Host-microbe interactions: How commensal immunogenicity shapes gut homeostasis and inflammation

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Abbreviations

AID	Autoimmune disease
ALDH	Aldehyde dehydrogenase
APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
Breg	Regulatory B cell
CD	Cluster of differentiation
CD	Crohn's disease
CLR	C-type lectin receptor
cLP	Colonic lamina propria
CTLA-4	Cytotoxic T-lymphocyte protein 4
DAI	Disease activity index
DC	Dendritic cell
DSS	Dextran sodium sulfate
EAE	Experimental autoimmune encephalomyelitis
GALT	Gut associated lymphoid tissue
GM-CMF	Granulocyte-macrophage colony stimulating factor
HCS	Histological colitis score
HD	Healthy donor
HEK	Human embryonic kidney
IBD	Inflammatory bowel disease
IFN	Interferon
IgA	Immunoglobulin A
IκB	Inhibitor of κB proteins
IKK	IκB kinase
IL	Interleukin
INAP	IL-1-inducible nuclear ankyrin-repeat protein
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MAIL	Molecule-possessing ankyrin repeats induced by lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MD2	Myeloid differentiation factor 2
MHC II	Major histocompatibility complex II
mLN	Mesenteric lymph node
mRNA	Messenger ribonucleic acid
NDR	NOD-like receptor

NFκB	Nuclear factor κ B
OS	oligosaccharide
PE	Phycoerythrin
PET	Positron emission tomography
PRR	Pattern recognition receptor
Rag1$^{-/-}$	Recombination-activating gene 1-deficient
ROR	Retinoid-related orphan nuclear receptor
SPF	Specific-pathogen-free
TLR	Toll-like receptor
Tfh	Follicular helper T cell
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
Treg	Regulatory T cell
UC	Ulcerative colitis
WT	Wildtype

Summary

The impact of the gut microbiota on the host's states of health and disease is indisputably large. Besides contributing to the host's physiological functions by digesting nutrients, providing vitamins and pathogen resistance, the gut microbiota educates and modulates immune responses. In healthy individuals, the microbiota is composed of a broad variety of different commensals that exhibit different immunogenicity and that reside within the host in a state of immune homeostasis. However, many immune-mediated or autoimmune diseases, e.g. inflammatory bowel disease (IBD), are associated with a shift in the normal microbiota termed dysbiosis. Gut immune responses can be influenced even by single bacterial strains. For instance, the model symbiont *Bacteroides vulgatus* exerts protective, anti-inflammatory functions in mouse models of experimental colitis, whereas the model pathobiont *Escherichia coli* rather promotes intestinal inflammation. An important link between the microbiota and the host's innate and adaptive immune system is represented by dendritic cells (DCs). These front-line antigen presenting cells at mucosal surfaces respond to the commensals by appropriate differentiation and maturation. Depending on their developed phenotype and created cytokine milieu, DCs mediate T cell differentiation towards a regulatory, anti-inflammatory or a pro-inflammatory T helper cell subset.

In this thesis, one of the most immunogenic bacterial antigens, lipopolysaccharide (LPS), is demonstrated to be the main mediator of immunogenicity of the mentioned model commensals. Due to its weak agonistic activity, LPS isolated from *B. vulgatus* is even able to ameliorate intestinal inflammation in a mouse model of experimental colitis, similar to administration of living *B. vulgatus.* This effect was associated with the induction of tolerogenic DCs, which contribute to intestinal homeostasis - in contrast to the fully mature DCs induced by *E. coli*, which promote inflammation. The transcription factor IκBζ is essential for the induction of an IL-17 secreting T helper cell subset, the Th17 cells, and was hypothesized to play a role in the commensal-specific DC effects. Th17 cells can be protective in mucosal infections under physiological conditions but are also associated with the pathology of autoimmune diseases and are increased in IBD. Here, it is demonstrated that the strong immunogenic *E. coli* promotes a significantly higher IκBζ expression in DCs, contributing to differentiation of pathologic Th17 cells, whereas *B. vulgatus* only induces low IκBζ expression in DCs and triggers differentiation of protective T helper cell subsets. However, *E. coli* also induces development of a regulatory B cell phenotype which counteracts on the strong immune response and can suppress inflammation in a mouse model of experimental colitis in dependence of the host's immune competence.

