signals requires the immune system to undergo many steps. Especially Th cells, as a core element of control of adaptive immunity, need to be kept in check. A high degree of inhibition is of importance (Littman and Rudensky, 2010), as is an intact mucosal barrier (Wehkamp et al., 2016). Autoimmune reactions can thus be viewed as a state of failed auto-tolerance.

1.3.1 Epidemiology and pathophysiology of T cell-mediated autoimmune diseases

T cell-mediated autoimmune diseases are common. By IBD, comprising Crohn's disease and ulcerative colitis, in Europe alone an estimated 2.2 million people are affected (Ananthakrishnan, 2015), while incidence and prevalence are increasing worldwide (Molodecky et al., 2012).

The pathophysiology of T cell-mediated autoimmune conditions such as IBD has not been completely unravelled so far. Aetiology seems to be multifactorial, starting with a genetically susceptible precondition, continuing with an aberrant gut microbiome and including environmental factors such as diet, physical activity, sleep, stress and smoking that influence immune system as well as microbiome (Ananthakrishnan, 2015). It is thought that dysfunctional immunological processes and mucosal barrier malfunction lead to inflammation of the intestines (Wehkamp and Frick, 2017). In Crohn's disease, this inflammation is mostly transmural and can occur in every part of the intestinal tract in a discontinuous way (Van Assche et al., 2010). In ulcerative colitis, intramucosal inflammation starts from the rectum and proceeds in oral direction, while usually only affecting the colon. Bacterial invasion of the mucosa and high counts of lymphocytes in the tissue are common (Herold, 2019). European twin studies suggest genetic involvement (Tysk et al., 1988, Thompson et al., 1996, Halme et al., 2006, Orholm et al., 2000). Genetic susceptibility is indicated by genetic association studies, as well (Liu et al., 2015, Cho, 2008). It seems to be based on defects of several immune axes. In context with Crohn's disease, involvement of the IL-23/IL-17 axis has been implied. The *IL23R* gene was associated with Crohn's disease in a genome-wide association study (Duerr et al., 2006), as well as other genes involved in Th17 regulation, such as *Nfkbiz*, the gene encoding transcription factor IκBζ (Liu et al., 2015). Involvement of the IL-23/IL-17 axis fits observations that chronic inflammation of intestinal mucosa is mostly mediated by Th1 and Th17 cells (Brand, 2009). Findings are similar for other T cell-mediated autoimmune conditions: IL-17 was detected in high levels in the synovial fluid of patients of RA, and both the cytokine IL-23 and Th17 cells induce EAE (Cua et al., 2003, Langrish et al., 2005).

Additionally, other studies point to involvement of the intestinal microbiota: Alterations of the microbiota in newly diagnosed underage IBD patients has been observed and correlated with severity of inflammation, showing a decrease in the presence of, e.g., *Bacteroidales* and *Clostridiales*, and an increased occurrence of, e.g., *Enterobacteriaceae* and *Fusobacteriaceae* (Gevers et al., 2014). It is yet unknown if these changes proceed disease onset or if they are a consequence

of inflammation. Mere microbial alterations do not seem to be sufficient for IBD development (de Souza and Fiocchi, 2016), but an increased risk of IBD has been associated with antibiotic treatment in early childhood and low LPS exposure during life (Hviid et al., 2011, Boneberger et al., 2011), while broad-spectrum antibiotics that alter the intestinal microbiota are successfully used in treatment (Perencevich and Burakoff, 2006). Some bacterial species have been attributed ameliorating effects in acute colitis in mice, e.g. *Faecalibacterium prausnitzii* (Zhou et al., 2018).

Previously discussed causes for IBD are joined by environmental factors. For instance, smoking and stress have negative effects on Crohn's disease and a diet high in soluble fibres shows positive effects (Ananthakrishnan, 2015).

All in all, aetiology of T cell-mediated inflammatory states is multifactorial, including genetic disposition, environmental factors and altered microbiota.

1.3.2 Treatment options in T cell-mediated autoimmune diseases

Until recently, T cell-mediated autoimmune diseases have been treated with corticosteroids and immunomodulators such as azathioprine and TNFα-blockers (Preiss et al., 2014). Insights in disease mechanisms of T cell-mediated inflammation are now applied in targeted therapies: E.g., anti-IL-12p40 and anti-IL-23p19, antibodies that aim at the two subunits of IL-23, and anti-IL-17 antibodies have been tested for several autoimmune conditions such as psoriasis and IBD, resulting in high disease clearance in psoriasis for all agents (Molinelli et al., 2017). IBD results are more confusing. When IL-17 was targeted, inflammation even worsened, suggesting a protective effect of low IL-17 levels (Gaffen et al., 2014, Hueber et al., 2012). Therefore, a differentiated use of biological agents might be beneficial in T cell-mediated autoimmune diseases. Alteration of the microbiota have been proposed as treatment option as well. So far, probiotics or faecal microbiota transplantation, containing potentially useful commensal bacteria, have not shown significant efficacy. Meanwhile, prebiotics, that contain nutrients favouring potentially beneficial symbionts, have shown promising reduction of intestinal inflammation in mice and reduced the risk of exacerbation in patients with Crohn's disease, but need additional studies to confirm these effects (Lane et al., 2017).

1.4 Previous Work

Our group aims at a more thorough understanding of host-microbiota interactions in inflammatory disorders, in order to develop new microbiota-derived components as useful therapeutic agents for inflammatory disorders.

In previous studies, it has been demonstrated, both *in vitro* and *in vivo,* that host interaction with the symbiont *B. vulgatus* mpk can drive immature DCs into a tolerant semimature phenotype that is not susceptible to stimulation with pathobiont *E. coli* mpk. This is accompanied by less Th1 cell activation by these DCs (Frick et al., 2006). *In vivo* treatment of gnotobiotic *IL-2*$^{-/-}$ mice with the symbiont *B. vulgatus* mpk was shown to prevent from pathobiont *E. coli* mpk-induced colitis (Waidmann et al., 2003). *IL-2*$^{-/-}$ mice have been used as model for a host that develops a colitis caused by microbiota-triggered T cell overreaction (Sadlack et al., 1995, Autenrieth et al., 1997).

Effects of *B. vulgatus* mpk on general Th cell development was also represented in lower $CD4^+$T cell counts in lamina propria of *Rag1*$^{-/-}$ mice treated with *B. vulgatus* mpk compared to mice treated with *E. coli mpk* (Steimle et al., 2016b). *Rag1*$^{-/-}$ mice are thought to model a chronic colitis mediated by a mixed Th1/Th17 response, as IFNγ and IL-17 are produced (Mombaerts et al., 1992, Ostanin et al., 2009). Additionally, it has been reported that this colitis can be induced by TGF-β-and-IL-6-polarized Th17 cells (Lee et al., 2009). Ameliorating effects of *B. vulgatus* mpk in *Rag1*$^{-/-}$ mice could be linked to the limited endotoxic potential of its surface LPS towards DCs (Steimle et al., 2019).

Another mouse model that points to involvement of the IL-23/IL-17 is based on *Il10*$^{-/-}$ mice. In this model, mice usually develop spontaneous colitis due to lack of IL-10-mediated regulation. But by crossing them with *Il23p19*$^{-/-}$ mice, that have no Th17 cells, colitis development is prevented (Yen et al., 2006).

One of the cytokines downstream of TLR-associated LPS signaling is IL-6. IκBζ, as a transcription factor of IL-6, was shown to play a role in context of murine colitis (Klameth, 2012). Other regulatory proteins, A20 and MCPIP-1, have been in focus in a project parallel to the study presented in this work (Birg, 2017).

1.5 Motivation and Aim of this Work

Autoimmune diseases show a considerable prevalence and increasing incidence (Molodecky et al., 2012). They pose a high burden for normal life and even are one of the prominent causes of death for young women (Cooper and Stroehla, 2003). Research in the field of underlying immunological processes is therefore highly important.

The pathophysiology of T cell-mediated autoimmune inflammation like IBD is nowadays thought to be triggered and sustained by a combination of Th17 and Th1 cells (Brand, 2009). Microbiota have been shown to be both promoting and protective in pathophysiology of autoimmune colitis (Wehkamp and Frick, 2017). *B. vulgatus* mpk is a promising candidate for work on preventive microbiota in face of autoimmune colitis (Frick et al., 2006). Based on the literature findings described above and previous work in our lab, it is of interest to investigate *B. vulgatus* mpk in the context of Th17 polarization.

Thus, this work aims at clarifying the influence of *B. vulgatus* mpk on Th17 induction by DCs on cytokine level and underlying regulatory processes. It investigates if *B. vulgatus* mpk-primed semimature DCs are less prone to induce Th17 cells compared to *E. coli* mpk-primed mature DCs. Possible related differences in cytokine levels, which are produced by DCs and involved in T cell differentiation, are addressed. Two in this context putative cytokine regulatory proteins in DCs, IκBζ and MST1, are checked for reactions on mRNA level to priming of the DCs with *B. vulgatus* mpk or *E. coli* mpk. In context of the role of semimature DCs in relation to Th17 cells, the last experiments investigate if there is a retrograde influence of Th17 signature cytokines on DCs.

2 Material and Methods

2.1 Material

2.1.1 Mice and bacteria

All mice used to isolate BMDCs for *in vitro* experiments were females of the inbred strain C57BL/6 purchased from Charles River. Their age ranged from three to six months. For isolation of Vβ5.1$^+$ naïve T cells, mice of the inbred line OT II/Bl6 were used (Barnden et al., 1998). Mice were kept under SPF conditions. The animal experiments were conducted within the frame of test numbers GVO 09.01.15 and H9/11.

Bacteria strains in use were *Bacteroides vulgatus* mpk (*B. vulgatus* mpk) and *Escherichia coli* mpk (*E. coli* mpk), both isolated from feces of a healthy mouse at the Max von Pettenkofer-Institute in Munich (Waidmann et al., 2003).

2.1.2 Equipment, chemicals and media

Table 2.1.1: Consumption items

material	manufacturer
1.5-mL/ 2-mL reaction tubes	Eppendorf, Hamburg, Germany
petri dishes	Greiner bio-one, Kremsmünster, Austria
Falcon tubes	Falcon, Corning, NY, USA
pipette tips	Sarstedt, Nümbrecht, Germany
10-mL syringes	B. Braun, Melsungen, Germany
27-gauge needles	BD Biosciences, San Jose, CA, USA
100-µm cell sieve	Greiner bio-one, Kremsmünster, Austria
plastic cell lifter	Corning Inc., Corning, NY, USA
6-well plates, 96-well plates	Falcon, Corning, NY, USA
flow cytometry tubes	Falcon, Corning, NY, USA
PCR 96-well plates	nunc, Roskilde, Denmark

Table 2.1.2: Technical devices

technical device	manufacturer
incubators	Thermo Fisher, Waltham, MA, USA; Heraeus, Haunau, Germany
incubator shaker	Infors AG, Bottmingen, Switzerland
laminar flow bench	BDK, Germany
DNA/RNA UV cleaner box	Biosan, Riga, Latvia
microscopes	Zeiss, Jena, Germany Olympus D., Hamburg, Germany
centrifuges	Eppendorf, Hamburg, Germany Heraeus, Hanau, Germany
flow cytometer LSRFortessa	BD Biosciences, San Jose, CA, USA
ELISA washer (M8/4R)	Tecan, Männedorf, Switzerland
photometer	Eppendorf, Hamburg, Germany
microplate reader	Tecan, Männedorf, Switzerland
Lightcycler 480	Roche, Basel, Switzerland
scales (1 mg – 210 g)	Sartorius, Göttingen, Germany

Table 2.1.3: Kits

kit	manufacturer
mouse IL-23 ELISA Kit	R&D, Minneapolis, MN, USA
mouse IL-6 ELISA Kit	BD Biosciences, San Jose, CA, USA
mouse IL-10 ELISA Kit	BD Biosciences, San Jose, CA, USA
mouse free active TGFβ1 ELISA Kit	Biolegend, San Diego, CA, USA
mouse naïve $CD4^+$ T cell isolation Kit	Miltenyi Biotec, Bergisch Gladbach, Germany
QuantiFastSYBRGreen RT-PCR Kit	Qiagen, Hilden, Germany
Anti-Rat/Anti-Hamster Ig κ Compensation Particles Set	BD Biosciences, San Jose, CA, USA
CBA Flex Set	BD Biosciences, San Jose, CA, USA
CBA Flex Set Master Buffer Kit	BD Biosciences, San Jose, CA, USA

Table 2.1.4: Chemicals

chemical	manufacturer
VLE RPMI 1640 cell culture medium	Biochrom, Berlin, Germany
fetal calf serum (FCS)	Sigma-Aldrich, München, Germany
phosphate buffered saline (PBS)	Gibco, Thermo Fisher, USA
Dulbecco's phosphate buffered saline (with Ca^{2+} and Mg^{2+}, DPBS)	Gibco, Thermo Fisher, USA
non-essential aminoacid solution	Biochrom, Berlin, Germany
penicillin/streptomycin (Pen/Strep)	Biochrom, Berlin, Germany
gentamycin	Biochrom, Berlin, Germany
β-mercaptoethanol	Biochrom, Berlin, Germany
granulocyte macrophage colony-stimulating factor (GM-CSF)	own production
AnaeroGen 2.5 L sachets	Thermo Scientific, MA, USA
Trypan Blue	Sigma-Aldrich, München, Germany
paraformaldehyde	Sigma- Aldrich, München, Germany
GolgiStop (contains monensin)	BD Biosciences, San Jose, CA, USA
Cytofix/Cytoperm fixation/permeabilization solution	BD Biosciences, San Jose, CA, USA
Perm/Wash buffer (contains saponin)	BD Biosciences, San Jose, CA, USA
chicken ovalbumin peptide (323-339) sequence: ISQAVHAAHAEINEAGR	EMC, Tübingen, Germany
leukocyte activating cocktail LAC (contains PMA, ionomycin, brefeldin A)	BD Biosciences, San Jose, CA, USA
rIL-17	Thermo Fisher, Waltham, MA, USA
rIL-22	R&D, Minneapolis, MN, USA

Cell culture

Table 2.1.5: DC medium

ingredient	volume of ingredient [mL]
RPMI	500
FCS	50
non-essential aminoacid solution	5
penicillin/streptomycin	5
β-mercaptoethanol	2.5
GM-CSF	30

Table 2.1.6: Erythrocyte lysis buffer

ingredient	mass of ingredient [g] in 1 L of purified water
NH_4Cl	8.3
$NaHCO_3$	1
Na_2EDTA	3.7

Bacterial culture

As liquid mediums, Luria-Bertani (LB) medium with and without bovine liver and Brain Heart Infusion (BHI) medium of in-house production were used.

Flow cytometry

Table 2.1.7: Staining mix for intra- and extracellular staining

ingredient	volume [µL] for 1 sample
Perm Wash buffer	50
antibody solution for surface antigens	0.5
antibody solution for intracellular antigens	1

Table 2.1.8: Staining mix for purely extracellular staining

ingredient	volume [µL] for 1 sample
PBS + 1% FCS	50
antibody solution for surface antigens	0.5

Table 2.1.9: Flow cytometry antibodies

antibody	fluorochrome	manufacturer
anti-mouse CD11c	APC, PE-Cy7	BD Biosciences, San Jose, USA
anti-mouse CD4	APC, Bv421	BD Biosciences, San Jose, USA
anti-mouse CD3	FITC	BD Biosciences, San Jose, USA
anti-mouse MHC II	FITC	BD Biosciences, San Jose, USA
anti-mouse IL-6	PE	BD Biosciences, San Jose, USA
anti-mouse IL-10	Bv510, APC	BD Biosciences, San Jose, USA
anti-mouse IL-17A	AF488, APC-Cy7	BD Biosciences, San Jose, USA
anti-mouse IL-21	PE	eBioscience, San Diego, CA, USA
anti-mouse IL-22	PerCP Efluor710	eBioscience, San Diego, CA, USA
anti-mouse IL-23p19	AF647	BD Biosciences, San Jose, USA
anti-mouse TGFβ1/LAP	Bv421	BD Biosciences, San Jose, USA
anti-mouse IL-17R	PE	eBioscience, San Diego, CA, USA
anti-mouse IL-21R	PE	BD Biosciences, San Jose, USA
anti-mouse IL-22R	FITC	biorbyt, Cambridge, UK
anti-mouse IFNγ	PE-Cy7	BD Biosciences, San Jose, USA
anti-mouse FOXP3	PE	BD Biosciences, San Jose, USA

Real-time qRT-PCR

Table 2.1.10: Real-time qRT-PCR mix

ingredient	volume [µL] for 1 well
2X QuantiFast SYBR Green RT-PCR Master Mix	5
forward primer (100 µM)	0.1
reverse primer (100 µM)	0.1
nuclease free water	3.7
QuantiFast RT Mix	0.1

For the total volume of the real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) mix the volumes given above need to be multiplied by the number of requested PCR samples. The QuantiFast RT Mix, containing reverse transcriptase, is only added right before use of the master mix.

Table 2.1.11: Primers for real-time qRT-PCR

primer (100 µM)	sequence	manufacturer
forward *Nfkbiz* (IκBζ)	gctccgactcctccgatttc	Biomers, Ulm, Germany
reverse *Nfkbiz*	gagttcttcacgcgaacacc	Biomers, Ulm, Germany
forward *Mst1* (MST1)	tgtggggctggttctgtatc	Biomers, Ulm, Germany
reverse *Mst1*	cctctttgccatggtatctgtaag	Biomers, Ulm, Germany
forward *Actb* (β-Actin)	ccctgtgctgctcaccga	Biomers, Ulm, Germany
reverse *Actb*	acagtgtgggtgaccccgtc	Biomers, Ulm, Germany

2.1.3 Software

Table 2.1.12: Software

software	manufacturer
FACSDiva	BD Biosciences, San Jose, CA, USA
FlowJo, X.0.7	FlowJo LLC, OR, USA
LightCycler 480, 1.5.0 SP3	Roche, Basel, Switzerland
Graphpad Prism, 5.04	Graphpad, La Jolla, CA, USA
Microsoft Excel, Version 1804	Microsoft, Redmont, WA, USA

2.2 Methods

2.2.1 Isolation of bone marrow-derived dendritic cells from mice for *in vitro* experiments

BMDCs, which are dendritic-like cells of a $CD11c^+$ phenotype, were generated out of murine bone marrow progenitor cells using an eight day protocol based on previous work from (Lutz et al., 1999). The mice were sacrificed on day 0 by exposure to CO_2 for several minutes and neck fracturing. Femur and Tibia were extracted, muscles removed, and joints cut away. The bone marrow was flushed out of the medullary cavity with a 27-gauge syringe and sterile DPBS, filtered through a 100 µm cell sieve and centrifuged at 400g for 5 min. After discarding the supernatant, cells were resuspended in BMDC cell culture medium. Viable cells were quantified using a Neubauer hemocytometer. $2x10^6$ cells per petri dish were seeded into 20 to 30 petri dishes per mouse filled with BMDC medium and incubated in an incubator at 37°C and 5% CO_2. At day 3, fresh BMDC cell culture medium was added. At day 6, 10 mL out of every petri dish was exchanged for fresh medium. The incubation was continued until day 7.

2.2.2 Cultivation of *B. vulgatus* mpk and *E. coli* mpk and stimulation of BMDCs with these bacteria or recombinant interleukines rIL-17 and rIL-22

As *Bacteroides* are slowly growing anaerobic bacteria, they were cultured for four days prior to use. 50 µL of *B. vulgatus* mpk stock suspension (stored at -80°C) was added to 5 mL of LB medium that contained a shred of bovine liver and kept at 37°C in an incubator under anaerobic conditions, using an air-tight container in which the oxygen was removed by an AnaeroGen 2.5 L sachet. After three days, 5 mL of the *B. vulgatus* mpk medium suspension was transferred into 45 mL of BHI medium and cultured as described previously.

50 µL of *E. coli* mpk stock suspension (stored at -80°C) was cultivated in 5 mL of LB medium under aerobic conditions over night at 37°C on an incubator shaker. 3-4 h before use, 400 µL of this suspension was added to 50 mL LB medium and cultured as before.

At day 7 of the BMDC isolation protocol, all petri dishes derived from one mouse were pooled into a falcon tube using a plastic cell lifter to detach adherent cells.

The cell suspension was spun down at 400g for 5 min and resuspended in 10 mL of DC medium before quantification with a Neubauer hemocytometer.