Zusammenfassung

Der Einfluss der Darmmikrobiota auf Gesundheit und Krankheit des Wirtsorganismus ist unbestreitbar groß. Sie unterstützt den Wirt nicht nur in physiologischen Vorgängen wie Nährstoffabbau, Vitaminproduktion oder Pathogenresistenz, sie trägt auch zur Entwicklung des Immunsystems bei und kann Immunantworten beeinflussen. In einem gesunden Wirt ist die Mikrobiota aus einer Vielzahl verschiedener Kommensalen aufgebaut, die alle unterschiedliche Immunogenitäten aufweisen und sich mit dem Wirt zusammen in einem Zustand der Immunhomöostase befinden. Viele immunvermittelte Krankheiten oder Autoimmunerkrankungen, wie z.B. chronisch entzündliche Darmerkrankungen (CED), sind mit einer Veränderung der normalen Mikrobiota, der sogenannten Dysbiose, verbunden. Darmimmunantworten können sogar von einzelnen Bakterienstämmen beeinflusst werden. Beispielweise übt der Modellsymbiont *Bacteroides vulgatus* schützende, anti-entzündliche Funktionen in Mausmodellen für experimentelle Kolitis aus, während der Modellpathobiont *Escherichia coli* die Darmentzündung eher fördert. Eine wichtige Verbindung zwischen der Mikrobiota und dem angeborenen und adaptiven Immunsystem stellen die dendritischen Zellen (DZs) dar. Diese antigenpräsentierenden Zellen sind Teil der ersten Verteidigungslinie an mukosalen Oberflächen und reagieren auf die Kommensalen durch angemessene Differenzierung und Reifung. Abhängig von ihren entwickelten Phänotypen und dem erzeugten Zytokinmilieu vermitteln die DZs die Differenzierung von T-Zellen in regulatorische, anti-entzündliche oder aber auch in entzündungsfördernde T-Zellsubtypen.

In dieser Arbeit wird gezeigt, dass eins der immunogensten Antigene der Bakterien, das Lipopolysaccharid (LPS), der Hauptmediator der Immunogenität der genannten Kommensalen ist. Aufgrund seiner schwachen agonistischen Aktivität kann das aus *B. vulgatus* isolierte LPS in einem Mausmodell für experimentelle Kolitis sogar die Darmentzündung lindern, ähnlich wie bei der Verabreichung von lebendem *B. vulgatus.* Dieser Effekt wurde mit der Induktion von tolerogenen DZs, die zur Darmhomöostase beitragen, in Zusammenhang gebracht - im Gegensatz zu den von *E. coli* induzierten vollreifen DZs, die die Entzündung fördern. Es wurde angenommen, dass der Transkriptionsfaktor IκBζ, welcher zur Entwicklung des IL-17-sekretierenden T-Helferzelltyps Th17 benötigt wird, eine Rolle bei diesen kommensalspezifischen Effekten spielt. Th17-Zellen tragen einerseits zur Abwehr von mukosalen Infektionen bei, spielen aber auch eine pathologische Rolle in Autoimmunerkrankungen und kommen erhöht bei CED vor. In dieser Arbeit wird gezeigt, dass der stark-immunogene Stamm *E. coli* eine signifikant höhere Expression von IκBζ verursacht, die zur Entwicklung von pathologischen Th17-Zellen beiträgt, während *B. vulgatus* nur eine geringe Expression von IκBζ in DZs auslöst und die

Differenzierung von protektiven T-Zellen fördert. *E. coli* trägt jedoch auch zur Entwicklung von regulatorischen B-Zellen bei, welche den starken Immunantworten entgegensteuern und auch Entzündungen in Kolitismausmodellen in Abhängigkeit der Wirtsimmunkompetenz unterdrücken können.

Publications

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2. **Michaelis, L.**, Treß, M., Löw, H.C., Klees, J., Klameth, C., Lange, A., Grießhammer, A., Schäfer, A., Menz, S., Steimle, A., Schulze-Osthoff, K., Frick, J.S.: Gut commensal-induced IκBζ expression in dendritic cells influences the Th17 response. Frontiers in Immunology 11, 612336 (2021)

3. Maerz, J.K., Trostel, C., Lange, A., Parusel, R., **Michaelis, L.**, Schäfer, A., Yao, H., Löw, H.C., Frick, J.S.: Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses. Frontiers in Immunology 10, 3093 (2020)

Contributions

Publication No.	**Accepted publication yes/no**	**Number of all authors**	Position of candidate in list of authors	Scientific ideas by the candidate (%)	Data generation by the candidate (%)	Analysis and Interpretation by the candidate (%)	Paper writing done by the candidate (%)
1	Yes	17	2*	10	10	20	50
2	Yes	12	1	90	90	90	90
3	Yes	9	5	20	20	10	10

**shared first authorship*

Introduction

1. The gut microbiota

The human body harbors abundant and diverse microbial communities consisting of bacteria, fungi, parasites and viruses - a mutual relationship that has been developed during millions of years of co-evolution [1]. The most densely populated site is the colon with an estimated number of 3.8 x 10^{13} bacteria [2, 3]. These bacteria, termed commensals, contribute to the host's physiological functions in many ways including digestion and fermentation of carbohydrates, production of vitamins, support in resistance against pathogens and promotion of the development and regulation of the innate as well as the adaptive immune system. In turn, the host provides nutrients and niches for microbial colonization and proliferation with constant temperature and regulates the microbiota composition through immune responses induced by endogenous bacteria [1, 4]. The gut microbiota is established immediately after birth and represents a dynamic ecosystem, influenced by various factors, such as age, diet, host immune factors and the use of antibiotics [4, 5]. It varies greatly between individuals and over the different habitats. However, the microbial metabolic and functional pathways are much more constant and evenly diverse in a healthy host population [6]. The gut microbiota is dominated by the two phyla of Bacteroidetes and Firmicutes, whereas Actinobacter and Proteobacteria are found less frequently [6]. A healthy microbiome is characterized by taxonomic and functional diversity, stability and resilience to perturbations [7]. Due to its tremendous influence on states of health and disease, research on microbiota gained increasing attention during the last years. Technical progress in culturing, DNA/RNA sequencing and other methods for microbiome analysis enabled deciphering the role of commensals in health and disease processes [8, 9].

1.1. Classification of commensals

A functioning cross-talk between the gut microbiota and the host's immune system is essential for maintenance of gut homeostasis. Intestinal bacteria can be classified according to their host immune-modulating effects into three major groups: Probiotics, autobionts/symbionts and pathobionts. All of them are non-pathogenic bacteria in a healthy host and are either transiently (probiotics) or permanently (symbionts, pathobionts) colonizing the gut [10]. Probiotics can be beneficial to the host when administered in adequate doses, as defined by the World Health Organization [11]. Besides indirect effects such as pathobiont and pathogen suppression, they can directly modulate the host immune system by inducing cytokine secretion or activation of innate immune receptors [10]. A prominent member of the probiotics is the *Escherichia coli* strain Nissle 1917, which has an anti-inflammatory effect in conditions of intestinal inflammation and was shown to promote