For the stimulation, several 6-well chambers for every test animal were seeded with $2x10^6$ cells, respectively. The wells were filled up to a volume of 2 mL with DC medium and incubated at 37°C and 5% CO_2 in an incubator for 1 h. Then a bacterial suspension of *B. vulgatus* mpk or *E. coli* mpk in DPBS was added in a multiplicity of infection (MOI) of 1, as well as 1 µL of gentamycin per mL medium to prevent bacterial growth. For the stimulation with the recombinant cytokines, rIL-17 or rIL-22 were added at an excess concentration of 100 ng/mL. This concentration was based on similar protocols in literature, e.g. (Sonder et al., 2011). In mock control wells, 100 ng/mL of DPBS was added instead. The wells were incubated at 37°C and 5% CO_2 for 4 - 16 h. For batches that were later stained intracellularly, 2 µL of GolgiStop were added to the wells 4 h prior to the stimulation endpoint, to trap newly produced secretory proteins inside the cell. After the stimulation period, cells were detached with a plastic cell lifter and the cell suspension was transferred into 2 mL test tubes for fixation and supernatant harvesting.

2.2.3 Isolation of naïve T cells and coculture of T cells with BMDCs in a T cell polarization assay

To isolate $CD3^+CD4^+CD25^{neg}CD45Rb^{hi}$ naïve T cells for a T cell proliferation assay, a naïve $CD4^+$ T Cell isolation Kit for mouse and a MACS-system (magnetic activated cell sorting) were used. All procedures were performed according to the manufacturer's manual.

Spleens from transgenic OT II/BL6 mice (Barnden et al., 1998) were extracted and strained through sterile sieves with ion-free PBS/ 1% FCS. The resulting cell suspension was filtered through a 100-µm sieve and spun down with 400g for 5 min. After discarding the supernatant, pellets were resuspended in 4 mL of erythrocyte lysis buffer and incubated on a shaker for 5 min at room temperature (RT). The cell suspension was filled up with 6 mL of PBS/ 1% FCS and after another centrifugation step all spleen cell pellets were pooled in 10 mL of PBS/ 1% FCS. While 10 µL of the suspension was used for viable cell counting the rest was spun down again. Supernatant was discarded, and the pellet

resuspended in 40 µL/10^7 cells of PBS/ 1% FCS. 10 µL/10^7 cells of biotin antibody cocktail were added and gently but thoroughly mixed. After 10 min of incubation on ice, 10 µL/10^7 cells of anti-CD44 microbeads, 20 µL/10^7 cells of anti-biotin microbeads and 20 µL/10^7 cells of PBS/ 1% FCS were added. The suspension was gently mixed and incubated on ice for 15 min.
The falcon containing the suspension was filled up completely with PBS/ 1% FCS, swayed to mix it gently, and centrifuged at 300g for 10 min at 4°C. The supernatant was aspirated, the pellet was resuspended in 4 mL of PBS/ 1% FCS. Four LS columns were arranged on a MACS separator and rinsed with 3 mL of PBS/ 1% FCS. 1 mL of cell suspension was filtered slowly through each column, respectively, adding 10 mL of ice-cold PBS/ 1% FCS for thorough flush out. All collected filtrate was centrifuged and pooled in 1 mL of pure PBS. Cells were counted, as described before, and adjusted to a target concentration of $5 \cdot 10^5$ cells/100 µL. 50 µL were used for a flow cytometry check of isolate purity, the major fraction for the T cell polarization assay.

BMDCs from WT mice of the strain C57BL/6 were primed with PBS, *B. vulgatus* mpk or *E. coli* mpk for 16 h as described in 2.2.2. Naïve Vβ5.1$^+$ T cells from transgenic OT II/BL6 mice that had been isolated as described above were added to the BMDCs and co-cultivated for three days. 6.5 h prior to the stimulation endpoint, OVA peptide 323-339, a part of chicken ovalbumin, was added in a concentration of 500 ng/mL to saturate MHC II molecules to present the OVA peptide 323-339 to its specific Vβ5.1 T cell receptors of the OT II mice and guarantee maximal stimulation throughout wells. The peptide sequence of this OVA peptide is ISQAVHAAHAEINEAGR. 2.5 h after adding the OVA peptide 323-339, 4 h prior to the endpoint of this coculture, 0.5 µL of a leukocyte activation cocktail (LAC), containing the phorbol ester PMA and ionomycin, ingredients widely used to stimulate leukocytes, was added to batches containing T cells for consistent activation. At the same time, 1 µL of GolgiStop was administered to batches that were designated for intracellular staining to stop protein secretion and promote intracellular cytokine accumulation. T cells and BMDCs were then fixed as described in 2.2.4 and analyzed by flow cytometry.

2.2.4 Analysis of proportions of BMDCs and T cells positive for Th17- or Treg-related proteins by flow cytometry

Stimulated cells or cells from a coculture batch were transferred into 2 mL test tubes and centrifuged for 5 min at 400g. Supernatant from batches that were not treated with GolgiStop was harvested and stored at -20°C, supernatant from GolgiStop-treated batches was discarded. Pellets were resuspended in 100 µL- 400 µL of Fc-block solution depending on the numbers of different stainings and number of controls. 100 µL for each staining were transferred into wells of a v-bottom 96-well plate. The rest of the suspensions was pooled and distributed as controls into a 96-well plate. After an incubation of 15 min at 4°C, cells were washed in 100 µL of PBS/ 1% FCS. To fix the cells, pellets for extracellular staining then were resuspended in 100 µL of 4% paraformaldehyde (PFA) and incubated at RT for 25 min, pellets for intracellular staining were resuspended in 200 µL of Cytofix/Cytoperm fixation/permeabilization solution and incubated for 20 min. After another centrifugation step, supernatant was removed under a laboratory hood and pellets were resuspended in 100 µL of PBS / 1% FCS for extracellular and Perm/Wash buffer for intracellular staining, sealed with parafilm and stored at 4°C until staining and analysis were performed. For each staining one master mix of fluorescently labelled antibodies and several control mixes were prepared in test tubes as shown in Table 2.1.7 and Table 2.1.8. Each staining required single-stained positive controls and fluorescence minus one (FMO) controls for each fluorescently labelled antibody, so control antibody mixes had to be prepared separately. For FMO control mixes, 0.5 µL of all extracellular antibodies and 1.0 µL of all intracellular antibodies present in this staining, except for the one antibody to be gated for, were added to the master mix. To each well of the 96-well plate filled with fixed cells 50 µL of the corresponding master mix or control mix was added, followed by an incubation of 30 min at 4°C. The plate was then centrifuged for 5 min at 400g, supernatant discarded, and pellets resuspended in 100 µL of PBS / 1% FCS. The suspension was transferred into flow cytometry analysis tubes.

Cytometer compensation beads for each fluorochrome were prepared as follows: One drop of anti-rat/anti-hamster Igκ compensation beads was filled into each

flow cytometry analysis tube. 1 µL of a fluorescently labelled anti-rat antibody was added. After 10 min of incubation measurements were started.

Multicolour flow cytometry was performed using a LSRFortessa equipped with three lasers (405 nm, 488 nm, 640 nm) and the software FACSDiva. Compensation was prepared using the compensation beads and unstained cell control.

Primary analysis was performed using the Software FlowJo. In the FSC/SSC plot cells were gated as shown in Figure 3.1.1. For analysis of BDMCs it was subsequently gated on $CD11c^+$ in a CD11c/FSC plot, for T cells on $CD3^+$ and $CD4^+$ in a $CD3^+/CD4^+$ plot. These populations were then analyzed for expression of the different regulators, cytokines or receptors using an FMO control gating strategy as shown in Figure 3.1.1. For MHC II it was gated on the high positive population.

2.2.5 Analysis of Th17- or Treg-related protein secretion from stimulated BMDCs and T cells by ELISA and flow cytometry

Supernatants of stimulated WT BMDCs, cocultures or stimulated $Nfkbiz^{-/-}$ and $Nfkbiz^{+/+}$ BMDCs (obtained from Christian Klameth) were analysed for several cytokines with mouse enzyme-linked immunosorbent assay (ELISA) kits and maxisorb plates according to the manufacturer's instructions. Washing was performed with an ELISA washer. Samples were diluted as shown in Table 2.2.1.

Table 2.2.1: Sample dilutions for ELISA

cytokine	dilution of samples
IL-6	1:50
IL-10	1:2
TGFβ1/ LAP	1:5
IL-23	only *E. coli* mpk-stimulated samples 1:20

To measure a broader range of cytokines from the same batches, supernatants were used in a cytometric bead array comprised of CBA Flex Sets that was analysed by flow cytometry.

The principle of such a bead immunoassay is similar to a sandwich ELISA. For each protein to be measured, there is one kind of bead that is coated with a fitting antibody and emits light in a specific red fluorescence intensity. The analytes are bound by the bead antibodies. Fluorescently labelled detection antibodies bind to free epitopes on the surface of the analyte. After washing away excess detection antibodies, the bead sandwiches are analysed in a flow cytometer. Beads for each protein of interest are recognized by their specific red fluorescence intensity in an APC/APC-Cy7 blot. The amount of analyte bound to these beads is determined via the detection antibody's reporter molecule PE. A standard serial dilution for each protein of interest is used to establish a standard curve for data interpretation.

The assay was prepared based on a protocol established by the William Agace group (Lund University, Sweden): Lyophilized standard spheres from each Flex Set were pooled and reconstituted in 2 mL of assay diluent from the CBA Flex Set Master Buffer Kit and incubated at RT for 15 min. For the standard curve, a serial dilution of seven 1:3 dilutions was prepared. A capture bead mix was prepared by mixing 0.15 µL per sample of capture beads from each Flex Set with 7.5 µL per sample of capture bead diluent from the Master Buffer Kit. Similarly, for the detection antibody mix, 0.15 µL per sample were added to 7.5 µL per sample of detection reagent diluent. Direct light exposure was avoided, as these antibodies and reagents are light-sensitive. The capture bead mix was vortexed for at least 5 sec and 7.5 µL respectively distributed into one well/sample of a v-bottom 96-well plate. 7.5 µL of standards or samples were added into corresponding wells, followed by 1h of incubation at RT in the dark. Then, 7.5 µL of the detection antibody mix was added to each well. The well contents were mixed gently and incubated for 1 h at RT in the dark. Then, 100 µL of wash buffer per well was added. The 96-well plate was centrifuged at 1200 rpm for 2 min, after which supernatants were carefully discarded and pellets resuspended in 200 µL of wash buffer.

The cytometer was set up using the software FACSDiva and cytometer setup beads according to the manufacturer's instructions. Standards and samples were measured using the preestablished setup.

2.2.6 Analysis of *Nfkbiz* and *Mst1* mRNA in stimulated BMDCs by real-time qRT-PCR

Samples of mRNA from a timeline experiment had been kindly prepared by Dr. Alex Steimle. DNA digestion was performed by Nadine Abele.

I prepared these DNA-free mRNA samples for the real-time qRT-PCR by diluting 1:10 with RNase free water. Standards were formed by pooling 1 µL of five samples respectively for the first standard and preparing a dilution series of the factors 1:5, 1:25 and 1:125. Out of each 1:10 diluted mRNA sample and the four standard dilutions 1 µL was pipeted into wells of a precooled 96-well PCR plate.

Actb mRNA, the mRNA of β-actin, was detected additionally as an internal control for normalization, on the assumption that expression of this housekeeping gene is independent of stimulus. For each primer set (forward and reverse primer) a separate PCR master mix was used (Table 2.1.10, Table 2.1.11). 9 µL of the corresponding PCR master mix were added to each well. The plate was sealed with a plastic foil and centrifuged for 1 min at 1000g prior to the real-time qRT-PCR. Real-time qRT-PCR was performed using a Roche LightCycler 480 and the program shown in Table 2.2.2.

Table 2.2.2: Real-time qRT-PCR program

stage	temperature	cycle duration	cycles
reverse transcription	50°C	10 min	1
first denaturation	95°C	5 min	1
polymerase chain reaction (PCR)			40
denaturation	95°C	10 sec	
annealing	60°C	30 sec	
melting curve analysis			1
denaturation	95°C	5 sec	
cooling	46°C	1 sec	
melt	95°C	0.06°C/sec	
cooling for storage	4°C		

The real-time qRT-PCR measurements were analysed with the software LightCycler 480. Melting curves of the standard samples were calculated using the tool “Tm calling”. From these a standard curve was computed as their second derivative using the tool “AbsQuant/2nd derivative”.

The efficiency E of the qRT-PCR was determined from the gradient of the curve:

$$\boldsymbol{E = 10^{-\frac{1}{m}}}$$

Ideally, E should be equal to 2, which means that every PCR cycle leads to duplication of DNA material.

Cp values (begin of exponential growth) of samples were calculated with high sensitivity setting by the tool “AbsQuant/2nd derivative”. Amounts of mRNA of the tested genes were standardized using E and the measurements for the housekeeping gene *Actb* and the unstimulated control of the genes of interest.

The following formula was used to calculate these relative transcriptional differences (R):

$$\boldsymbol{R = \frac{E_{tested\ gene}^{Cp(control)_{tested\ gene} - Cp(sample)_{tested\ gene}}}{E_{housekeeping\ gene}^{Cp(control)_{housekeeping\ gene} - Cp(sample)_{housekeeping\ gene}}}}$$

2.2.7 Statistics

Statistical analysis was performed with the software Graphpad Prism. Samples derived from different mice were considered to be biologically independent. For comparisons between two treatment groups, means were determined, and the unpaired student's t-test was used. For P-values beneath $p < 0.05$ statistical significance was assumed. Error bars show ± one standard deviation (SD).

3 Results

3.1 Influence of Commensal Bacteria on Th17/Treg cell Balance via Dendritic Cells

At the mucosal interface, DCs are important APCs, acting as a link between commensal bacteria and T cells (see 1.2.1).To examine the effect of the symbiont bacterium *B. vulgatus* mpk and the pathobiont bacterium *E. coli* mpk on the polarization balance of murine naïve $CD4^+$ T cells into Th17 or Treg cells by mediation of DCs, a coculture experiment was performed.

$CD11c^+$ BMDCs serve as a well-established source for *in vitro* experiments with dendritic cells (1.2.1 and 2.2.1). These cells were primed with either *B. vulgatus* mpk or *E. coli* mpk and then co-cultivated with naïve T cells from transgenic OT II/BL6 mice (Figure 3.1.1a). T cells of OT II/BL6 mice all have the same $V\beta5.1^+$ T cell receptor. Its ligand is an OVA peptide. This ligand was added in excess to the cells to achieve a maximum of MHC II antigen presentation in all batches to effectively activate T cells. T cells and BMDCs were analyzed by flow cytometry according to Figure 3.1.1 and Figure 3.1.2.

The capability of BMDCs to activate T cells and influence their polarization depends on their maturation state. A MHC $II^{int}CD11c^+$ phenotype has been shown to reflect the semimature state that BMDCs adopt in contact with the symbiont *B. vulgatus* mpk, in contrast to full maturation with $CD11c^+$ BMDCs that have high numbers of MHC II (subsequently named MHC II^{hi} $CD11c^+$ BMDCs) when stimulated with *E. coli* mpk (see 1.2.1). Here, to check the maturation state of BMDCs, the percentage of MHC II^{hi} $CD11c^+$ BMDCs was determined by flow cytometry. *B. vulgatus* mpk-primed samples show a similar proportion of MHC II^{hi} $CD11c^+$ BMDCs than unstimulated ones with means of about 20%, while *E. coli* mpk priming leads to a significantly increased proportion of this subset with a mean of about 60% of $CD11c^+$ cells (Figure 3.1.1b).

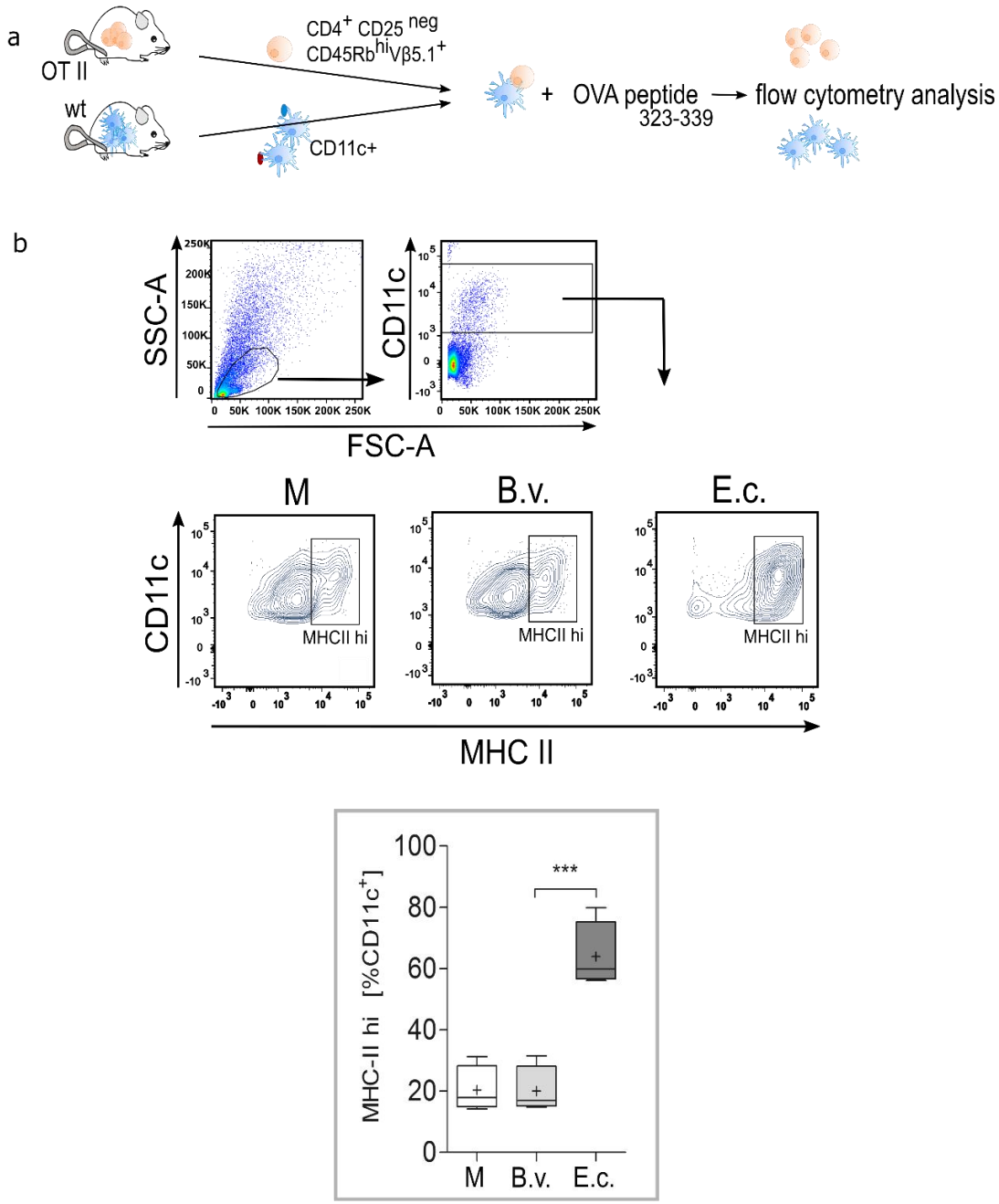

Figure 3.1.1: Maturity of BMDCs after priming with symbiont *B. vulgatus* mpk or pathobiont *E. coli* mpk and coculture with naïve T cells

(a) Overview of experimental design: BMDCs from WT mice of the strain C57BL/6 were primed with PBS (mock), B. vulgatus mpk and E. coli mpk for 16 h. Naïve T cells ($CD3^+CD4^+CD25^{neg}$ $CD45Rb^{hi}V\beta5.1^+$ T cells) from transgenic OT II/BL6 mice were co-cultivated with those BMDCs and OVA peptide for 3 days and subsequently analysed by flow cytometry.
(b) Analysis of MHC II high positive ($MHC\ II^{hi}$) subpopulation of $CD11c^+$ BMDCs to determine their maturity status after priming with B. vulgatus mpk or E. coli mpk and subsequent coculture with T cells. Panels show the gating strategy and flow cytometry results of one mouse as an example, the graph depicts the proportion of $MHC\ II^{hi}$ cells of $CD11c^+$ BMDCs (n=3). M stands for Mock, B.v. for B. vulgatus mpk, E.c. for E. coli mpk. Statistical analyses by student's t test, boxes indicate 50% interval, whiskers minimum to maximum, vertical lines represent medians, pluses indicate means, asterisks stand for the p-value: $***\leq 0{,}001$.