maintenance of the remission phase in ulcerative colitis (UC) patients [12]. Furthermore, our group could demonstrate with the help of an experimental mouse model of acute colitis, that it is able to ameliorate intestinal inflammation by immunomodulation due to a distinct structure of its flagellin, the monomer that makes up the bacterial flagellum [13]. Symbionts, as members of the regular microbiota, promote the state of health and immune balance by influencing immune cell development, differentiation and effector function [10]. The mouse commensal *Bacteroides vulgatus* mpk was demonstrated to prevent *E. coli*-induced colitis in gnotobiotic *Il-2*$^{-/-}$ mice by promoting anti-inflammatory immune responses, and is thus frequently used as a model symbiont by our group and in the work presented in this thesis [14]. In contrast, pathobionts are usually present at low levels in the microbiota and harmless to a healthy host but can expand when other commensals are eliminated or immune homeostasis is impaired. Under these conditions, they can contribute to disease development in a genetically or environmentally predisposed host [10]. *E. coli* mpk, as the respective model pathobiont in this thesis, was shown to promote inflammation in mouse models of colitis [14, 15]. The two model commensals were shown to interact differently with host immune cells. To name one pathway influenced by these commensals and analyzed by our group: *B. vulgatus* mpk helps to maintain physiological levels of host cathepsin S activity in dendritic cells (DCs), thus promoting gut homeostasis. On the contrary, *E. coli* mpk induces high cathepsin S activity in DCs leading to a pro-inflammatory immune response and promoting intestinal inflammation in mice [15].

1.2. The role of the gut microbiota in health and disease

Chronic or acute perturbations of the microbiota can contribute to dysbiosis, a functional and compositional alteration of the microbiota characterized by a bloom of pathobionts, loss of commensals and diversity. Due to the close link between host immune system and microbiome, it is not surprising that dysbiosis has been associated with many immune-mediated and autoimmune diseases [16]. Numerous studies showed a connection between dysbiosis and inflammatory bowel disease (IBD), indicating the impact of the gut microbiota on local immune responses. Especially an increased abundance of members of the Enterobacteriaceae family and depletion of *Firmicutes* and *Bacteroidetes* has been reported for patients with IBD [17-20]. But also extra-intestinal, systemic immune-mediated disorders, such as rheumatoid and psoriatic arthritis, type 1 diabetes or multiple sclerosis, have been associated with a disturbed microbiota composition, making the microbiota an interesting target for therapeutic approaches in autoimmune diseases [21-24].

Gnotobiotic mice serve as a useful tool for the dissemination of microbe-host interactions mediated by defined strains while keeping control of host genetics, diet and other

environmental factors. In IBD research, wildtype (WT) or genetically predisposed germ-free mice, either healthy or as a model for experimental colitis, are inoculated with a single strain or defined bacterial consortia. This allows examination of the bacterial influence on e.g. host immune system development and contribution to or prevention of inflammatory processes without being biased by effects mediated by a complex microbiota as it is found in specific-pathogen-free (SPF) mice [25]. Furthermore, transplanting a human microbiome into germ-free mice can be applied to associate the human microbiota composition to a disease phenotype. This way, experimental manipulations of the microbiota can be used for the development of microbiota-targeted therapeutic approaches. To mention one example, studies by Britton *et al.* could demonstrate that in gnotobiotic mice, association with the microbiota of IBD patients induces higher numbers of intestinal Th17 cells, which are relevant for development of intestinal inflammation, than the microbiota from healthy donors (HD) [26, 27]. Additionally, transplantation of HD-microbiota into gnotobiotic mice exhibiting the IBD-microbiota restored the balance of Th17 and regulatory T cells (Tregs) (cell types explained in 3.1) and enabled identification of one single disease-promoting strain [27]. These studies once more demonstrate the large contribution of the microbiota to IBD-pathogenesis which will be further analyzed in this thesis.

2. The gut mucosal immune system

The gut mucosal barrier has the challenging task to separate the luminal environment and the host's internal milieu in order to impede the entrance of harmful antigens and microorganisms, while at the same time allowing the transport of nutrients and immune sensing [28]. A dynamic interplay between different structural components and molecular interactions is required for maintenance of intestinal integrity and homeostasis. The intestinal barrier function is provided by three main players interacting with each other : i) The commensal microbiota promotes colonization resistance and acts as the biological barrier, ii) the immune barrier consisting of gut associated lymphoid tissue (GALT), recruited and residing T and B cells, macrophages, innate lymphoid cells and DCs in the lamina propria (LP), and iii) the mechanical barrier made up of tightly lined epithelial cells covered by the mucus layer [28, 29]. Disruption and impairment of the intestinal barrier are associated with gastrointestinal diseases and infections. In the following paragraphs, the function of the gut mucosal immune system will be described with a focus on microbial influences on immune cell responses and the impact of a dysbiotic microbiota on immune-mediated diseases. Figure 1 gives a broad overview on gut barrier components in states of homeostasis and inflammation including characteristics of both states mentioned below.

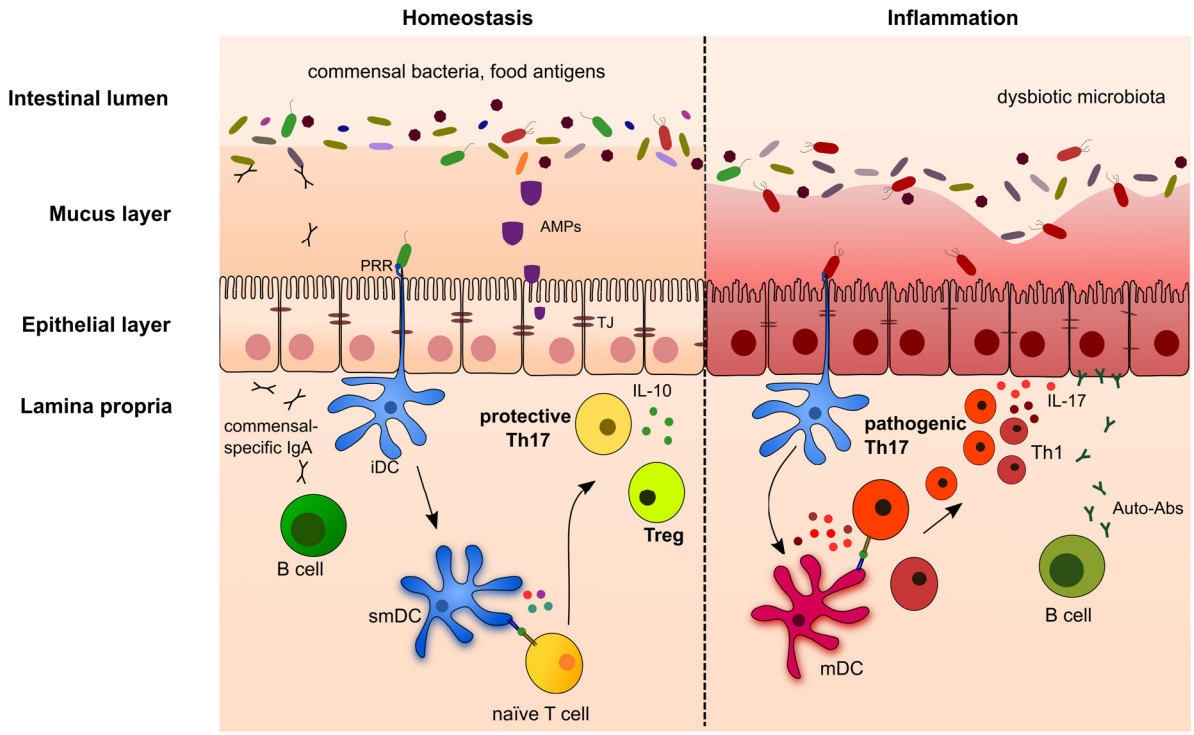

Figure 1: The gut mucosal barrier in homeostasis and inflammation. AMP= antimicrobial peptides, PRR= pattern recognition receptor, TJ= tight junction proteins, iDC= immature dendritic cells, smDC= semi-mature DCs, mDCs= mature DCs, Auto-Abs= auto-antibodies