T cell analysis focused on the cytokines IL-17A and IFNγ as key cytokines secreted by pathologic Th17 cells and FOXP3 as key transcription factor of Treg cells (Figure 3.1.2c). The amount of key Th17 transcription factor RORγt was tested, as well, but due to a faulty flow cytometry antibody no reliable results can be shown. Protein production was indicated as percentage of $CD3^+CD4^+$ cells being positive for the cytokine, e.g. $IL\text{-}17A^+$ [$\%CD3^+CD4^+$].
As seen in Figure 3.1.2c, stimulation with *B. vulgatus* mpk does not lead to a significant increase in $IL\text{-}17A^+CD3^+CD4^+$ T cells and $IL\text{-}17A^+IFN\gamma^+$ $CD3^+CD4^+$ T cells compared to unstimulated BMDCs, while *E. coli* mpk-stimulated BMDCs initiate an increase in the proportion of $IL\text{-}17A^+CD3^+CD4^+$ T cells.
Regardless of type of stimulation, about 30% of $CD3^+CD4^+$ T cells are positive for FOXP3. This indicates that polarization into $FOXP3^+$ Treg cells is independent of stimulation of BMDCs by *B. vulgatus* mpk or *E. coli* mpk in this setting.

Taken together, the results in Figure 3.1.2 suggest that the balance between Th17 and Treg cells is not shifted in favour of proinflammatory Th17 cells in cocultures of naïve T cells and BMDCs stimulated with the symbiont *B. vulgatus* mpk, as it is after stimulation with pathobiont *E. coli* mpk.
Polarization of naïve T cells is particularly dependent on cytokines acting on the T cells. Thus, the cytokine environment is of further interest.

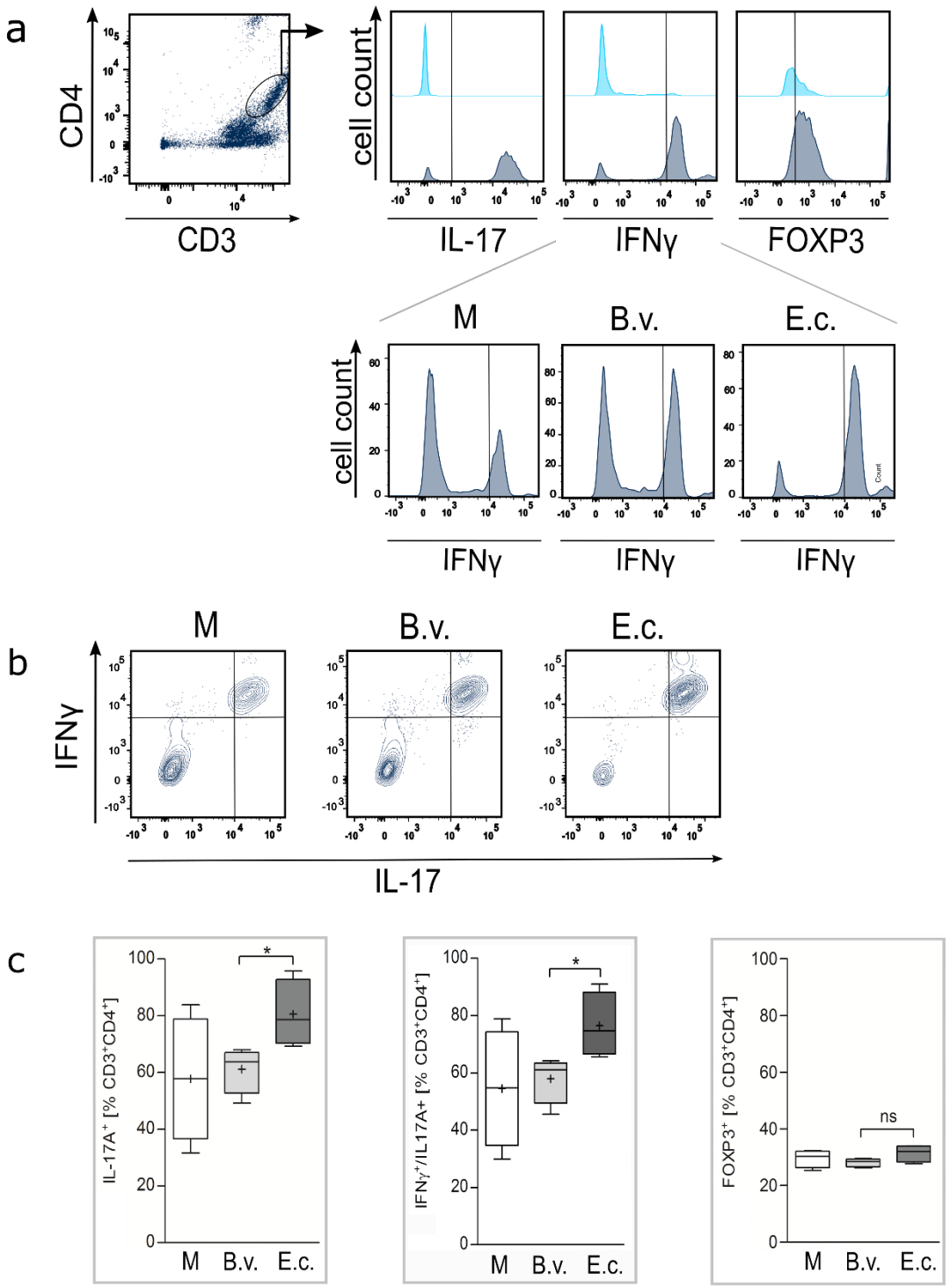

Figure 3.1.2: Polarization of naïve T cells after coculture with BMDCs primed with symbiont *B. vulgatus* mpk or pathobiont *E. coli* mpk

(a) Panels show the gating strategy for the determination of IL-17$^+$, IFNγ$^+$ or FOXP3$^+$ subsets of CD3$^+$CD4$^+$CD25^{neg}CD45Rb^{hi+}Vβ5.1$^+$ T cells in grey under undyed control in blue in histograms. (b) Panels show the gating of IL-17$^+$/IFNγ$^+$ double positive subpopulation in a density plot. (c) Graphs depict the analysis of IL-17$^+$, FOXP3$^+$ and IL-17$^+$/IFNγ$^+$ subpopulations of CD3$^+$CD4$^+$ T cells (n=4). M stands for Mock, B.v. for B. vulgatus mpk, E.c. for E. coli mpk. Statistical analyses by student's t test, boxes indicate 95% interval, whiskers minimum to maximum, vertical lines are medians, pluses indicate means, asterisks are p-values: $*\leq 0{,}05$.

3.2 Comparison of Th17- and Treg-inducing Cytokine Amounts Produced by Dendritic Cells Stimulated with Commensal Symbiont *B. vulgatus* mpk or Pathobiont *E. coli* mpk

In chapter 3.1 the influence of stimulation of BMDCs with symbiont *B. vulgatus* mpk and pathobiont *E. coli* mpk on polarization of naïve T cells in coculture was investigated. For a better understanding of the mechanism in which symbionts benefit their hosts in Th17-relevant situations, a closer look at their effects on DCs seems prudent. In addition to the capability of DCs to display antigens, DCs are important cytokine producers and facilitate T cell polarization in this way. In this context, cytokines of high interest are: IL-6, TGFβ1 and IL-23 for Th17 differentiation, as well as TGFβ1 and IL-10 for Treg differentiation (see 1.2.2). Production of these four key cytokines was explored in BMDCs under the influence of symbiont *B. vulgatus* mpk versus pathobiont *E. coli* mpk by flow cytometry.

Similarly to the analysis in chapter 3.1, for this experiment, the maturation state of BMDCs was investigated, as well. A comparison of MHC II^{hi} cell subsets between *B. vulgatus* mpk- and *E. coli* mpk-stimulated BMDCs shows no significant difference at 4 h of stimulation, but a significantly lower amount of MHC II^{hi} cells in *B. vulgatus* mpk-primed BMDCs (Figure 3.2.1) after 16 h of stimulation compared to *E. coli* mpk stimulation.

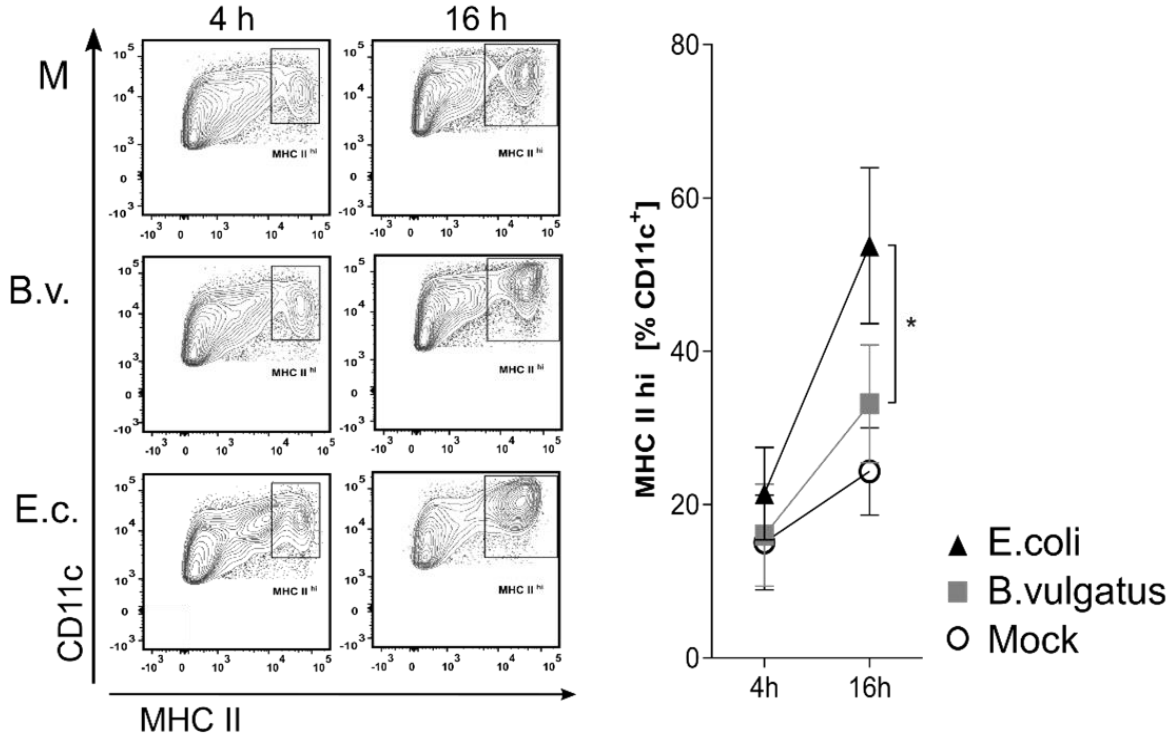

Figure 3.2.1: Maturity of BMDCs after stimulation with symbiont *B. vulgatus mpk* or pathobiont *E. coli mpk* at early and late timepoint

Gating strategies as depicted in Figure 3.1.1b. These will be used throughout the following analyses. Analysis of MHC II high positive (MHC II^{hi}) subpopulation of CD11c^{+} BMDCs to show their maturity status after priming with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) for 4 h and 16 h. Unprimed control is labelled mock (M). Left panels show exemplary flow cytometry results of one mouse, the graph depicts the proportion of MHC II^{hi} cells of CD11c^{+} BMDCs (n=3). Statistical analyses by student's t test, dots represent mean, error bars standard deviation (SD), asterisk stands for p-value: $*\leq 0{,}05$.

Analysis of BMDCs after 4 h of stimulation shows no significant difference in the proportion of IL-6^{+} cells between *B. vulgatus* mpk and *E. coli* mpk stimulation (Figure 3.2.2). Both are at a similar level as the unstimulated control at about 10% of CD11c^{+} cells. After 16 h of stimulation, *E. coli* mpk priming increases proportions 4-fold to about 40% of cells. IL-6^{+} cell amounts after *B. vulgatus* mpk stimulation less than double to a value of 16% of CD11c^{+} cells, resulting in a significant difference between the two groups. The fraction of IL-6^{+} cells in unstimulated batches increases to 25% of CD11c^{+} cells. Thus, *B. vulgatus* mpk stimulation of BMDCs leads to the lowest proportion of IL-6^{+} cells.

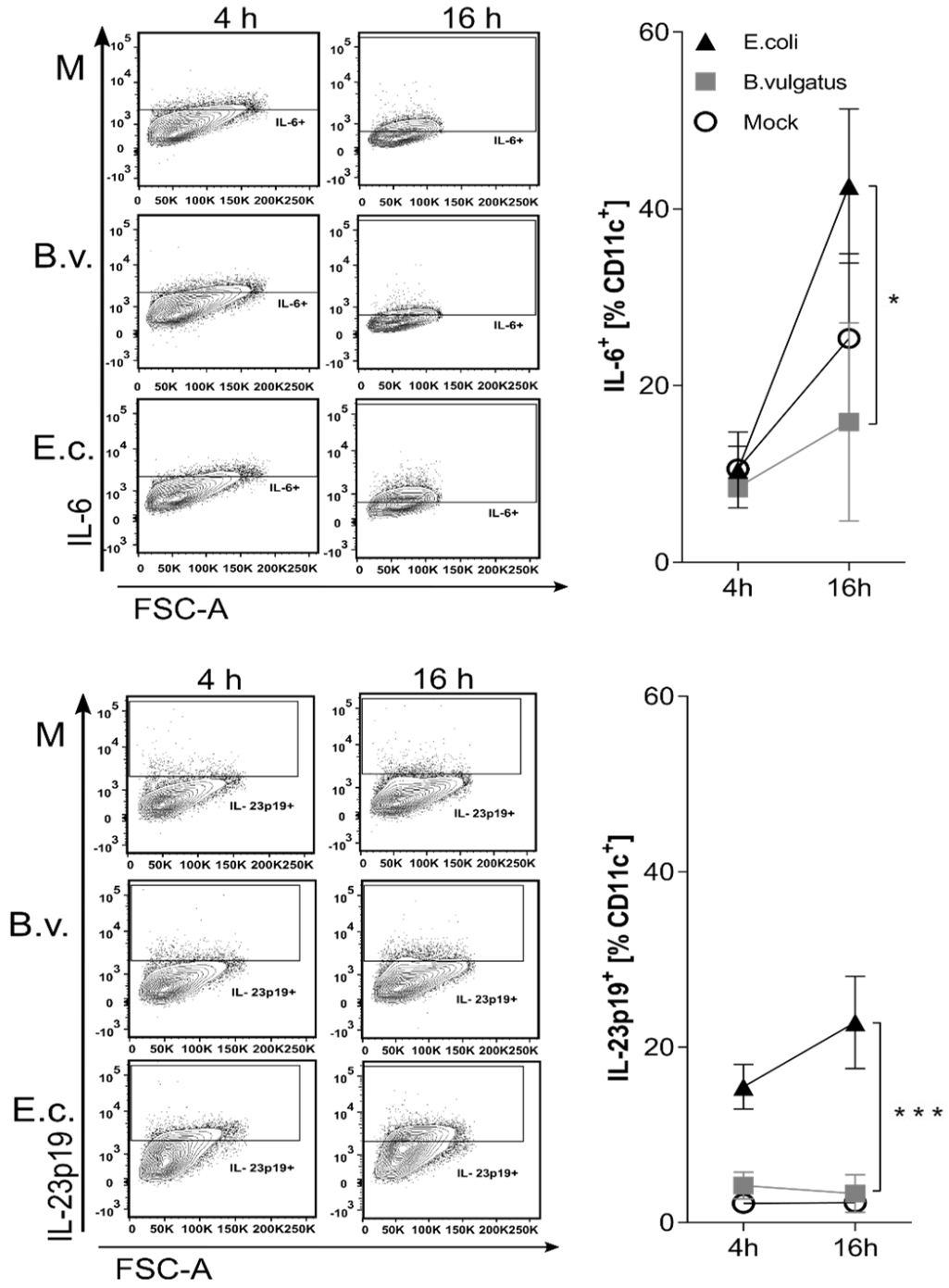

Figure 3.2.2: BMDC production of Th17-related cytokines IL-6 and IL-23 after stimulation with symbiont *B. vulgatus mpk* or pathobiont *E. coli mpk*

Analysis of cytokine positive subpopulations (IL-6, IL-23) of CD11c⁺ BMDCs after stimulation with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) for 4 h and 16 h. Unprimed control is labelled mock (M). IL-23 is analyzed in form of its unique subunit IL-23p19. Left panels show exemplary flow cytometry results of one mouse, the graphs depict the proportion of cytokine⁺ cells of CD11c⁺ BMDCs for each cytokine (IL-6 n=3; IL-23 n=4). Statistical analyses by student's t test, dots represent mean, error bars SD, asterisks stand for p-values: $*\leq 0{,}05$, $***\leq 0{,}001$.

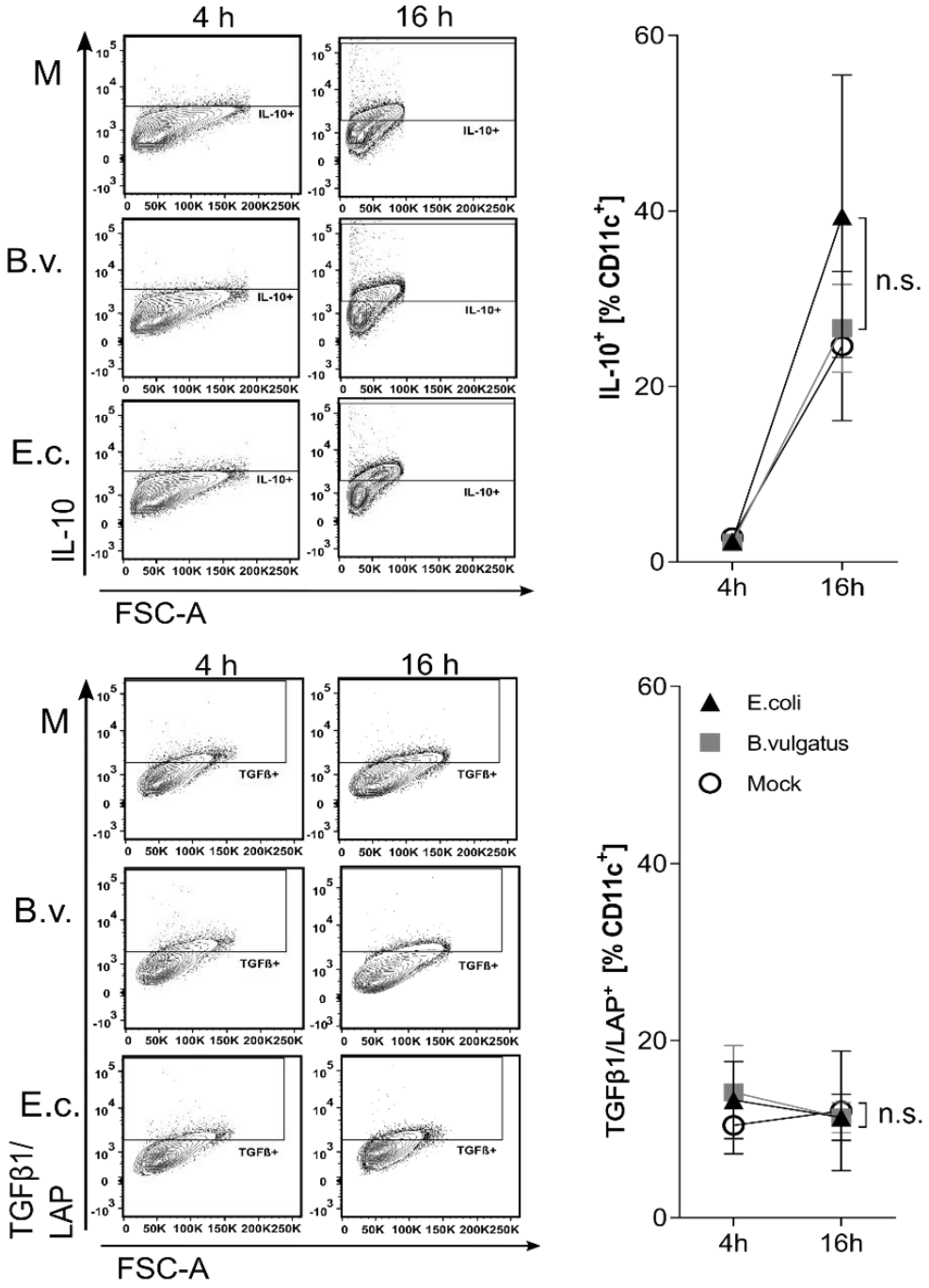

Figure 3.2.3: BMDC production of Treg-related cytokine IL-10 and Th17/Treg-related TGFβ1 after stimulation with symbiont *B. vulgatus mpk* or pathobiont *E. coli mpk*

Analysis of cytokine positive subpopulations (IL-10, TGFβ1/LAP) of CD11c⁺ BMDCs after stimulation with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) for 4 h and 16 h. Unprimed control is labelled mock (M). TGFβ1/LAP refers to the complex of TGFβ1 and latency associated peptide (LAP) it forms for secretion before splitting the dimer. Left panels show exemplary flow cytometry results of one mouse, the graphs depict the proportion of cytokine⁺ cells of CD11c⁺ BMDCs for each cytokine (IL-10 n=3; TGFβ n=4). Statistical analyses by student's t test, dots represent mean, error bars SD, n.s. means non-significant p-value $\geq 0{,}05$.