2.1. Microbe-host interactions

In order to appropriately respond to infections by pathogens or translocation of bacteria, as well as to discriminate between harmless commensals and pathogenic bacteria, the host's immune system is required to quickly recognize molecules derived from bacteria. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NDRs) and C-type lectin receptors (CLRs) expressed in and on host cells, recognize conserved microbial components, the so-called microbe-associated molecular patterns (MAMPs) [30, 31]. In this thesis, microbe-induced TLR signaling will be examined in more detail. TLRs are single-pass transmembrane proteins harboring extracellular, N-terminal leucine-rich repeats for MAMP recognition, a membrane-spanning domain and a cytoplasmic, C-terminal Toll/Interleukin (IL)-1 receptor domain for downstream signaling [32]. In humans, 11 members of the TLR family (in mice even 13 members) can detect distinct MAMPs derived from bacteria, fungi, viruses and protozoa [33]. For example, TLR4 is an important receptor for recognition of gram-negative bacteria. Liberated lipopolysaccharide (LPS), the major cell wall component in gram-negative bacteria, is bound by the acute phase protein LPS-binding protein (LBP) which then presents it to the cluster of differentiation (CD) 14. Existing in two forms, either soluble or bound to the cell surface, CD14 mediates LPS-transfer to the TLR4 complexed with the accessory protein myeloid differentiation factor 2 (MD2). Binding of LPS to the TLR4/MD2 complex results in dimerization of the receptors initiating a downstream

TLR4 signaling cascade and eventually leading to the host immune response [30, 34]. Since MAMPs are not uniquely carried by pathogens but also by harmless commensals, the host must distinguish a threatening from an innocuous structure [35]. In the case of LPS, its structure differs between bacteria, thus, its biological activity defining the immunogenicity and the resulting host immune response is also varying [30]. However, the differences are not always clearly discriminable, so the amount and localization of PRR-signaling additionally helps to perceive pathogenic bacteria. As an example, TLR5, which recognizes bacterial flagellin, is expressed mainly on the basolateral but not on the apical surface of intestinal epithelial cells. Consequently, only invasive or translocating flagellated bacteria induce TLR5 signaling followed by an appropriate inflammatory immune response, whereas commensal flagellated bacteria in the lumen do not activate TLR5 [36].

2.2. Main players of the mucosal immune system

Several immune cells of the innate and adaptive immune system are patrolling and residing in the intestinal tissue, mediating immune homeostasis or inflammation. The interplay between DCs as antigen-presenting cells (APCs), T cells and B cells is representing the adaptive immune system, which will be described in detail in the following part.

2.2.1. Intestinal dendritic cells

As a link between innate and adaptive immune system, DCs play a major role in the gut mucosal immunity [37]. They serve as first-line APC at the mucosal surface, encountering a variety of gut microbes and responding to luminal antigens with induction of immune tolerance towards harmless commensal- or nutrient-derived antigens or inflammatory responses to potential pathogens. These hematopoietic, phagocytic cells are present in the GALT including Peyer's patches and throughout the lamina propria, the connective tissue underlying the intestinal epithelium [38, 39]. By extending their dendrites and reaching through the epithelial cell layer, immature DCs can sample microbes directly from the intestinal lumen. As described above, MAMP recognition by DCs is mediated by PRRs such as TLRs. Upon sampling of these antigens, an intracellular signaling pathway induces DC maturation facilitating efficient endocytosis, antigen processing and upregulation of major histocompatibility complex II (MHC II) as well as T cell costimulatory molecules such as CD40, CD80, CD86 [37]. In just one day, DCs change their main aspect of antigen sampling to antigen presentation [40]. Mature DCs migrate from intestinal tissue to mesenteric lymph nodes (mLN) where they present sampled and processed antigens *via* MHC II, accompanied by secretion of pro- and/or anti-inflammatory cytokines to naïve $CD4^+$ T cells [37]. Elucidating the mechanism of how DCs can discriminate between harmless commensals and hostile pathogens or pathobionts is part of this thesis. In contrast to fully mature DCs, so-called

semi-mature DCs are characterized by an intermediate MHC II expression, low expression of T cell costimulatory molecules and pro-inflammatory cytokines [15, 37]. This phenotype is potent tolerant and tolerogenic, meaning it is unresponsive towards a secondary, stronger stimulus in terms of maturation, T cell priming and TNFα secretion, and induces Tregs while inhibiting pro-inflammatory T cell proliferation. Thus, this type of DCs is important in preventing an inflammatory response and maintenance of immune homeostasis [37, 41]. Our group could show that the model symbiont *B. vulgatus* mpk induces semi-mature DCs whereas the model pathobiont *E. coli* mpk induces fully mature DCs, thereby explaining the promotion of homeostasis or inflammation, respectively [15, 41, 42].