For my analysis of IL-23, I focused only on the unique subunit IL-23p19. In contrast to IL-6, the amount of IL-23p19$^+$ cells differs after 4 h significantly (Figure 3.2.2). Stimulation with *B. vulgatus* mpk amounts to 5% and *E. coli* stimulation to 15% of CD11c$^+$ BMDCs. After 16 h, the gap becomes more apparent: 5% of cells are IL-23p19$^+$ after priming with *B. vulgatus* mpk and 20% with *E. coli* mpk.

Similarly to IL-6$^+$ cells, after 4 h of stimulation, all batches irrespective of stimulation type show a fraction of 10% of CD11c$^+$ cells that are LAP$^+$, and thus TGFβ$^+$, as LAP and TGFβ are still a unit inside the cell (see 1.2.2). This remains the same for a stimulation period of 16 h, showing no differences between priming with *B. vulgatus* mpk and *E. coli* mpk (Figure 3.2.3).
The amount of IL-10$^+$ cells increases in all stimulation groups from a level of <5% of CD11c$^+$ cells to 25-40% of CD11c$^+$ cells, showing no significant differences due to high standard deviation (Figure 3.2.3).

In summary, stimulation of DCs with symbiont *B. vulgatus* mpk results in a significantly lower percentage of cells producing the cytokines IL-6 and IL-23, but not IL-10, compared to stimulation with pathobiont *E. coli* mpk. Concerning TGFβ1 production, cells show no reaction to our bacterial stimulation.

The flow cytometry analysis in Figure 3.2.2 and Figure 3.2.3 displays differences between DCs primed with the symbiont *B. vulgatus* mpk and the pathobiont *E. coli* mpk. It shows which proportion of $CD11c^+$ cells produce certain cytokines and allows conclusions about relations between bacterial stimuli and cytokine production of DCs. It does not, though, give information on which amount of each cytokine is released and therefore effectively available to act on T cells. To shed light on this issue, amounts of secreted proteins were determined with ELISA experiments for the cytokines IL-6, IL-23, TGFβ1 and IL-10 at the timepoints 4 h and 16 h (Figure 3.2.4).

Results show that BMDCs react to *E. coli* mpk stimulation with a high release of approximately 30,000 pg/mL of IL-6 as early as 4 h after stimulation. After 16 h of stimulation, secretion is almost doubled to about 55,000 pg/mL. Priming with the symbiont *B. vulgatus* mpk is followed by significantly lower IL-6 secretion at both timepoints compared to stimulation with *E. coli* mpk. After 4 h, about 320 pg/mL of IL-6 is released, comparable to secretion by cells in the unstimulated control. After 16 h, secretion increases to about 5300 pg/mL of IL-6, while the mock control remains at about 170 pg/mL.
IL-23 concentrations detected in supernatants of *E. coli* mpk-stimulated BMDCs are high at 4 h, with 4,000 pg/mL, more than doubling at 16 h of stimulation, with 9,400 pg/mL of IL-23. After *B. vulgatus* mpk stimulus, IL-23 secretion by BMDCs is significantly lower than after priming with *E. coli* mpk. The 4 h value is at the low level of <20 pg/mL, resembling the unstimulated control. After 16 h, it increases to 600 pg/mL of secreted IL-23, with the control remaining at about 25 pg/mL. All in all, the amount of secreted IL-23 is more than six times lower as the amount of released IL-6 for all bacterial stimulation.

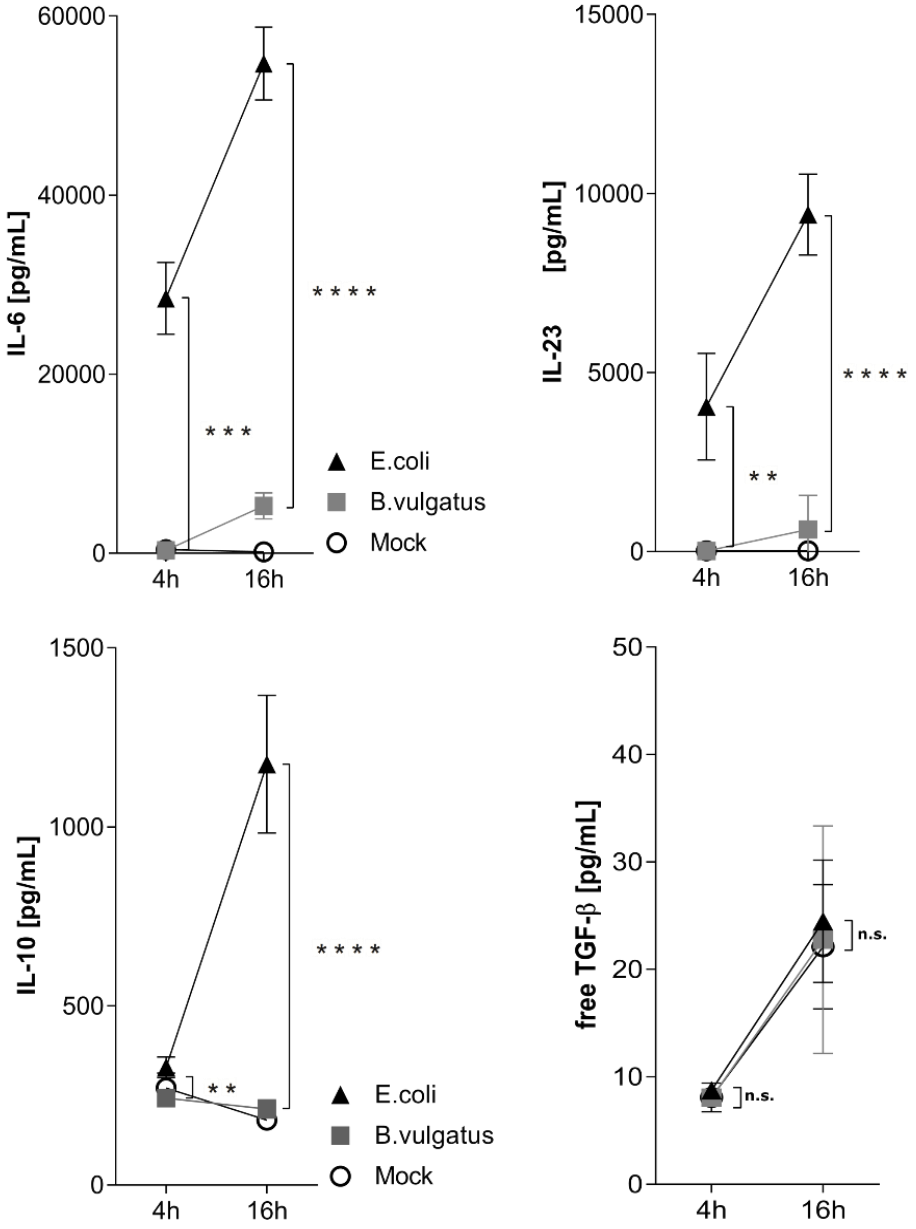

Figure 3.2.4: BMDC secretion of Th17/Treg-related cytokines after stimulation with symbiont *B. vulgatus* mpk or pathobiont *E. coli* mpk

ELISA analysis of secreted protein amounts of cytokines important for Th17 induction (IL-6, free TGFβ, IL-23) or Treg induction (IL-10, free TGFβ). Free TGFβ refers to TGFβ1 after splitting the dimer with latency associated peptide (LAP) that it forms for secretion. Assays were performed with supernatant collected from BMDC batches after stimulation with B. vulgatus mpk (B. vulgatus) or E. coli mpk (E. coli) for 4 h and 16 h. Unprimed control is labelled Mock. Free TGFβ assay was performed by Annika Bender. Graphs show the concentration of each cytokine in the supernatant in pg/mL (4 h n=3, 16 h n=4 for all cytokines). Note that axes of ordinates of different graphs have different maxima in order to better display differences between stimulation types within the graphs. Statistical analyses by student's t test, dots represent mean, error bars correspond to SD, asterisks stand for p-values: $**\leq 0{,}01$, $***\leq 0{,}001$, $****\leq 0{,}0001$, *n.s. means non-significant p-value* $\geq 0{,}05$.

IL-10 concentrations that we measured are generally lower than IL-6 and IL-23 concentrations in the 4 h supernatant. Nevertheless, IL-10 concentrations in the pathobiont *E. coli* mpk-stimulated batches are with about 330 pg/mL still significantly higher than in the ones stimulated with the symbiont *B. vulgatus* mpk which amount to about 240 pg/mL of IL-10. Only *E. coli* mpk stimulation for 16 h results in a distinct increase of IL-10 secretion up to about 1200 pg/mL, while *B. vulgatus* mpk-stimulated cells show stagnant secretion of about 200 pg/mL of IL-10.
Concentrations of active TGFβ1 were measured in these samples. Levels of free TGFβ1 in the supernatants are low regardless of stimulation type, with <10 pg/mL of free TGFβ after 4 h and approximately 20 pg/mL after 16 h of stimulation.

To sum this up, IL-6 secretion by BMDCs increases after stimulation with *B. vulgatus* mpk ten times less than after stimulation with *E. coli* mpk, but there is a positive dynamic over time. IL-23 secretion by BMDCs is very low after 4 h of stimulation with symbiont *B. vulgatus* mpk, increasing after 16 h. Compared to stimulation with pathobiont *E. coli* mpk, the measured IL-23 concentration after stimulation with symbiont *B. vulgatus* mpk is significantly lower. Concentrations of IL-10 secreted by BMDCs after stimulation by symbiont *B. vulgatus* mpk do not increase with time and are significantly lower than after stimulation with pathobiont *E. coli* mpk. Concentrations of free TGFβ1 show a trend for increase independent of stimulation type throughout time, but on a very low level.

3.3 Influence of Transcription Factor IκBζ and Kinase MST1 on Production of Th17-inducing Cytokines in Dendritic Cells Stimulated with Commensal Symbiont *B. vulgatus* mpk or Pathobiont *E. coli* mpk

Considering the association of transcription factor IκBζ with susceptibility to autoimmune diseases and its function in upregulation of IL-6 (see 1.2.2), IκBζ is investigated more closely in context with Th17-related cytokine production by BMDCs.

On that account, first an earlier experiment of our group was repeated, measuring *Nfkbiz* mRNA, the mRNA of IκBζ, in WT BMDCs after priming with *B. vulgatus* mpk or *E. coli* mpk (Klameth, 2012). Figure 3.3.1a shows relative amounts of *Nfkbiz* mRNA in WT BMDCs obtained by real-time qRT-PCR. The mRNA isolates date from 2 h of stimulation with *B. vulgatus* mpk or *E. coli* mpk. The relative amounts shown are standardized using mRNA amounts of the housekeeping gene *Actb* and unstimulated controls (see 2.2.6). In accordance with earlier findings of our group (Klameth, 2012), priming with the symbiont *B. vulgatus* mpk leads to significantly lower relative *Nfkbiz* mRNA expression than stimulation with the pathobiont *E. coli* mpk.

To investigate the influence of IκBζ on the production of the Th17-inducing cytokines IL-6 and IL-23 in BMDCs, concentrations of these cytokines in the supernatant of *Nfkbiz*$^{-/-}$ BMDCs with cytokine concentrations in supernatants of WT BMDCs are compared. IL-6 concentrations in the supernatant of *Nfkbiz*$^{-/-}$ BMDCs stimulated with *B. vulgatus* mpk or *E. coli* mpk for 24 h are contrasted with respective cytokine concentrations from WT BMDCs (Figure 3.3.1b). Unstimulated BMDCs secrete about 140 pg/mL of IL-6 with no difference between *Nfkbiz*$^{-/-}$ and WT cells. Priming with *B. vulgatus* mpk is met by a slight, but not significant increase of IL-6 compared to mock in *Nfkbiz*$^{-/-}$ and WT cells. There is no significant difference between *Nfkbiz*$^{-/-}$ and WT groups stimulated with *B. vulgatus* mpk, with values of 550 pg/mL versus 730 pg/mL. Secretion of IL-6 after *E. coli* mpk stimulation is higher, with a significant difference between *Nfkbiz*$^{-/-}$ and WT, with about 13,200 pg/mL and 36,600 pg/mL, respectively.

When measuring amounts of IL-23, only the expected differences due to stimulus are apparent: For *B. vulgatus* mpk-primed batches, the concentration of IL-23 is <15 pg/mL with no significant difference to unstimulated cells. After stimulation with *E. coli* mpk, concentrations are significantly higher. However, there is no significant difference between *Nfkbiz* $^{-/-}$ cells and WT cells, with about 600 pg/mL versus 500 pg/mL.

Taken together, the data of Figure 3.3.1 shows no difference in IL-23 secretion for WT and *Nfkbiz*$^{-/-}$ BMDCs. It indicates, however, a significant difference between WT and *Nfkbiz*$^{-/-}$ BMDCs in the secretion of IL-6 by BMDCs that have been primed with the pathobiont *E. coli* mpk. In contrast, stimulation with the symbiont *B. vulgatus* mpk leads to a higher secretion than in the control but not to a significant difference between IL-6 secretion of WT and *Nfkbiz*$^{-/-}$ BMDCs. This indicates a role of IκBζ in IL-6 regulation in BMDCs but does not explain the observed differences fully. Other regulatory proteins might be equally important.

Thus, a second protein known for IL-6 regulation, was looked upon: MST1. It is reported to influence Th17 differentiation by negatively regulating IL-6 production in DCs (see 1.2.2). Here, it was brought into context with bacterial stimulation of BMDCs. Additionally, it was investigated if MST1 might play a role in IL-23 regulation. Thus, mRNA levels of *Mst1* were examined at different time points of stimulation of BMDCs with *B. vulgatus* mpk or *E. coli* mpk to elucidate if the protein product MST1 is involved in bacterial regulation of IL-6 (Figure 3.3.2). *Nfkbiz* mRNA timelines have been shown in previous works of our lab (Klameth, 2012, Birg, 2017). In this work the real-time qRT-PCR measurements were repeated for a comparison to *Mst1* mRNA.

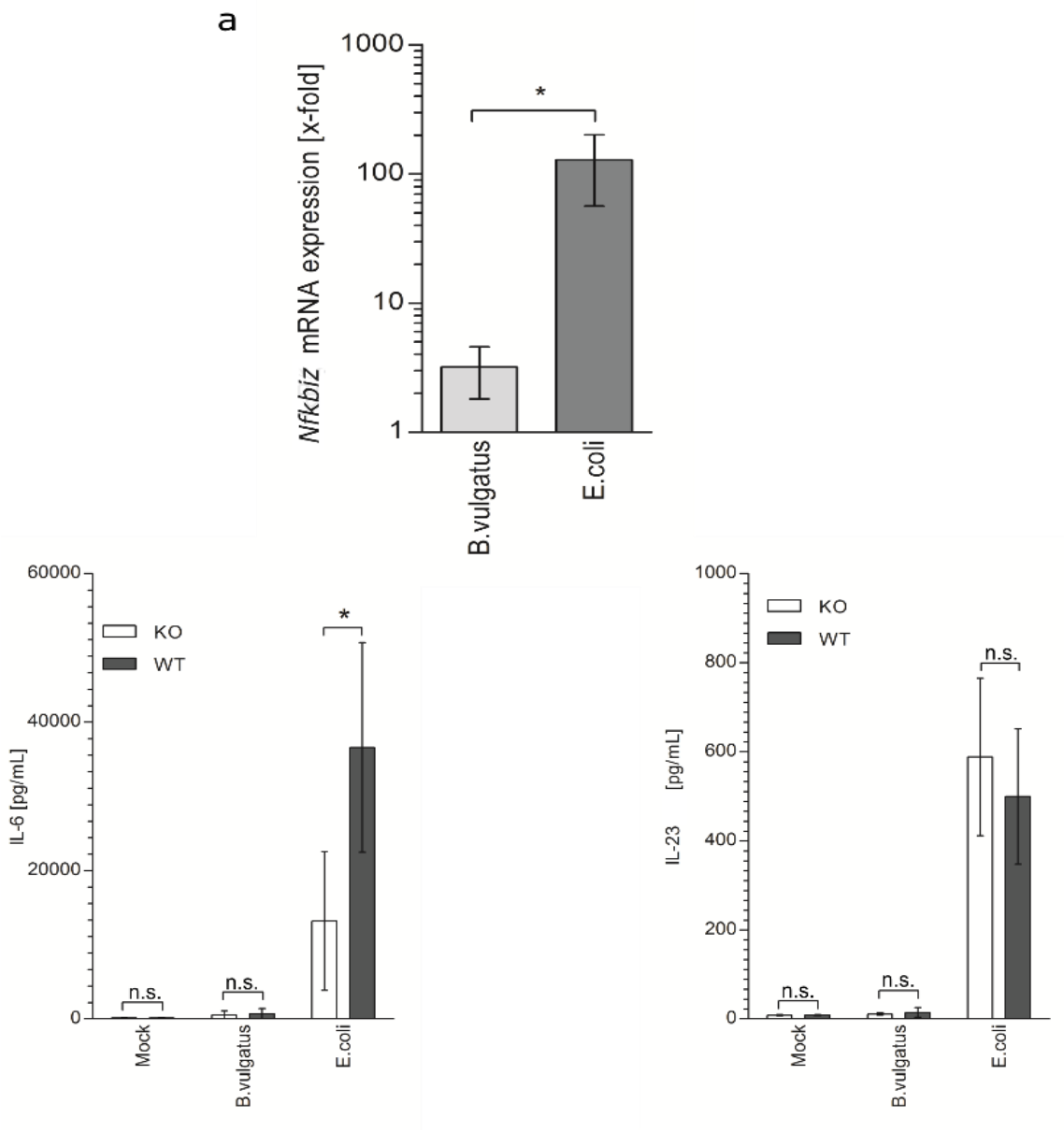

Figure 3.3.1: BMDC secretion of Th17-inducing cytokines in *Nfkbiz*$^{-/-}$ cells after stimulation with symbiont *B. vulgatus* mpk or pathobiont *E. coli* mpk

(a) Real-time qRT-PCR analysis of Nfkbiz mRNA levels in WT BMDCs stimulated with B. vulgatus mpk (B. vulgatus) or E. coli mpk (E. coli) for 2 h. The graph shows relative amounts of Nfkbiz mRNA (calculation: see 2.2.6) (n=4). It serves for discussion of (b), its data is also used in the timeline in 3.3.2a. Similar data has been shown before by our group.

(b) ELISA analysis of secreted protein levels of the cytokines IL-6 and IL-23 in WT mice and Nfkbiz$^{-/-}$ *(KO) mice. Assays were performed with supernatant from BMDC batches that had been stimulated with B. vulgatus mpk (B. vulgatus) or E. coli mpk (E. coli) for 24 h both from WT and KO mice by Christian Klameth. Unprimed control is labelled mock. Graphs show the cytokine concentration in pg/mL (n=5). Note that axes of ordinates of the two graphs have different maxima in order to better display differences between stimulation types within the graphs. Statistical analyses by student's t test, columns represent mean, error bars correspond to SD, asterisks stand for p-values:* $*\leq 0{,}05$, *n.s. means non-significant p-value* $\geq 0{,}05$.

Mst1 mRNA shows no regulation for different timepoints depending on different type of stimulation (Figure 3.3.2b). *B. vulgatus* mpk-stimulated cells as well as *E. coli* mpk-stimulated ones produce *Mst1* mRNA on the same level as unstimulated controls for all tests. In contrast, mRNA of *Nfkbiz* shows a distinct peak in *E. coli* mpk-stimulated cells after 2 h of stimulation and a mild, but not significant, increase in *B. vulgatus* mpk-stimulated cells (Figure 3.3.2a).