2.2.2. Lamina propria T cells

In adaptive immunity, a diverse group of $CD4^+$ T cells named T helper (Th) cells play a crucial role by assisting B cells and cytotoxic T cells and thus enhance protection against a broad range of pathogens [43]. Upon activation by APCs, naïve $CD4^+$ T cells differentiate into functionally distinct Th cells which express gut-homing receptors enabling migration to their effector site, the gut. Depending on their unique cytokine profile and network of transcriptional regulators, Th cells are categorized into distinct lineages that either weaken or promote gut homeostasis [44, 45]. Classical Th cell subsets include Th1, Th2, Th9, Th17, follicular helper T cells (Tfh) and Tregs. Details on the main Th cell subsets, their characteristic cytokines and transcription factors as well as their function under physiologic conditions are shown in Table 1.

One of the $CD4^+$ Th subsets most abundant at steady state in the intestinal LP and addressed in more detail in this thesis are the IL-17 secreting Th17 cells. This cell type is crucial for the immune response against mucosal pathogens and is induced during steady state conditions by luminal commensals, such as the *Clostridia*-related segmented filamentous bacteria in the small intestine or *Clostridia* in the colon [46, 47]. However, Th17 cells also play a pathogenic role in inflammatory disorders which will be further described in section 3.1. Another $CD4^+$ Th subset highly abundant in the LP are the Tregs, which are crucial for mucosal tolerance towards commensal- and nutrition-derived antigens. They suppress and restrict pro-inflammatory effector T cell responses and thus prevent excessive tissue damage, e.g. by secretion of anti-inflammatory IL-10 or expression of the negative regulator cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [48, 49]. Presence of certain bacteria, such as *Bacteroides fragilis* and members of commensal *Clostridia* in the intestine, was shown to induce Tregs [50, 51]. Since Th17 cells and Tregs have antagonistic functions, a balanced activity of these cell types is required for an appropriate immune response to external challenges [47]. The impact of commensals on Th differentiation and the underlying

molecular mechanisms of determining the differential ability to do so remained largely unknown and will be investigated in this thesis [52].

Table 1: Classical CD4$^+$ T helper cell subsets (adapted from [43, 53, 54])

Th subset	Major cytokines driving differentiation	Master transcriptional regulator	Major cytokines produced	Function
Th1	IFNγ, IL-12	T-bet	IFNγ	Intracellular infections, help B cells
Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	Extracellular infections and parasites, tissue repair
Th9	IL-4, TGFβ	PU.1	IL-9	Extracellular infections and parasites, promote IgG and IgE production by B cells
Th17	IL-1β, IL-23, IL-6, TGFβ	RORγt	IL-17A/F, IL-22	Bacterial, fungal and viral infections, mucosal homeostasis, neutrophil response
Tfh	IL-21, IL-6 (?)	Bcl-6	IL-21	Extracellular infections, B cell activation
Treg	TGFβ	FoxP3	IL-10	Maintenance of peripheral tolerance, regulation of inflammation, intestinal homeostasis