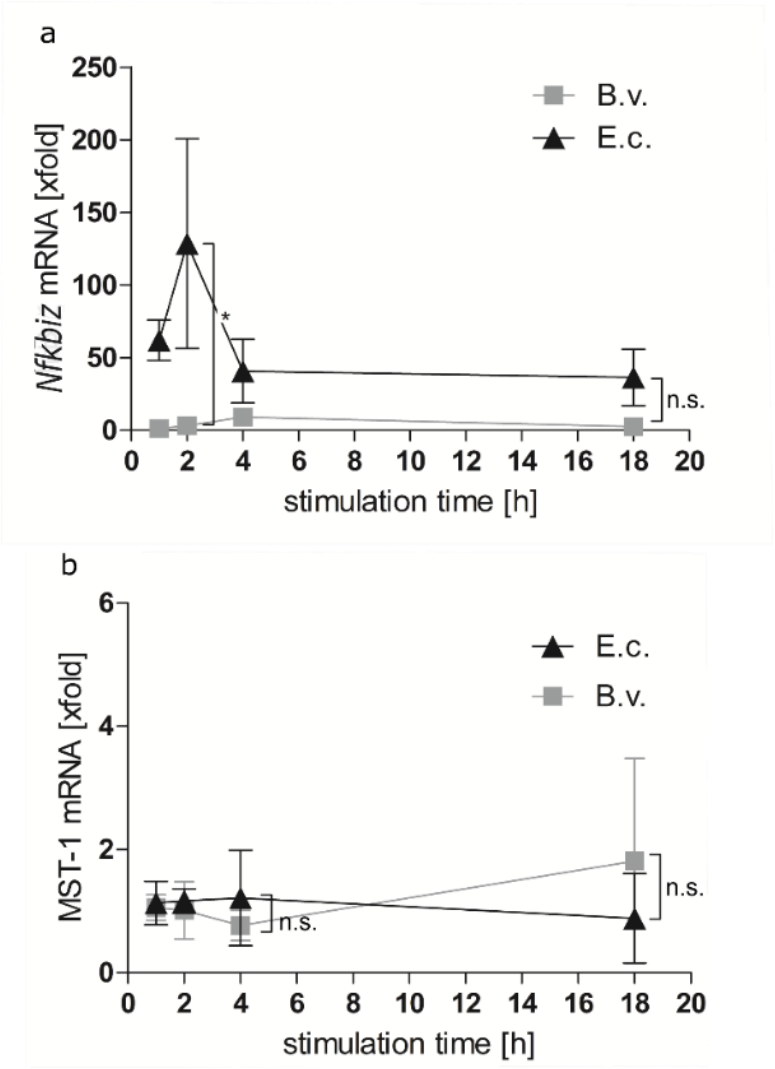

Figure 3.3.2: mRNA levels of *Nfkbiz* and *Mst1* in BMDCs after stimulation with symbiont *B. vulgatus* mpk and pathobiont *E. coli* mpk

Real-time qRT-PCR analyses of (a) Nfkbiz and (b) Mst1 mRNA levels in WT mice. The mRNA was isolated from BMDCs stimulated with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) for early timepoints 1 h, 2 h, 4 h and late timepoint 18 h to form a timeline. Values for 2 h are the same as depicted in 3.3.1a. Graphs show relative amounts of mRNA (calculation: see 2.2.6) (n=5). Statistical analyses by student's t test, dots represent mean, error bars correspond to SD, asterisk stands for p-value: $*\leq 0{,}05$*, n.s. means non-significant p-value* $\geq 0{,}05$.

3.4 Retrograde Influence of Th17 Signature Cytokines IL-17 and IL-22 on Dendritic Cells

Th17 cells release downstream Th17 cytokines such as IL-17, IL-21 and IL-22 close to DCs (see 1.2.2). It is therefore possible that these cytokines exert an influence on DCs in a feedback loop, modulating cytokine production of DCs and interfering with the bacterial influence on DCs that is the focus of this work.

First, endogenous production of IL-17, IL-21 and IL-22 of BMDCs was assessed by flow cytometry analysis (Figure 3.4.1a). This analysis shows that 2-4% of $CD11c^+$ BMDCs are $IL\text{-}17^+$ at 4 h, independently of stimulation with *B. vulgatus* mpk or *E. coli* mpk. The fraction of $IL\text{-}21^+$ BMDCs is 16-17% at 4 h. $IL\text{-}22^+$ subpopulations at 4 h diverge, with 4% of $CD11c^+$ BMDCs in the mock control, 8% in *B. vulgatus* mpk-primed batches and with 17% significantly more in *E. coli* mpk-stimulated cells.

Additionally, BMDCs were checked for cytokine receptor expression (Figure 3.4.1b). For IL-17R, the percentages of $IL\text{-}17R^+CD11c^+$ BMDCs are 15-18% after 4 h, indifferent of stimulation type. The fraction of $IL\text{-}21R^+$ cells after 4 h is significantly lowest for *E. coli* mpk stimulation with about 10%, and about 15% for stimulation with *B. vulgatus* mpk or no stimulation. For IL-22R, after 4 h all types of stimulation lead to less than 2% of $IL\text{-}22R^+$.

Complementarily, secretion of IL-17 and IL-22 was measured in supernatants of BMDCs stimulated for 16 h. Secreted IL-17 and IL-22 were detected using a flow cytometric bead assay. Despite good standard curves, unexpectedly effectively no IL-17 and IL-22 in the supernatant samples could be detected (data not shown).

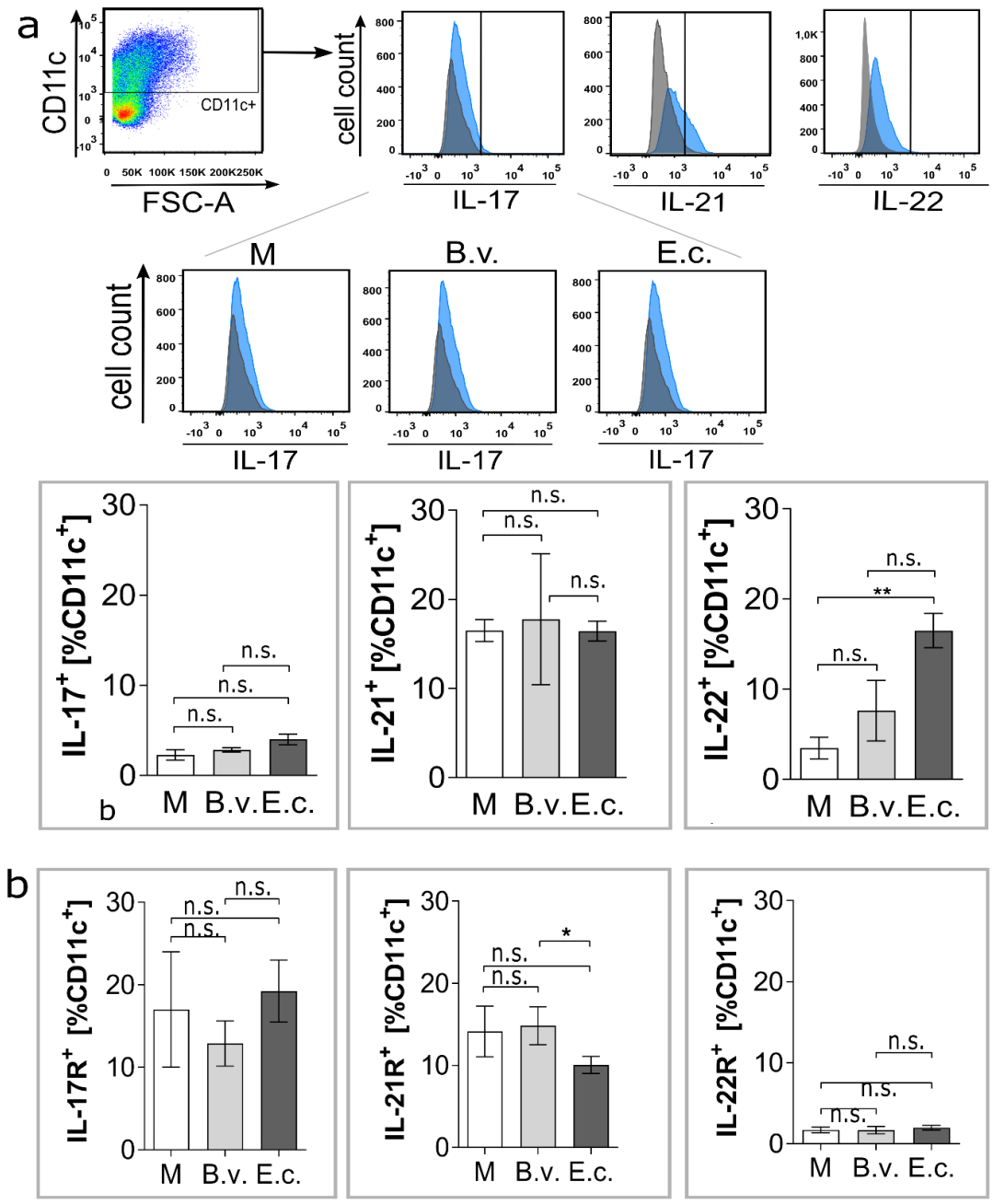

Figure 3.4.1: Endogenous production of Th17 signature cytokines and cytokine receptor expression in BMDCs after stimulation with symbiont *B. vulgatus* mpk and pathobiont *E. coli* mpk

(a) Inherent Th17 effector cytokine production of BMDCs with IL-17A$^+$, IL-21$^+$, IL-22$^+$ subpopulations of CD11c$^+$ BMDCs after priming with B. vulgatus mpk (B.v.) or E-coli mpk (E.c.) for 4 h. Unprimed control is labelled mock (M). Similar gating strategy used for (b). Upper plots show gating strategy up to cytokine$^+$ populations. Lower histograms depict exemplary flow cytometry results of one mouse for IL-17A in blue over undyed control in grey. Column graphs show the percentage of cytokine$^+$ subpopulations after stimulation (n=3).

(b) Inherent cytokine receptor expression on BMDC surfaces. Graphs show the proportion of receptor$^+$ cells (IL-17R$^+$, IL-21R$^+$, IL-22R$^+$) of CD11c$^+$ BMDCs after priming for 4 h with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) (n=3). Unprimed control is labelled mock (M).

Statistical analyses by student's t test, bars represent mean, error bars SD, asterisks stand for p-values: $*\leq 0{,}05$, $**\leq 0{,}01$, n.s. means non-significant p-value $\geq 0{,}05$.

Furthermore, to examine if activation of IL-17R or IL-22R has an influence on IL-6 and IL-23 production in DCs, and thus indirectly on Th17 differentiation, it was measured how much of IL-6 and IL-23 was produced after stimulation with recombinant IL-17 and IL-22 (rIL-17 and rIL-22). Secreted IL-6 and IL-23 levels were determined by ELISA (Figure 3.4.2b). In general, the amount of secreted IL-6 is about 100 times higher than the one of IL-23. However, for both cytokines there is no significant change in secretion induced by either rIL-17 or rIL-22 compared to no stimulation.

The level of maturation that was induced by rIL-17 and rIL-22 stimulation was also investigated, by determining the fraction of MHC $II^{hi}CD11c^{+}$ BMDCs. Similarly to IL-6 and IL-23 levels, the amount of BMDCs that produce high amounts of MHC II and are therefore in a higher state of maturity are not increased by stimulation with 100 ng/mL of either rIL-17 or rIL-22 compared to no stimulation (Figure 3.4.2a). In contrast, stimulation with *E. coli* mpk significantly enlarges the MHC $II^{hi}CD11c^{+}$ population. Thus, both recombinant cytokines do not increase this marker of maturation of BMDCs in this setting.

Figure 3.4.2c displays the results of an experiment in which BMDCs were first primed for 24 h with *B. vulgatus* mpk or *E. coli* mpk and then subjected to a re-stimulation with either PBS (mock) or rIL-17 for 4 h. As in Figure 3.4.2a, maturation of the BMDCs was estimated by determining the percentage of MHC II^{hi} cells. There are no significant differences in the percentage of mature cells between mock and rIL-17 re-stimulation.

All in all, the experiments described in this chapter, with the chosen settings, do not show differences in the MHC $II^{hi}CD11c^{+}$ BMDC population or differences in Th17-inducing cytokines IL-6 and IL-23 produced by BMDCs after addition of Th17 effector cytokines IL-17 and IL-22 to BMDCs compared to no stimulation.

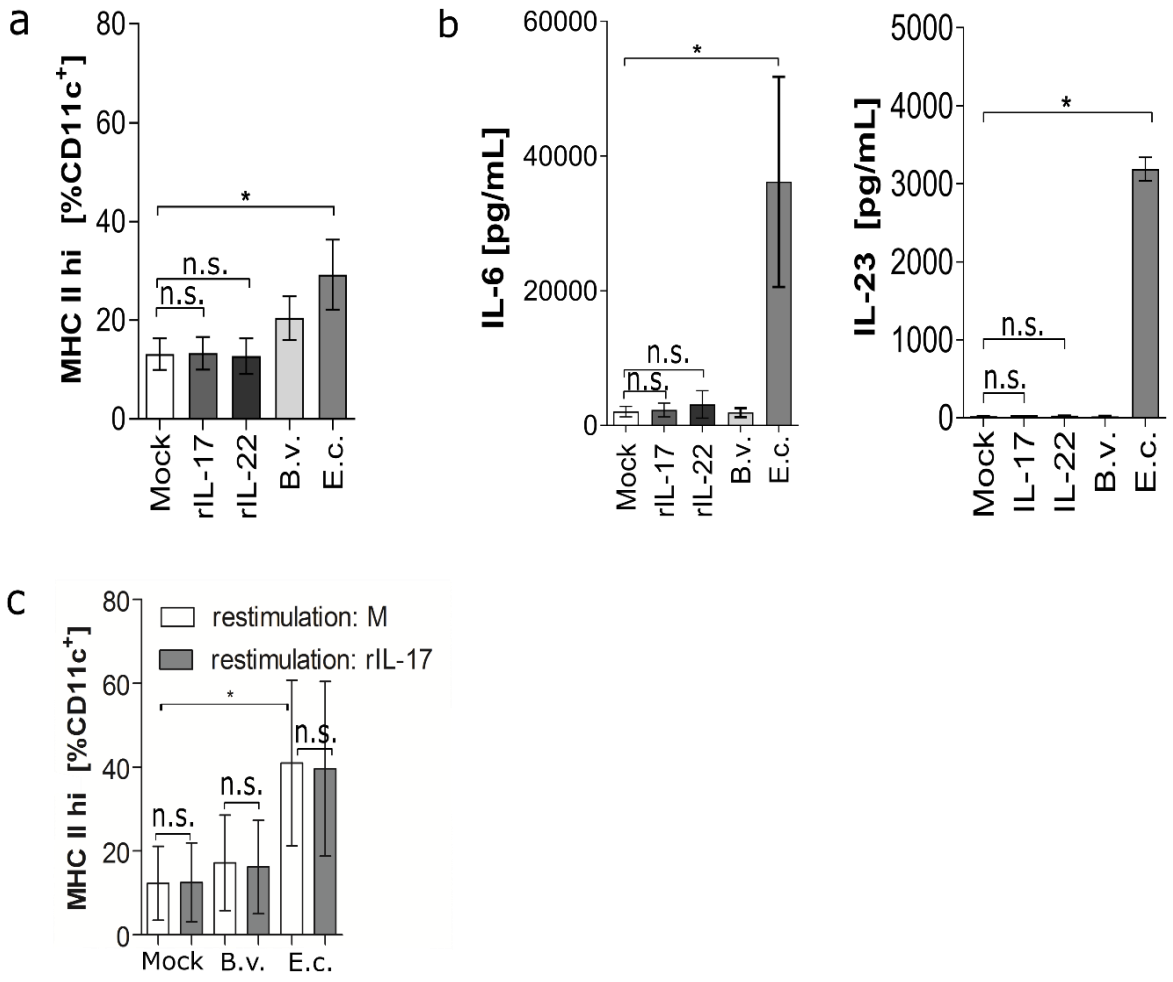

Figure 3.4.2: Maturity of BMDCs and production of Th17-inducing cytokines by BMDCs after stimulation with cytokines IL-17 and IL-22 and maturity of BMDCs challenged with IL-17 after priming with symbiont *B. vulgatus* mpk and pathobiont *E. coli* mpk

(a) and (b) show an analysis of a direct effect of recombinant IL-17 or IL-22 (rIL-17 or rIL-22) on BMDCs, while (c) is a re-stimulation of bacteria-primed BMDCs with rIL-17 to investigate maturation behaviour.

(a) BMDCs were stimulated with B. vulgatus mpk (B.v.), E. coli mpk (E.c.), or 100 µg/mL of rIL-17 or rIL-22 for 16 h. Unprimed control is labelled mock (M). Effect on BMDC maturation after 16 h was examined by flow cytometry. Gating strategy as demonstrated in Figure 3.1.1b. Graph shows the proportion of MHC II^{hi} cells of CD11c^{+} BMDCs (n=3).

(b) Capacity of Th17 induction was measured via ELISA for IL-6 and IL-23 after 4 h of stimulation. Graphs depict the amount of cytokine in the supernatant in pg/mL (n=3).

(c) Re-stimulatory effect of rIL-17 on maturation of primed BMDCs. BMDCs were primed with B. vulgatus mpk (B.v.) or E. coli mpk (E.c.) for 24 h and re-stimulated for 4 h with rIL-17, then subjected to flow cytometry. Unprimed control is labelled mock. Gating strategy as demonstrated in Figure 3.1.1b. Graph represents the proportion of MHC II^{hi} cells of CD11c^{+} BMDCs (n=3). Statistical analyses by student's t test, bars represent mean, error bars SD, asterisks stand for p-values: $*\leq 0{,}05$, n.s. means non-significant p-value $\geq 0{,}05$.

4 Discussion

The human body contains billions of microbes, mostly bacteria (Luckey, 1972, Sender et al., 2016). The biggest population of bacteria is hosted by the colon (Savage, 1977). Interestingly, the human host organism and its cohabitating bacteria seem to exert mutual influence, among other on immune processes (Mazmanian et al., 2005, Macpherson et al., 2000, Zaph et al., 2008) (see also 1.1). The same is true for other mammals (Ley et al., 2008).
As I described in the introduction, colitis in some mouse models such as the *Rag1*$^{-/-}$ model is seen as a partly Th17-mediated inflammation. The commensal *B. vulgatus* mpk ameliorates and even prevents formation of this inflammation (Gronbach et al., 2014, Steimle et al., 2019). Thus, it is of interest to investigate *B. vulgatus* mpk in the context of Th17 polarization.

Therefore, in this thesis, I investigated how *B. vulgatus* mpk modifies Th17-mediated inflammation. I could show correlation between DCs in a semimature state, induced by stimulation with *B. vulgatus* mpk, and a decreased proportion of Th17 cell in DC-T cell coculture. My results indicate that differences in the cytokine milieu created by DCs which are primed with the symbiont *B. vulgatus* mpk or the pathobiont *E. coli* mpk, play a role. Additionally, they indicate that these differences, namely in IL-6 production, are linked at least partly to coregulatory transcription factor IκBζ, while not to the negatively cytokine-regulating kinase MST1. Besides, a confounding retrograde influence of Th17 cytokines on the cytokine production of DCs, is not indicated.

4.1 Commensal Symbiont *B. vulgatus* mpk and Pathobiont *E. coli* mpk Influence Th17/Treg Cell Balance *in vitro* via Dendritic Cells

Based on the previous work described in chapter 1.4, putative influence of the symbiont *B. vulgatus* mpk on BMDC-mediated polarization of murine naïve $CD4^+$ T cells into Th17 or Treg cells was investigated and compared to influence of the pathobiont *E. coli* mpk. BMDCs were first primed with *B. vulgatus* mpk or *E. coli* mpk and then co-cultivated with naïve $CD4^+V\beta5.1^+$ T cells in order to analyse resulting T cell differentiation. Consistent with earlier findings of our group, *B. vulgatus* mpk priming results in significantly lower expression of MHC II on BMDCs than *E. coli* mpk stimulation (Figure 3.1.1), representing a semimature state of DCs (Waidmann et al., 2003, Frick et al., 2006, Muller et al., 2008). To compare Th17 and Treg polarization in the co-cultivated T cells, $CD3^+CD4^+$ T cells were analyzed for positivity for IL-17, the Th17 signature cytokine (Harrington et al., 2005, Park et al., 2005), compared to the fraction of cells positive for FOXP3, a typical Treg-regulating transcription factor (Fontenot et al., 2005). The amount of RORγt, the key transcription factor of Th17 cells, was meant to be examined as well, but I cannot show any reliable results due to a faulty flow cytometry antibody.

As presented in chapter 3.1, stimulation of BMDCs with *B. vulgatus* mpk does not lead to a significant increase in IL-$17A^+$ T cells compared to unstimulated BMDCs, while *E. coli* mpk-stimulated BMDCs initiate effective induction of Th17 cells as demonstrated by the high proportion of IL-$17A^+$ T cells.

In addition to determining the percentage of IL-17^+ T cells, cells were stained for IFNγ, an important cytokine in Th1 cells, but also secreted by a fraction of Th17 cells which are associated with severe inflammation in mice as well as humans. These IL-17^+IFNγ$^+$ T cells have been detected, e.g., in the intestines of patients with Crohn`s disease (Annunziato et al., 2007). Their occurrence has been linked to IL-12 and IL-23 (Ahern et al., 2010) and they likely represent a particularly pathogenic subpopulation of Th17 cells (Lee et al., 2009, Annunziato et al., 2007,

Boniface et al., 2010, Hirota et al., 2011), which makes them worth investigating in the context of this work.

The difference in amounts of IL-17$^+$IFNγ$^+$ T cells between *B. vulgatus* mpk-primed cocultures and *E. coli* mpk-primed cocultures is significant and results are very similar to the ones of IL-17$^+$ T cells. IL-17$^+$IFNγ$^+$ T cells have been described in literature as a fraction of T cells, being present mainly at chronically inflammatory sites (Ahern et al., 2010). Thus, such a high fraction is in line with expectations for the pathobiont *E. coli* mpk-primed batches. However, after priming with *B. vulgatus* mpk, I would have expected a lower fraction of IL-17$^+$IFNγ$^+$ T cells than IL-17$^+$ T cells, coherent with the less inflammatory conditions. If this correspondence between IL-17$^+$IFNγ$^+$ T cells and IL-17$^+$ T cells is valid or due to experimental conditions favouring differentiation towards IL-17$^+$IFNγ$^+$ T cells over IL-17$^+$ T cells or to methodical flaws like fluorescence spread in this multicolour flow cytometry setting, could be discerned by repetition under other staining conditions.

A surprising finding is that stimulation with *B. vulgatus* mpk and no stimulation results in already high percentages of IL-17$^+$ as well as IL-17$^+$IFNγ$^+$ T cells. Since these cells are described as associated with inflammatory conditions in literature, there seems to be no reason for higher amounts in an *in vitro* setting that lacks these inflammatory stimuli. So, in this experiment, I would have expected lower levels of IL-17$^+$IFNγ$^+$ T cells after stimulation with *B. vulgatus* mpk and in the unstimulated control. One explanation for the high levels in this experiment might be false positive events because of fluorescence spread. Such an error was minimized by use of FMO controls in the data analysis, but it is possible that the gate was set too permissively. It is also imaginable that, in this *in vitro* setting, influences of other factors than stimulation type, like autocrine stimulation of the T cells, play a role. Nevertheless, the gating criteria for all batches were the same, which makes the significant difference in percentage of IL-17A$^+$ T cells and IL-17$^+$IFNγ$^+$ T cells between symbiont *B. vulgatus* mpk- and pathobiont *E. coli* mpk-primed cocultures most likely valid, indicating that

B. vulgatus mpk-driven in contrast to *E. coli* mpk-driven T cell differentiation is more balanced between Th17 cells and Treg cells.

Regardless of type of stimulation, about 30% of $CD3^+CD4^+$ cells are $FOXP3^+$. Consequently, naïve $CD4^+$ T cells seem to be polarized into $FOXP3^+$ Treg cells independently of priming of BMDCs with either *B. vulgatus* mpk or *E. coli* mpk under these conditions.
The measurements in this experiment were performed after three days of coculture. In literature such a timespan has been used before for differentiation of naïve $CD4^+$ T cells into iTreg cells by stimulation with anti-CD3, splenic APCs and TGFβ (Chen et al., 2003), but mostly five or more days are used (Bettelli et al., 2006). It is possible that bacterial influences on Treg polarization become apparent only after a longer time of coculture. This could be investigated with longer coculture experiments.
Beside this, in literature, there are findings that short chain fatty acids produced by *B. fragilis* directly induce Treg cells (Smith et al., 2013) and correlations that indicate that *B. vulgatus* is able to produce short chain fatty acids itself (Yang et al., 2013). As *B. fragilis* is a relatively close relative of *B. vulgatus* (Lange et al., 2016), even though in the current setting an influence is not indicated, further experiments, also on direct effect of *B. vulgatus* on Treg development might be interesting.

One cannot compare the findings for IL-17^+ cells and $FOXP3^+$ cells directly, of course, as IL-17 is an effector protein and FOXP3 a transcription factor. However, both are commonly used as markers for Th17 or Treg cells in literature, e.g. in this publication published in 2011 (Hirota et al., 2011). Thus, the observation of differences between batches primed with *B. vulgatus* mpk or *E. coli* mpk points into the direction that only the pathobiont *E. coli* mpk tilts the balance between Treg and Th17 cells towards Th17.

Notably, after the complete gating process of the flow cytometry data, the total numbers of events, interpreted as total number of cells, are partly below

100 events/graph. This evokes the question of how biologically relevant the results of such low numbers are. According to a publication dedicated to this subject (Roederer, 2008), there is no ultimate lower threshold event number for a truly positive sample, though a higher degree of certainty can be achieved by carefully applying negative and positive controls and by repetition.

Taken together, the results of this coculture experiment suggest that *B. vulgatus* mpk stimulation results in more semimature BMDCs and less differentiation into Th17 cells (IL-17$^+$ T cells and IL-17$^+$IFNγ$^+$ T cells) than stimulation with pathobiont *E. coli* mpk. Thus, there is no shift towards proinflammatory Th17 cells in the Th17/Treg balance by *B. vulgatus* mpk.

4.2 Dendritic Cells Produce Th17- or Treg-inducing Cytokines Differentially, when Stimulated with Commensal Symbiont *B. vulgatus* mpk and Pathobiont *E. coli* mpk

In vivo as well as in *in vitro* coculture experiments, APCs, such as DCs, form a link between bacteria and T cells. Consequently, special attention was paid to these cells, studying BMDCs in more detail in the context of Th 17 and Treg differentiation.

The comparison of MHC II^{hi} cell subsets in Figure 3.2.1a is a measure of maturity of these cells. In accordance to earlier data, the significantly lower amount of MHC II^{hi} cells in *B. vulgatus* mpk-primed BMDCs compared to *E. coli* mpk stimulation, is thought to go along with a semimature phenotype (Frick et al., 2006, Waidmann et al., 2003, Muller et al., 2008).

How this semimaturity reflects on production of the key cytokines for Th cell differentiation, is addressed in Figure 3.2.2 and Figure 3.2.3. This is of importance for the lineage commitment of naïve T cells, as their cytokine environment directs and upholds differentiation.

IL-6 and TGFβ1, e.g., induce Th17 polarization (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). But only under the influence of IL-23, Th17 cells become a more active, or even destructive subset (McGeachy et al., 2009). IL-10, on the other hand, is the signature cytokine of Treg cells (Noack and Miossec, 2014, Fontenot et al., 2005). It is not only produced by T cells, though. Being

released by APCs, together with TGFβ1, it drives polarization of Tregs (Chen et al., 2003, Hsu et al., 2015). So, one can state that these four cytokines are key players in establishing a balance between Th17 cells and their antagonists, iTreg cells, by directing T cell polarization.

A similar characterization for Th1-inducing cytokines in BMDCs primed with either the symbiont *B. vulgatus* mpk or the pathobiont *E. coli* mpk has been presented previously by our group (Frick et al., 2010), as well as partly for Th17-inducing cytokines (Birg, 2017). Broadening the picture, the experiments discussed here characterize the fraction of *B. vulgatus* mpk- or *E. coli* mpk-primed BMDCs that are positive for Th17-inducing cytokines for an early and late timepoint and adds two cytokines that are involved in iTreg differentiation, TGFβ1 and IL-10.

First, the fractions of $CD11c^+$ BMDCs that contained the cytokines of interest were determined by flow cytometry analysis. This method focuses on cytokine production in the cells, not cytokine release, and therefore sheds a light on how many cells are able to produce a certain cytokine. Two time-points were analysed: One early one at 4 h of stimulation and a late one at 16 h of stimulation. These were chosen based on observations of our group concerning the kinetics of cytokine release. Additionally, in an earlier study on the kinetics of cytokine release the time intervals of 3-5 h and 12-18 h have been found to be of special interest in the kinetics of dendritic IL-6 production after stimulation with bacterial LPS (Langenkamp et al., 2000).

Results shown in chapter 3.2 differ for every cytokine: Amounts of IL-$23p19^+$ cells are the only ones that are increased in pathobiont *E. coli* mpk-stimulated DCs compared to *B. vulgatus* mpk-primed DCs at the early timepoint of 4 h. For IL-6, at this early timepoint, stimulation does not lead to differences in the fraction of cells capable to produce this cytokine. At the later timepoint of 16 h, stimulation of DCs with *B. vulgatus* mpk results in significantly lower amounts of cells producing the cytokines IL-6 and IL-23 compared to stimulation with *E. coli* mpk, which is in accordance with the lower polarization rate of IL-17^+ T cells shown in chapter 3.1.

The fraction of IL-10$^+$ BMDCs starts at a very low level and increases for all stimulation types over time. There is no significant difference, possibly due to high standard deviation. However, a clear trend is indicated, with *B. vulgatus* mpk-primed DCs showing only half as many IL-10$^+$ cells than *E. coli* mpk-stimulated DCs.
The amount of TGFβ$^+$ BMDCs, as indicated by flow cytometry data for LAP, the protein with which TGFβ1 is associated prior to and while release, shows no reaction to this bacterial stimulation.

So far, the fractions of cytokine producing cells were investigated. The question remained how much of these cytokines was actually released in this *in vitro* setting, and thus able to act on T cells in coculture. This was addressed through measuring the concentrations of the cytokines IL-6, IL-23, TGFβ1 and IL-10 in cell culture supernatant by ELISA (Figure 3.2.4).
The experiment showed much lower IL-6 secretion after stimulation with the symbiont *B. vulgatus* mpk than after stimulation with the pathobiont *E. coli* mpk, for both timepoints. So, considering the number of IL-6$^+$ cells from the flow cytometry analysis (Figure 3.2.2), IL-6 production and secretion seems to be strongly initiated by *E. coli* mpk early after challenge in a few IL-6 producing BMDCs, as IL-6 concentration is high after 4 h stimulation time, before the number of IL-6$^+$ cells increase. This highly active fraction of IL-6$^+$ cells explains the apparent contradiction between the flow cytometry results of few IL-6$^+$ cells at 4 h of stimulation time and descriptions of increased IL-6 production in literature plus the ELISA results (Langenkamp et al., 2000). Again taking into account the proportion of IL-6$^+$ cells, a low amount of IL-6$^+$ cells produces low amounts of IL-6 after *B. vulgatus* mpk stimulation, with a lower increase. Earlier findings (Frick et al., 2006) demonstrated an IL-6 secretion of about 2000 pg/mL after 20 h of incubation for both *B. vulgatus* mpk and *E. coli* mpk. In contrast to those findings, results presented here show comparable concentrations of IL-6 detected after stimulation with *B. vulgatus* mpk, but substantially higher concentrations after *E. coli* mpk stimulation, leading to a significant difference.

The experimental design was slightly different. In the earlier experimental setting, bacteria were viable for 1 h, then gentamycin was added, and the BMDCs were incubated with the devitalized bacteria for another 19 h. In the current setting, gentamycin was added right away, and incubation was done with these devitalized bacteria for 4 h and 16 h. But I would not expect large differences due to these slight experimental changes. So, at this point, I cannot explain the discrepancy to the earlier data. Conclusions drawn from my data contradict the previous conclusions in so far, as a lower production of IL-6 in BMDCs that have been primed by *B. vulgatus* mpk is indicated. On the other hand, this is in line with the lower polarization rate of Th17 cells, for which IL-6 is an inducing cytokine.

After 4 h of stimulation with *B. vulgatus* mpk, IL-23 secretion by BMDCs is about six times lower than IL-6 secretion, increasing about 30fold after 16 h. It is also significantly lower than after stimulation with pathobiont *E. coli* mpk. This corresponds well to the generally lower proportions of IL-23p19$^+$ cells (Figure 3.2.2), and the activating role of IL-23 in late directing and maintaining of Th17 differentiation, that has been described before (Bettelli et al., 2006, McGeachy et al., 2009, McGeachy and Cua, 2007). Reflecting the semimaturity of BMDCs primed with *B. vulgatus* mpk, here, IL-23 is released only by a low number of BMDCs in low amounts (Figure 3.2.2 and Figure 3.2.4).

IL-10 concentrations in the supernatant of symbiont B. vulgatus mpk-stimulated batches are significantly lower than in the ones stimulated with pathobiont E. coli mpk (Figure 3.2.4). Stimulation by the symbiont B. vulgatus mpk does not trigger an increase in the amount of secreted IL-10. Interestingly, when looking at the cell proportions (Figure 3.2.3), IL-10 producing cells multiply over time with all types of stimulation. So, after 16 h there seems to exist a higher population of CD11c$^+$IL-10$^+$ cells, but only in BMDCs stimulated with the pathobiont E. coli mpk this is echoed in increased IL-10 secretion. The same trend has been observed before, in literature as well as in our group. It has been suggested that IL-10 production of APCs increases under inflammatory conditions as a means of (auto-)regulation (Fiorentino et al., 1991, de Waal Malefyt et al., 1991, Frick et al., 2006).

TGFβ1 is a pleiotropic cytokine that can be produced, e.g., by macrophages, DCs and lymphocytes. In this experiment, only active TGFβ1 was measured in the supernatants. This reflects the TGFβ1 produced by BMDCs that is able to act on T cells in an *in vitro* setting like the one in the coculture experiment addressed in 3.1 and 4.1, and thus most interesting in this context. The low level of activated TGFβ1 that shows a similar small trend of increase over time for all stimulation types, is consistent with findings of the cell proportions (Figure 3.2.3 and

Figure 3.2.4) that present low proportions of about 10% for all stimulation types. B. vulgatus mpk stimulation does not seem to take influence on TGFβ1 levels secreted and activated by BMDCs in this *in vitro* setting. These findings cannot, though, be generalised for *in vivo* research. A necessary next step in this direction would be to determine the overall amount of secreted TGFβ1, ligated as well as free. The lack of bacterial regulatory effect on TGFβ1 shown in this *in vitro* setting, would not be surprising *in vivo* as well, as this cytokine is known to be abundant in the intestinal lamina propria and already low levels of TGFβ are key to differentiation of both Th17 and Treg cells (Manel et al., 2008, Yang et al., 2008a). I would, thus, rather expect other cytokines, such as IL-6, to relay an influence of symbionts like *B. vulgatus* mpk on the balance of Th17 and Treg cells.

To sum this up, data from flow cytometry and ELISA together reveal that symbiotic stimulation by *B. vulgatus* mpk turns only few BMDCs into producers of IL-6, IL-23, TGFβ1 or IL-10 and overall production of these cytokines is low compared to pathobiont stimulation by *E. coli* mpk. Fittingly, *B. vulgatus* mpk has previously been identified to be a symbiont with anti-inflammatory effects associated to promotion of a tolerant semimature DC phenotype that proved unsusceptible to subsequent challenge with other bacteria (Frick et al. 2006, Waidmann et al. 2003, Muller et al. 2008).

One must bear in mind that the experiments discussed above show the reactions of BMDCs and T cells towards priming with symbiotic or pathobiontic bacteria. They show differences that result from these different stimulations. However, to prove undeniably that stimulation by the symbiont *B. vulgatus* mpk differs from

no effect in having a positive effect on Th17-mediated reactions, in future, experiments with re-stimulations of symbiont *B. vulgatus* mpk-primed cells with pathobiont *E. coli* mpk and Th17-related read-outs would be useful. To show a positive effect of *B. vulgatus* mpk, I would expect *B. vulgatus* mpk-primed cells to react less to a re-stimulation with *E. coli* mpk than the unprimed control. Besides, *in vitro* settings are never as complex as *in vivo* cell environments: *In vivo* cells encounter a lot of other cell types in near vicinity, a much more complex biochemical milieu and a totally different 3D tissue architecture than in petri dishes. Thus, *in vivo* experiments are necessary to complement the *in vitro* findings presented here.

All in all, the results discussed so far indicate that symbiont *B. vulgatus* mpk-primed semimature BMDCs balance polarization of naïve T cells into Th17 and Treg cells through lower induction of IL-6 and IL-23, especially at a later timepoint. These findings are similar to the effect of semimature BMDCs on Th1-related cytokines like IL-12 (Frick et al., 2010) and explain the difference in Th17/Treg cell ratio demonstrated in chapter 3.1 and part of the effect of *B. vulgatus* mpk-primed semimature BMDCs on colitis *in vivo (Gronbach et al., 2014)*. Consequently, that brings up the question, how the production of these cytokines in BMDCs is regulated by bacterial influence.

4.3 Production of IL-6 by Dendritic Cells after Stimulation with Commensal Symbiont *B. vulgatus* mpk and Pathobiont *E. coli* mpk can be Linked to Transcription Factor IκBζ and not to Regulatory Kinase MST1

In our group, we have previously addressed a promising regulatory protein of IL-6: Nuclear IκBζ, which is known to be an important regulatory protein for IL-6 in T cells and macrophages. IκBζ is an atypical IκB protein, that can both inhibit certain NF-κB pathways and act as a positive coregulatory transcription factor for other NF-κB target genes. This coregulatory function is exhibited in cooperation with the NF-κB subunit p50 (Yamamoto et al., 2004, Okamoto et al., 2010). The gene coding for IκBζ is known as *Nfkbiz* (see 1.2.2).

Our group has demonstrated before that *Nfkbiz* is expressed on a low level by BMDCs stimulated with the symbiont *B. vulgatus* mpk compared to higher levels

in pathobiont *E. coli* mpk-stimulated BMDCs (Klameth, 2012). In the present work, these findings were reaffirmed and brought into context with the Th17 immune network.

Results presented in Figure 3.3.1b shed a light on the impact of IκBζ on secretion of the cytokines IL-6 and IL-23 by BMDCs. For this purpose, cytokine secretion of BMDCs from *Nfkbiz*$^{-/-}$ and WT BMDCs are compared. In short, stimulation with the pathobiont *E. coli* mpk results in a significantly lower secretion of IL-6 by *Nfkbiz*$^{-/-}$ BMDCs compared to WT, while priming with *B. vulgatus* mpk does not lead to a significant difference in IL-6 secretion between these groups.
These findings emphasize the relevance of IκBζ for IL-6 production in BMDCs, as *Nfkbiz*$^{-/-}$ BMDCs stimulated with pathobiont *E. coli* mpk are not able to react with an IL-6 production comparable to WT cells. Together with the observation shown in Figure 3.3.2, that only *E. coli* mpk, but not *B. vulgatus* mpk stimulation leads to high transcription of *Nfkbiz* mRNA in BMDCs, this indicates that pathobiont *E. coli* mpk stimulation leads to high IκBζ expression associated with high IL-6 secretion in BMDCs, while symbiont *B. vulgatus* mpk exposure is followed by fewer IκBζ production associated with a level of IL-6 secretion in BMDCs that is higher, though not significantly, than in unstimulated cells. It is, therefore, plausible that IκBζ acts as a transcription factor of IL-6 in DCs that are in contact with commensals, in this case being activated to a lower level by the symbiont *B. vulgatus* mpk than by the pathobiont *E. coli* mpk. Similar, TLR-mediated IL-6 expression has been described in literature for T cells (Okamoto et al., 2010). Experiments by this group also showed an impaired IL-6 production in *Nfkbiz*$^{-/-}$ DCs stimulated with LPS, showing the same trend we observed in *E. coli* mpk-stimulated DCs (Okamoto et al., 2010).

A similar analysis, this time for IL-23 production by BMDCs, was done. IL-23 production in BMDCs has been shown to be triggered among others by TLR4 stimulation (Roses et al., 2008). In another study, TLR4 response in *Nfkbiz*$^{-/-}$ cells was impaired. This suggests a possible link of IL-23 production in BMDCs via TLR4 stimulation and IκBζ activation. The results show the previously described

difference in the concentration of secreted IL-23 between stimulation with symbiont *B. vulgatus* mpk or pathobiont *E. coli* mpk, with lower secretion after *B. vulgatus* mpk stimulation. There is, however, no difference in the level of IL-23 between *Nfkbiz*$^{-/-}$ and WT cells upon stimulation. Thus, it is indicated that *B. vulgatus* mpk and *E. coli* mpk influence IL-23 production in DCs in a way independent of IκBζ.

Notably, IL-6 production in *Nfkbiz*$^{-/-}$ BMDCs stimulated with the pathobiont *E. coli* mpk is still higher than in WT BMDCs stimulated with the symbiont *B. vulgatus* mpk. Additionally, in another study LPS-stimulated *Nfkbiz*$^{-/-}$ DCs that showed impaired IL-6 production were still able to induce Th17 cells on the same level as WT DCs (Okamoto et al., 2010). Thus, IκBζ does not seem to be the only factor involved in regulation of IL-6 production induced by commensal stimulation.

So, another regulatory protein was considered: MST1 is a kinase involved in several important cell pathways (see 3.3). It contributes to such different processes as regulation of cell cycle and apoptosis, Treg cell function and ROS production in phagocytes (Li et al., 2017). Recently it has been shown to play a crucial role in blocking Th17 differentiation as a negative regulator of IL-6 production in DCs (Li et al., 2017). So, in this context, it is explored if MST1 is also involved in regulation of IL-6 production of DCs by contact with *B. vulgatus* mpk by analysis of mRNA levels of *Mst1* at different time points after stimulation.

For IκBζ, *Nfkbiz* mRNA timelines have been established previously by our lab (Klameth, 2012, Birg, 2017). Here, these PCR measurements are reaffirmed and contrasted with a *Mst1* mRNA timeline. As has been shown before, *Nfkbiz* mRNA in *E. coli* mpk-stimulated BDMCs shows a high increase in the first four hours of stimulation, before it decreases to a moderate long-term level. BDMCs stimulated with symbiont *B. vulgatus* mpk show a mild, non-significant, increase in *Nfkbiz* mRNA. This is in line with the postulated effects of *B. vulgatus* mpk on IL-6 production by BMDCs via IκBζ.

In contrast, *Mst1* mRNA levels do not differ relating to stimulation type. This is an indication that, unlike IκBζ, the negative MST1 regulation pathway is not part of IL-6 regulation in BMDCs primed with symbiont *B. vulgatus* mpk by change of transcription rate. Post-translational changes to MST1 could mediate another kind of regulation. However, in literature, I could not find evidence for such post-translational regulation of MST1.

Hence, the question of further regulatory proteins involved in this process remains unanswered by this work. Other proteins of interest have been investigated by our group in similar context, such as the endoribonuclease MCPIP-1, that is able to degrade *Nfkbiz* mRNA and *IL-6* mRNA, or A20, a negative regulator of NF-κB. They seem to be involved in regulation of NF-κB signaling taking influence on semimaturation of BMDCs (Birg, 2017). Further work will be necessary to evolve a comprehensive picture of the mechanisms involved in semimaturation of DCs and subsequent regulation of Th17 differentiation.

4.4 Th17 Signature Cytokines IL-17 and IL-22 Have no Retrograde Effect on Naïve Dendritic Cells

So far, influence of the symbiont *B. vulgatus* mpk, and for comparison, pathobiont *E. coli* mpk on the cytokine repertoire of DCs and their capability to induce Th17 or Treg cells has been the focus of this work.

This last chapter of the discussion complements that with investigating influence from a source which is located in some way at the end of the process: The activated Th cells. Activated Th17 cells release downstream Th17 cytokines such as IL-17, IL-21 and IL-22 in the vicinity of DCs (see 1.2.2). For different Th cells it has been shown that they exert influence on their own differentiation and survival by autocrine feedback loops (Becskei and Grusby, 2007, Nurieva et al., 2007). Moreover, for IL-21, a negative effect on the production of Th1 inducing cytokines by DCs has been proposed (Strengell et al., 2006, Gharibi et al., 2016). To investigate if there exist other effects of Th17 effector cytokines on DCs that would have to be discerned from commensal effects on BMDCs in future, experiments with recombinant cytokines and BMDCs are looked upon.

Cells are only able to respond to stimuli if they contain suitable receptors. In this case, surface cytokine receptors such as receptors of the IL-17R family are most likely the partners of interaction of the soluble cytokines. Figure 3.4.1b depicts a measurement of the percentage of $CD11c^+$ BMDCs that express IL-17R, IL-21R and IL-22R after stimulation with *B. vulgatus* mpk or *E. coli* mpk. In all batches there was a fraction of $CD11c^+$ cells that were IL-$17R^+$, IL-$21R^+$ or IL-$22R^+$, but with very low levels of below 2% of $CD11c^+$ cells for IL-22R. For IL-17R, the 15-18% IL-$17R^+$ BMDCs correspond well with findings in another study, where $CD11c^+$ BMDCs contained a fraction of IL-$17R^+$ cells of about 20% (Antonysamy et al., 1999). For IL-21R, it has been shown to be expressed in BMDCs before as well (Brandt et al., 2003). My findings of about 10-15% of IL-$21R^+$ cells correspond to this trend. The low fraction of 2% of IL-$22R^+CD11c^+$ cells are in line with literature findings, as *IL-22R1* gene expression was not upregulated in DCs in a study on human *ex vivo* immune cells (Wolk et al., 2004).

To investigate an effect on these receptors by Th17 effector cytokines, a stimulation of BMDCs with recombinant IL-17 (rIL-17) or IL-22 (rIL-22) was conducted. IL-22 was included to complete the picture, even though the very low percentage of IL-$22R^+$ BMDCs and the literature findings mentioned above (Wolk et al., 2004) were suggestive for the assumption that BMDCs are not reactive to IL-22. Stimulation with rIL-21 could not be investigated due to low protein availability at the time. A closer look would certainly be interesting, though, as I detected about ten times more IL-$21R^+$ BMDCs than IL-$22R^+$ BMDCs, with a significantly lower percentage in *E. coli* mpk-primed cells than in *B. vulgatus* mpk-primed cells. As it has been shown in literature that exposure to IL-21 can lead to a less Th1-inducing profile of LPS-induced cytokines produced by DCs (Strengell et al., 2006), a higher expression of IL-21R, and hence higher responsivity to the IL-21 feedback loop, in *B. vulgatus* mpk-primed semimature DCs is conclusive. Furthermore, amounts of inherent cytokine production of BMDCs were generally higher for IL-21 than for IL-17 and IL-22. This, however, is unexpected, as in contrast to my findings, in an earlier study low-dose LPS-stimulated BMDCs showed no inherent IL-21 production (Brandt et al., 2003). It

is possible, though, that our bacterial stimulation influenced IL-21 upregulation in another way than by TLR activation.

As representation for an effect of Th17 effector cytokines on BMDCs I chose to measure the amounts of IL-6 and IL-23 secreted by BMDCs after stimulation with rIL-17 or rIL-22. This mimics an influence of the effector cytokines on their own induction. Additionally, BMDCs were checked for the maturity marker MHC II, similarly to the tests in 3.1 and 3.2. In this experimental setting and with the chosen concentration, neither stimulation with rIL-17 nor with rIL-22 does lead to an increase in production IL-6 or IL-23 in BMDCs or an increase in maturity marker MHC II, indicating no retrograde effect of Th17 effector cytokines on the cytokines for their own induction.

Even an additional re-stimulation with rIL-17 shows no increase in IL-6 or IL-23 above levels of unstimulated cells.

For stimulation with rIL-22, this is in line with other findings in literature. E.g., in an earlier study on *ex vivo* human immune cells, cultured peripheral blood mononuclear cells did not react to stimulation with IL-22 (Wolk et al., 2004). For stimulation and re-stimulation with rIL-17, particularly the lack of increase in MHC II is unexpected, as other studies have shown an increase in maturity markers such as CD40 after stimulation of BMDCs with lower concentrations of rIL-17 (Antonysamy et al., 1999, Bai et al., 2009).

Considering this apparent contradiction and that more than 10% of BMDCs are IL-17R$^+$ and IL-21R$^+$, the questions remains if there is a purpose for this. So, it might be interesting to modulate the experimental setting, or have a closer look at other effects than IL-6 and IL-23 induction that might be triggered by activation of these receptors.

4.5 Conclusion and Outlook

To address the influence of *B. vulgatus* mpk on Th17 cell induction by DCs, it is shown that stimulation of DCs with *B. vulgatus* mpk results in a lower fraction of Th17 cells in coculture with those DCs, compared to stimulation of DCs with *E. coli* mpk. Correspondingly, analysis of the expression of Th17-inducing cytokines and Treg-inducing cytokines reveals that after stimulation with the symbiont *B. vulgatus* mpk BMDCs produce less Th17-inducing cytokines than pathobiont *E. coli* mpk-stimulated BMDCs. Thus, they seem to tilt Th cell differentiation away from potentially destructive activation of Th17 cells. These findings support the thesis that *B. vulgatus* mpk aids intestinal homeostasis. This is especially important in the context of immune overreaction and autoimmunity.

To further characterize the way *B. vulgatus* mpk influences Th17 induction by DCs, regulation of IL-6 and IL-23 as key Th17-inducing cytokines was highlighted. Two known molecular regulators of IL-6 in several immune cells, IκBζ and MST1 (see 1.2.2), were investigated. It was shown that BMDCs of *Nfkbiz*$^{-/-}$ mice show impaired IL-6 production after stimulation with the pathobiont *E. coli* mpk compared to WT BMDCs. These findings support the assumption that IκBζ plays an important role in IL-6 regulation in BMDCs. Fitting the cytokine analysis described above, stimulation with the symbiont *B. vulgatus* mpk leads to lower and not significantly differing levels of IL-6 production in *Nfkbiz*$^{-/-}$ and WT mice, as IL-6 production is not upregulated to such an extend as by *E. coli* mpk stimulation. For IL-23, the data show no regulation through bacterial stimulation. A comparison of mRNA levels over time of expressed genes encoding the proteins IκBζ and MST1 reveals that, in contrast to *Nfkbiz* transcription, *Mst1* transcription is consistent throughout time for stimulation with *B. vulgatus* mpk or *E. coli* mpk. Therefore, this indicates no commensal influence via MST1 on BMDCs under these settings.

Another way to regulate cells is a feedback loop. Th17 cells release signature cytokines like IL-17, IL-21 and IL-22. It was checked if BMDCs in contact with bacteria and in vicinity to T cells would be affected by these cytokines. Under the

investigated settings, a retrograde influence of stimulation with rIL-17 or rIL-22, or re-stimulation with rIL-17, on production of Th17-inducing cytokines by BMDCs can be excluded.

All in all, this shows that influence of symbiont *B. vulgatus* mpk on Th17 cell differentiation in context with intestinal homeostasis is a promising mechanism for approaching treatment options of autoimmune disorders. To find to a profound understanding of the mechanisms through which this influence is exerted, additional regulatory pathways need to be addressed in future research.

Another challenge to T cell research has emerged recently; possibly the starting point of fruitful new insights: It has become evident that T cells are cells of high plasticity. All Th cells, but especially Th17 cells, are able to change their differentiation programs, extend the range of expressed proteins or switch to another differentiation type altogether (see 1.2.2). The paradigm has shifted from the idea of terminal differentiation of T cells towards plasticity throughout the whole cell life cycle. This adds a new layer of complexity to T cell-related immunological research, which will surely stay a fascinating field of science.

5 Abstract

5.1 English Abstract

Autoimmune diseases such as inflammatory bowel disease (IBD) are accompanied by severe limitations to living a normal life. Hence, a thorough understanding of the underlying immunological events is crucial to develop new therapies. T helper17 (Th17) cells act at the core of adaptive immune responses that facilitate autoimmune disorders. They are known to have a close reciprocal relationship with regulatory T cells, forming a balance of pro- and anti-inflammatory actions under physiological conditions. Apart from that, the immune system is in close contact to commensal microbes. Especially the intestinal microbiota have been linked closely to T cell-mediated autoimmunity. Symbiotic bacteria such as the strain *Bacteroides vulgatus* mpk (*B. vulgatus* mpk), have been shown to positively influence the action of T cell-activating dendritic cells (DCs). *B. vulgatus* mpk has the capacity to drive DCs into a semimature state and to ameliorate murine colitis, a model for IBD in mice.

If and how this ability of *B. vulgatus* mpk influences Th17 induction is not known. For this purpose, effects of *B. vulgatus* mpk on DCs and on co-cultivated naïve T cells are compared to effects of the model pathobiont *E. coli* mpk. Stimulation of DCs with symbiont *B. vulgatus* mpk results in a lower fraction of co-cultivated T cells differentiating into Th17 cells compared to priming with pathobiont *E. coli* mpk. Analysis of cytokines IL-6 and IL-23, that induce and maintain pathogenic Th17 cells, reveals that after stimulation with *B. vulgatus* mpk DCs produce less Th17-inducing cytokines compared to stimulation with the pathobiont *E. coli* mpk.

So, two known molecular regulators of IL-6 in immune cells, IκBζ and MST1, are investigated in context with regulation of IL-6 and IL-23 in DCs after stimulation with *B. vulgatus* mpk. A lower concentration of IL-6 associated with a lower transcription of *Nfkbiz*, the gene of IκBζ, in DCs can be affirmed. In contrast to IκBζ, *Mst1* mRNA analysis shows no symbiont *B.vulgatus* mpk- and pathobiont *E.coli* mpk-dependent regulation.

Additionally, the expression of Th17-inducing cytokines by DCs could be influenced retrogradely by feedback loops of cytokines secreted by Th17 cells. To investigate this, the effect of recombinant IL-17 and IL-22 stimulation on DC cytokine production is explored. The results suggest that ability of DCs to induce Th17 cells is not regulated by retrograde exposure to Th17 signature cytokines under these settings.

Taken together, my results indicate that contact of DCs with the symbiotic bacterium *B. vulgatus* mpk in contrast to contact with pathobiont *E. coli* mpk indirectly tilts T helper cell differentiation away from a potentially destructive activation of Th17 cells, giving evidence for its role in intestinal homeostasis. This is facilitated by a lower secretion of Th17-inducing cytokines IL-6 and IL-23 by DCs. For IL-6, this lower secretion is correlated with lower production of its regulator IκBζ in DCs. Thus, stimulation of DCs with *B. vulgatus* mpk is a promising mechanism for approaching treatment options and is especially important in the context of immune overreaction and autoimmunity.

5.2 Deutsche Zusammenfassung

Autoimmunerkrankungen wie chronisch entzündliche Darmerkrankungen (CED) gehen für Patienten einher mit langfristig schweren Einschränkungen im Alltag. Um gezielte Therapie-Ansätze zu entwickeln, ist es unumgänglich, die zu Grunde liegenden immunologischen Prozesse besser zu verstehen. Th17-Zellen, eine Art von T-Helferzellen, bilden den Kern von adaptiven Immunreaktionen, die Autoimmunerkrankungen begünstigen. Diese T-Zell-vermittelten Immunreaktionen sind von einer engen reziproken Beziehung zwischen Th17-Zellen und regulatorischen T-Zellen bestimmt. Deren proinflammatorische und anti-inflammatorische Einflüsse stehen unter physiologischen Bedingungen in einer Balance. Außerdem wurde in den letzten Jahren immer deutlicher, wie eng der Kontakt des Immunsystems mit kommensalen Bakterien ist. Besonders die Darm-Mikrobiota beeinflusst T-Zell-vermittelte autoimmune Reaktionen. Für symbiotische Bakterien wie *B. vulgatus*, insbesondere den Stamm *B. vulgatus* mpk, wurde ein positiver Einfluss auf dendritische Zellen (DCs), wichtige Antigen-präsentierende Zellen für T-Zellen, gezeigt. *B. vulgatus* mpk kann diese in einen semimaturen Zustand versetzen und mildert die induzierte murine Colitis, ein Maus-Modell für CED.

Ob diese Fähigkeiten einen Einfluss auf die Induktion autoimmun-assoziierter Th17-Zellen haben, ist bislang nicht geklärt. Daher untersucht diese Arbeit, inwiefern *B. vulgatus* mpk durch Beeinflussung von DCs einen Einfluss auf die Differenzierung von naïven T-Helferzellen zu Th17-Zellen hat. Dafür werden die Effekte des Symbionten *B. vulgatus* mpk mit denen eines Pathobionten, *E. coli* mpk, verglichen. Es zeigt sich, dass die Stimulation von DCs mit *B. vulgatus* mpk zu einem geringeren Anteil an Th17-Zell-Differentierung in Co-Kulturen von naïven T-Helferzellen mit diesen DCs führt. Analysen der Zytokine IL-6 und IL-23, die bekannt für ihre induzierende Wirkung auf potentiell destruktive Th17-Zellen sind, ergeben, dass *B. vulgatus* mpk-stimulierte DCs weniger dieser Zytokine bilden als DCs, die mit *E. coli* mpk stimuliert werden.
Um diesen Einfluss von *B. vulgatus* mpk auf die Regulation von IL-6 und IL-23 in DCs zu charakterisieren, werden IκBζ und MST1 untersucht, zwei zuvor in

anderem Kontext mit IL-6 beschriebene Regulatoren. Bei Kontakt mit *B. vulgatus* mpk kann eine geringere Konzentration von IL-6 in Zusammenhang mit einer geringeren Transkription von *Nfkbiz*, dem Gen, das IκBζ codiert, in DCs bestätigt werden. Die Transkription von *Mst1* zeigt sich hingegen unbeeinflusst von der Stimulation mit Symbiont *B. vulgatus* mpk oder Pathobiont *E. coli* mpk.

Außerdem ist es denkbar, dass Feedbackschleifen Th17 Zytokinen die Bildung Th17-induzierender Zytokine in DCs beeinflussen. Um einen solchen Einfluss zu untersuchen, wird der Effekt durch rekombinantes IL-17 und IL-22 auf die Bildung von Zytokinen in DCs betrachtet. Es zeigt sich, dass die Fähigkeit von DCs Th17 Zellen zu induzieren, unter den untersuchten Bedingungen nicht von retrograd wirkenden charakteristischen Th17 Zytokinen beeinflusst wird.

Zusammenfassend deuten meine Ergebnisse darauf hin, dass der Kontakt von DCs mit symbiotischen Bakterien wie *B. vulgatus* mpk die Differenzierung potenziell destruktiv wirkender Th17-Zellen vermindert, was ein Hinweis auf ihre Rolle in der intestinalen Homöostase ist. Dies wird durch eine geringere Ausschüttung Th17-induzierender Zytokine wie IL-6 und IL-23 durch DCs vermittelt. Für IL-6 ist diese geringere Sekretion mit einer geringeren Produktion des regulatorisch wirkenden IκBζ in DCs korreliert. Die Stimulation von DCs mit *B. vulgatus* mpk ist daher ein vielversprechender Ansatz für zukünftige Therapien im Kontext von Überreaktionen des Immunsystems und Autoimmunität.

6 Bibliography

Aggarwal, S.et al. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem,* 278**,** 1910-4.

Ahern, P. P.et al. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity,* 33**,** 279-88.

Almquist, H. J.et al. 1938. Synthesis of the Antihemorrhagic Vitamin by Bacteria. *Proceedings of the Society for Experimental Biology and Medicine,* 38**,** 336-338.

Ananthakrishnan, A. N. 2015. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol,* 12**,** 205-17.

Annes, J. P.et al. 2003. Making sense of latent TGF beta activation. *Journal of Cell Science,* 116**,** 217-224.

Annunziato, F.et al. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med,* 204**,** 1849-61.

Antonysamy, M. A.et al. 1999. Evidence for a role of IL-17 in organ allograft rejection: IL-17 promotes the functional differentiation of dendritic cell progenitors. *J Immunol,* 162**,** 577-84.

Autenrieth, I. B.et al. 1997. Cytokine mRNA expression in intestinal tissue of interleukin-2 deficient mice with bowel inflammation. *Gut,* 41**,** 793-800.

Backhed, F.et al. 2005. Host-bacterial mutualism in the human intestine. *Science,* 307**,** 1915-20.

Bai, H.et al. 2009. IL-17/Th17 promotes type 1 T cell immunity against pulmonary intracellular bacterial infection through modulating dendritic cell function. *J Immunol,* 183**,** 5886-95.

Bamba, T.et al. 1995. The pathogenic role of Bacteroides vulgatus in patients with ulcerative colitis. *J Gastroenterol,* 30 Suppl 8**,** 45-7.

Barnden, M. J.et al. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunology and Cell Biology,* 76**,** 34-40.

Becskei, A. and Grusby, M. J. 2007. Contribution of IL-12R mediated feedback loop to Th1 cell differentiation. *FEBS Lett,* 581**,** 5199-206.

Bentley, R. and Meganathan, R. 1982. Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol Rev,* 46**,** 241-80.

Beriou, G.et al. 2009. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood,* 113**,** 4240-9.

Bettelli, E.et al. 2006. Reciprocal developmental pathways for the generation of pathogenic effector T(H)17 and regulatory T cells. *Nature,* 441**,** 235-238.

Bhaumik, S. and Basu, R. 2017. Cellular and Molecular Dynamics of Th17 Differentiation and its Developmental Plasticity in the Intestinal Immune Response. *Front Immunol,* 8**,** 254.

Birg, A. 2017. *Microbiota-dependent regulation of the expression of Th17 mediating signature cytokines.* Master Thesis, University of Tübingen.

Blauvelt, A. 2008. T-helper 17 cells in psoriatic plaques and additional genetic links between IL-23 and psoriasis. *J Invest Dermatol,* 128**,** 1064-7.

Blekhman, R.et al. 2015. Host genetic variation impacts microbiome composition across human body sites. *Genome Biology,* 16.
Boneberger, A.et al. 2011. Endotoxin levels in house dust samples and juvenile inflammatory bowel disease - a case-control study. *J Crohns Colitis,* 5**,** 525-30.
Boniface, K.et al. 2010. Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. *J Immunol,* 185**,** 679-87.
Brand, S. 2009. Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut,* 58**,** 1152-1167.
Brandt, K.et al. 2003. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood,* 102**,** 4090-8.
Chen, W.et al. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med,* 198**,** 1875-86.
Cho, J. H. 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol,* 8**,** 458-66.
Coombes, J. L.et al. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med,* 204**,** 1757-64.
Cooper, G. S. and Stroehla, B. C. 2003. The epidemiology of autoimmune diseases. *Autoimmun Rev,* 2**,** 119-25.
Creasy, C. L. and Chernoff, J. 1995. Cloning and characterization of a human protein kinase with homology to Ste20. *J Biol Chem,* 270**,** 21695-700.
Cua, D. J.et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature,* 421**,** 744-8.
De Filippo, C.et al. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 14691-14696.
de Souza, H. S. and Fiocchi, C. 2016. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol,* 13**,** 13-27.
de Waal Malefyt, R.et al. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med,* 174**,** 1209-20.
Duerr, R. H.et al. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science,* 314**,** 1461-1463.
Eyerich, K.et al. 2008. Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. *J Invest Dermatol,* 128**,** 2640-5.
Fiorentino, D. F.et al. 1991. IL-10 inhibits cytokine production by activated macrophages. *J Immunol,* 147**,** 3815-22.
Fontenot, J. D.et al. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity,* 22**,** 329-41.
Frank, D. N.et al. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A,* 104**,** 13780-5.

Frick, J. S.et al. 2010. Immunomodulation by semi-mature dendritic cells: a novel role of Toll-like receptors and interleukin-6. *Int J Med Microbiol,* 300**,** 19-24.
Frick, J. S.et al. 2006. Colitogenic and non-colitogenic commensal bacteria differentially trigger DC maturation and Th cell polarization: an important role for IL-6. *Eur J Immunol,* 36**,** 1537-47.
Fujii, S.et al. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med,* 199**,** 1607-18.
Fujino, S.et al. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut,* 52**,** 65-70.
Gaffen, S. L.et al. 2014. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol,* 14**,** 585-600.
Geng, J.et al. 2015. Kinases Mst1 and Mst2 positively regulate phagocytic induction of reactive oxygen species and bactericidal activity. *Nat Immunol,* 16**,** 1142-52.
Gevers, D.et al. 2014. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe,* 15**,** 382-392.
Gharibi, T.et al. 2016. Biological effects of IL-21 on different immune cells and its role in autoimmune diseases. *Immunobiology,* 221**,** 357-67.
Goodrich, J. K.et al. 2014. Human genetics shape the gut microbiome. *Cell,* 159**,** 789-99.
Goto, Y.et al. 2014. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity,* 40**,** 594-607.
Graves, J. D.et al. 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *EMBO J,* 17**,** 2224-34.
Gronbach, K.et al. 2014. Endotoxicity of lipopolysaccharide as a determinant of T-cell-mediated colitis induction in mice. *Gastroenterology,* 146**,** 765-75.
Gulen, M. F.et al. 2010. The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. *Immunity,* 32**,** 54-66.
Halme, L.et al. 2006. Family and twin studies in inflammatory bowel disease. *World J Gastroenterol,* 12**,** 3668-72.
Harrington, L. E.et al. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol,* 6**,** 1123-32.
Harrison, O. J. and Powrie, F. M. 2013. Regulatory T cells and immune tolerance in the intestine. *Cold Spring Harb Perspect Biol,* 5.
Herold, G. (ed.) 2019. *Innere Medizin,* Köln, 480-481.
Hirota, K.et al. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nature Immunology,* 12**,** 255.
Hold, G. L.et al. 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. *Fems Microbiology Ecology,* 39**,** 33-39.
Hornef, M. 2015. Pathogens, Commensal Symbionts, and Pathobionts: Discovery and Functional Effects on the Host. *Ilar Journal,* 56**,** 159-162.

Hsu, P.et al. 2015. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. *Journal of Immunology,* 195**,** 3665-3674.
Huang, W.et al. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis,* 190**,** 624-31.
Hueber, W.et al. 2012. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut,* 61**,** 1693-700.
Human Microbiome Project, C. 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486**,** 207-14.
Hunter, C. A. and Jones, S. A. 2015. IL-6 as a keystone cytokine in health and disease. *Nat Immunol,* 16**,** 448-57.
Hviid, A.et al. 2011. Antibiotic use and inflammatory bowel diseases in childhood. *Gut,* 60**,** 49-54.
Inaba, K.et al. 1983. Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J Exp Med,* 158**,** 2040-57.
Ivanov, I. I.et al. 2006. The orphan nuclear receptor ROR gamma t directs the differentiation program of proinflammatory IL-17(+) T helper cells. *Cell,* 126**,** 1121-1133.
Jernberg, C.et al. 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *Isme Journal,* 1**,** 56-66.
Johansson, C. and Kelsall, B. L. 2005. Phenotype and function of intestinal dendritic cells. *Semin Immunol,* 17**,** 284-94.
Johansson, M. E.et al. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A,* 105**,** 15064-9.
Katagiri, K.et al. 2009. Mst1 controls lymphocyte trafficking and interstitial motility within lymph nodes. *EMBO J,* 28**,** 1319-31.
Kirkham, B. W.et al. 2006. Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis - A two-year prospective study (the DAMAGE study cohort). *Arthritis and Rheumatism,* 54**,** 1122-1131.
Kitamura, H.et al. 2000. MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. *FEBS Lett,* 485**,** 53-6.
Klameth, C. 2012. *Untersuchung der Rolle von IκB-ζ bei der Entstehung semimaturer dendritischer Zellen und der Aufrechterhaltung der intestinalen Homöostase* Medical Doctorate Thesis, Eberhard Karls University Tuebingen.
Koenen, H. J. P. M.et al. 2008. Human CD25 high Foxp3 pos regulatory T cells differentiate into IL-17–producing cells. *Blood,* 112**,** 2340-2352.
Lane, E. R.et al. 2017. The microbiota in inflammatory bowel disease: current and therapeutic insights. *J Inflamm Res,* 10**,** 63-73.
Lange, A.et al. 2016. Extensive Mobilome-Driven Genome Diversification in Mouse Gut-Associated Bacteroides vulgatus mpk. *Genome Biol Evol,* 8**,** 1197-207.

Langenkamp, A.et al. 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol,* 1**,** 311-6.
Langrish, C. L.et al. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med,* 201**,** 233-40.
Lee, Y. K.et al. 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity,* 30**,** 92-107.
Ley, R. E.et al. 2005. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A,* 102**,** 11070-5.
Ley, R. E.et al. 2008. Evolution of mammals and their gut microbes. *Science,* 320**,** 1647-1651.
Li, C. X.et al. 2017. Dendritic cell MST1 inhibits Th17 differentiation. *Nature Communications,* 8.
Li, M. O.et al. 2006. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol,* 24**,** 99-146.
Libermann, T. A. and Baltimore, D. 1990. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol,* 10**,** 2327-34.
Littman, D. R. and Rudensky, A. Y. 2010. Th17 and Regulatory T Cells in Mediating and Restraining Inflammation. *Cell,* 140**,** 845-858.
Liu, J. Z.et al. 2015. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet,* 47**,** 979-86.
Low, D.et al. 2013. Animal models of ulcerative colitis and their application in drug research. *Drug Design Development and Therapy,* 7**,** 1341-1356.
Luckey, T. D. 1972. Introduction to Intestinal Microecology. *American Journal of Clinical Nutrition,* 25**,** 1292-1294.
Lutz, M. B.et al. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods,* 223**,** 77-92.
Macpherson, A. J.et al. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science,* 288**,** 2222-6.
Maerz, J. K.et al. 2017. Outer membrane vesicles blebbing contributes to B. vulgatus mpk-mediated immune response silencing. *Gut Microbes***,** 1-12.
Manel, N.et al. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol,* 9**,** 641-9.
Mangan, P. R.et al. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441**,** 231-4.
Matusevicius, D.et al. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Multiple Sclerosis,* 5**,** 101-104.
Mazmanian, S. K.et al. 2005. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell,* 122**,** 107-18.
Mazmanian, S. K.et al. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature,* 453**,** 620-5.

McGeachy, M. J.et al. 2007. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nature Immunology,* 8**,** 1390-1397.
McGeachy, M. J.et al. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nature Immunology,* 10**,** 314-324.
McGeachy, M. J. and Cua, D. J. 2007. The link between IL-23 and Th17 cell-mediated immune pathologies. *Semin Immunol,* 19**,** 372-6.
Mellman, I. and Steinman, R. M. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell,* 106**,** 255-8.
Milner, J. D.et al. 2008. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature,* 452**,** 773-6.
Molet, S.et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol,* 108**,** 430-8.
Molinelli, E.et al. 2017. Biologic Therapy in Psoriasis (Part II): Efficacy and Safety of New Treatment Targeting IL23/IL-17 Pathways. *Current Pharmaceutical Biotechnology,* 18**,** 964-978.
Molodecky, N. A.et al. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology,* 142**,** 46-54 e42; quiz e30.
Mombaerts, P.et al. 1992. Rag-1-Deficient Mice Have No Mature Lymphocytes-B and Lymphocytes-T. *Cell,* 68**,** 869-877.
Mosmann, T. R.et al. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136**,** 2348-57.
Mucida, D.et al. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science,* 317**,** 256-60.
Muller, M.et al. 2008. Intestinal Colonization of IL-2 Deficient Mice with Non-Colitogenic B. vulgatus Prevents DC Maturation and T-Cell Polarization. *Plos One,* 3.
Noack, M. and Miossec, P. 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev,* 13**,** 668-77.
Nurieva, R.et al. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature,* 448**,** 480-U8.
Oeckinghaus, A. and Ghosh, S. 2009. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol,* 1**,** a000034.
Ohnmacht, C.et al. 2009. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med,* 206**,** 549-59.
Okamoto, K.et al. 2010. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature,* 464**,** 1381-5.
Olszak, T.et al. 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science,* 336**,** 489-93.
Onishi, R. M. and Gaffen, S. L. 2010. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology,* 129**,** 311-21.

Orholm, M.et al. 2000. Concordance of inflammatory bowel disease among Danish twins - Results of a nationwide study. *Scandinavian Journal of Gastroenterology,* 35**,** 1075-1081.
Ostanin, D. V.et al. 2009. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physiol,* 296**,** G135-46.
Ott, S. J.et al. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut,* 53**,** 685-93.
Park, H.et al. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol,* 6**,** 1133-41.
Perencevich, M. and Burakoff, R. 2006. Use of antibiotics in the treatment of inflammatory bowel disease. *Inflamm Bowel Dis,* 12**,** 651-64.
Pierre, P.et al. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature,* 388**,** 787-92.
Preiss, J. C.et al. 2014. Updated German clinical practice guideline on "Diagnosis and treatment of Crohn's disease" 2014. *Z Gastroenterol,* 52**,** 1431-84.
Rescigno, M. and Di Sabatino, A. 2009. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest,* 119**,** 2441-50.
Ridaura, V. K.et al. 2013. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. *Science,* 341**,** 1079-U49.
Roederer, M. 2008. How many events is enough? Are you positive? *Cytometry A,* 73**,** 384-5.
Roses, R. E.et al. 2008. Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists. *J Immunol,* 181**,** 5120-7.
Rudner, X. L.et al. 2007. Interleukin-23 (IL-23)-IL-17 cytokine axis in murine Pneumocystis carinii infection. *Infect Immun,* 75**,** 3055-61.
Sadlack, B.et al. 1995. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. *Eur J Immunol,* 25**,** 3053-9.
Sakaguchi, S.et al. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev,* 212**,** 8-27.
Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol,* 31**,** 107-33.
Sender, R.et al. 2016. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell,* 164**,** 337-40.
Smith, P. M.et al. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science,* 341**,** 569-73.
Sonder, S. U.et al. 2011. IL-17-induced NF-kappa B Activation via CIKS/Act1 physiologic significance and signaling mechanism. *Journal of Biological Chemistry,* 286**,** 12881-12890.
Song, H.et al. 2010. Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression. *Proc Natl Acad Sci U S A,* 107**,** 1431-6.

Steimle, A.et al. 2016a. Structure and function: Lipid A modifications in commensals and pathogens. *Int J Med Microbiol,* 306**,** 290-301.
Steimle, A. and Frick, J. S. 2016. Molecular Mechanisms of Induction of Tolerant and Tolerogenic Intestinal Dendritic Cells in Mice. *J Immunol Res,* 2016**,** 1958650.
Steimle, A.et al. 2016b. Symbiotic gut commensal bacteria act as host cathepsin S activity regulators. *J Autoimmun,* 75**,** 82-95.
Steimle, A.et al. 2019. Weak Agonistic LPS Restores Intestinal Immune Homeostasis. *Mol Ther,* 27**,** 1974-1991.
Steinman, R. M. and Cohn, Z. A. 1973. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice. *Journal of Experimental Medicine,* 137**,** 1142-1162.
Strengell, M.et al. 2006. IL-21 enhances SOCS gene expression and inhibits LPS-induced cytokine production in human monocyte-derived dendritic cells. *J Leukoc Biol,* 79**,** 1279-85.
Thompson, N. P.et al. 1996. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ,* 312**,** 95-6.
Tsoi, L. C.et al. 2015. Enhanced meta-analysis and replication studies identify five new psoriasis susceptibility loci. *Nat Commun,* 6**,** 7001.
Turnbaugh, P. J.et al. 2009. A core gut microbiome in obese and lean twins. *Nature,* 457**,** 480-U7.
Tysk, C.et al. 1988. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut,* 29**,** 990-6.
Tzartos, J. S.et al. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *American Journal of Pathology,* 172**,** 146-155.
Van Assche, G.et al. 2010. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. *J Crohns Colitis,* 4**,** 7-27.
Veldhoen, M.et al. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity,* 24**,** 179-89.
Waidmann, M.et al. 2003. Bacteroides vulgatus protects against Escherichia coli-induced colitis in gnotobiotic interleukin-2-deficient mice. *Gastroenterology,* 125**,** 162-77.
Wehkamp, J. and Frick, J. S. 2017. Microbiome and chronic inflammatory bowel diseases. *J Mol Med (Berl),* 95**,** 21-28.
Wehkamp, J.et al. 2016. Inflammatory Bowel Disease. *Deutsches Arzteblatt International,* 113**,** 72-+.
Wolk, K.et al. 2004. IL-22 increases the innate immunity of tissues. *Immunity,* 21**,** 241-54.
Wu, G. D.et al. 2011. Linking long-term dietary patterns with gut microbial enterotypes. *Science,* 334**,** 105-8.
Yamamoto, M.et al. 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature,* 430**,** 218-22.

Yamazaki, S.et al. 2001. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem,* 276**,** 27657-62.
Yang, J.et al. 2013. In vitro characterization of the impact of selected dietary fibers on fecal microbiota composition and short chain fatty acid production. *Anaerobe,* 23**,** 74-81.
Yang, L.et al. 2008a. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature,* 454**,** 350-2.
Yang, X. O.et al. 2008b. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity,* 29**,** 44-56.
Yang, X. O.et al. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem,* 282**,** 9358-63.
Yen, D.et al. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest,* 116**,** 1310-6.
Zaph, C.et al. 2008. Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med,* 205**,** 2191-8.
Zheng, Y.et al. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature,* 445**,** 648-51.
Zhou, L.et al. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature,* 453**,** 236-40.
Zhou, L.et al. 2018. Faecalibacterium prausnitzii Produces Butyrate to Maintain Th17/Treg Balance and to Ameliorate Colorectal Colitis by Inhibiting Histone Deacetylase 1. *Inflamm Bowel Dis*.

7 Declaration of Own Contribution

Diese Arbeit wurde am Institut für Medizinische Mikrobiologie und Hygiene des Universitätsklinikums Tübingen und der Eberhard-Karls-Universität Tübingen unter Betreuung von Prof. Dr. med. Julia-Stefanie Frick durchgeführt.
Die Konzeption der Experimente erfolgte in Zusammenarbeit mit Dr. Alexander Steimle, Postdoc der Arbeitsgruppe.
Die Versuche zu Figure 3.1.1, 3.1.2, 3.3.1, 3.3.2 und 3.4.2 und die IL-23p19-Versuche für Figure 3.2.4 wurden nach Einarbeitung durch Dr. Steimle von mir durchgeführt. Die Versuche zu Figure 3.2.1, 3.2.2, 3.2.3 und Figure 3.4.1, sowie die Versuche zu IL-6 und IL-10 für Figure 3.2.4 wurden mit Unterstützung durch Alexander Birg durchgeführt, der zu diesem Zeitpunkt sein Großpraktikum zu Studienende als Vorbereitung für seine Masterarbeit absolvierte. Die Daten für TGFβ für Figure 3.2.4 stammen aus einem Versuch von Annika Bender. Daten aus den mit Unterstützung durch Alexander Birg durchgeführten Versuchen wurden von diesem in seiner Masterarbeit aufgegriffen.
Für meine Analyse in 3.3.1b wurden von mir *Nfkbiz*$^{-/-}$ und Wildtyp- Überstände von Christian Klameth verwendet, für 3.3.2 Überstände von Dr. Alex Steimle, deren DNA-Verdau von Nadine Abele durchgeführt wurde. Die real-time qRT-PCR Analyse der mRNA levels für die Analyse führte ich durch.
Die Auswertung, inklusive der statistischen Auswertung, erfolgte durch mich.
Ich habe die vorgelegte Dissertation selbst verfasst, keine anderen als die ausdrücklich bezeichneten Quellen und Hilfsmittel genutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet. Unterstützungsleistungen, die ich von anderen Personen erhalten habe, wurden in der Dissertationsschrift als solche benannt.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form in einem anderen universitären Prüfungsverfahren vorgelegt.

Tübingen, den 28.06.2021

Acknowledgements

First of all, I dearly thank Prof. Julia-Stefanie Frick for the opportunity to work in your group and to write this thesis under your supervision. Thank you especially for being such a supportive, approachable, and down-to-earth advisor. I thank Prof. Weber, for being the best 2nd supervisor I could wish for, even during pandemic conditions.

Prof. Dr. Ingo Autenrieth and Prof. Bernd Pichler I thank for the permission to earn my doctorate at the Faculty of Medicine of the University of Tübingen.

Deep gratitude I owe Dr. Alex Steimle for your committed, challenging, and at the same time supportive supervision, your careful introduction into the laboratory works and your project management. I am heartily grateful to soon-to-be Dr. Lena Michaelis for taking over the supervision at the stage of writing, for your encouragement, our scientific discussions, and your careful proof-reading of several versions of the work. Likewise, thank you a lot, my dear friend Verena, for your brilliant advice and finding even the smallest punctuation error while proof-reading.

Of course, I also want to appreciate all team members of the Frick Lab, especially Annika and Andi, Alex Birg, Biggi, Jan, Raphael, Anna, Thomas, Nadine and all others for being a true team, for amiably including me as a lab member from day one and making the work place a fun place. Thank you for all explanations, help with experiments and other support, as well.

I thank Dr. Dirk Kraus for your dedicated mentorship and for inciting the idea of a doctorate at the Institute of Medical Microbiology and Hygiene and applying for the scholarships and grants of the DZIF and IZKF. Many thanks also to the members of my IZKF Research Training Group and Dr. Inka Montero and Prof. Dr. Marlies Knipper for your guidance and priceless advice. Our meetings motivated me greatly. Additionally, I am grateful for the research grant of the DZIF that helped me to afford spending 6 months in the lab.

Above all, huge thanks to my friends, who supported and encouraged me especially during difficult times of the thesis process, my great family for your unconditional love, backing and belief in me, and, last but not least, Philip, for your unwavering faith in me, for being my match and for your love.