2.2.3. B cells

As another main player of the adaptive immune system, B cells are required for the maintenance of intestinal immune homeostasis by producing secretory immunoglobulin A (IgA) antibodies. IgA can be targeted to bacteria or specific bacterial structures, e.g. flagellin, especially to commensals in close mucosal proximity to the intestinal epithelium which are colitogenic in intestinal inflammation [55]. Naïve B cells are mainly activated through microbial antigen presentation by Th cells. Upon recognition by the B cell receptor, the antigen is engulfed *via* endocytosis, degraded and prepared for peptide presentation on the B cell surface on the MHC II complex. T cell receptors specific for the presented antigen

epitope can recognize the antigen:MHC II complex and induce B cell proliferation, immunoglobin class switching and somatic hypermutation by binding of CD40L to CD40 on B cells and cytokine secretion, e.g. of IL-4 and IL-21 [56, 57]. Interaction with antigen-loaded DCs in the mLN instructs B cells to produce commensal-specific IgA [58]. Coating of bacteria "neutralizes" the bacteria, limiting their growth in the lumen and hindering them from penetrating the gut barrier [58].

However, B cells not only act as inducers of immune responses against infections, they can also possess an immune-suppressive function and contribute to gut homeostasis. In the mouse model of experimental autoimmune encephalomyelitis (EAE), which resembles a Th17-driven multiple sclerosis-like phenotype, mice developed a more severe disease phenotype upon genetically removing B cells [59]. According to their regulatory ability, the responsible B cells were later named regulatory B cells (Bregs) and extensively studied in many mouse models of inflammatory and autoimmune diseases. It is now widely accepted that impaired function and/or reduced numbers of Bregs are associated with the pathology of many autoimmune disorders, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis or IBD [60, 61]. Furthermore, the development of Bregs was demonstrated to be influenced by the intestinal microbiota since commensals enhance the number and function of IL-10-producing Bregs in ex-germfree mice, one of the most widely studied Breg subset [61, 62]. A link between dysbiosis, impaired Breg function and various immune disorders seems thus to be reasonable and worth to be investigated.

3. Autoimmune diseases: Inflammatory bowel disease in focus

Dysregulation and dysfunction of immune responses mediated by the cell types mentioned above lead to the loss of immune homeostasis and self-tolerance, and thus promote the development of autoimmune diseases (AIDs) [63]. Due to the close relationship between gut microbiota and the immune system as well as their local proximity, the work presented in this thesis will focus on the influence of certain gut commensals on IBD as an AID of interest.

3.1. Molecular and cellular background of autoimmune diseases

The inability of the immune system to distinguish between self and non-self and, thus, induction of an inflammatory response to the body's own cells and tissue is the basis of AIDs. This loss of tolerance is promoted by genetic susceptibility and environmental risk factors and can develop at any age, ranging from fighting specific tissues to organ-non-specific or systemic reactivity. Yet the exact etiology is not fully understood [64]. Tissue damage mediated by autoantibody-producing B cells and autoreactive T cells can be caused by local inflammation, destruction of the antigen-bearing cell or by immune complex formation of

antibodies responding to soluble antigens [65]. To mention one example, an increased prevalence of autoantibodies against intestinal goblet cells has been detected in IBD patients [66]. Characteristic for most AIDs is the differentiation and pathogenic activity of an IL-17-secreting $CD4^+$ T helper cell subset called Th17 cells, accompanied by an imbalance between Tregs and Th17 cells. Upon stimulation by APCs and recognition of cytokines IL-6, IL-1β and IL-23, as well as transforming growth factor β (TGFβ), naïve $CD4^+$ T cells upregulate the expression of the lineage-defining transcription factor retinoid-related orphan nuclear receptor γt (RORγt) [67, 68]. Subsequent production and secretion of signature cytokines IL-17A, IL-17F and IL-22 by Th17 cells is crucial for mucosal host defense against extracellular microbes under physiological conditions since they fortify the intestinal barrier by instructing epithelial cells to produce antimicrobial peptides as well as tight junction claudin proteins, and recruiting neutrophils [69, 70]. However, Th17 cells are not a stable Th lineage but possess the ability to convert into other Th subsets. With this plasticity, Th17 cells are adapting to the surrounding microenvironment [43]. Under inflammatory conditions providing the pro-inflammatory cytokines IL-1β and IL-23, secreted e.g. by DCs, a potentially pathogenic Th17 phenotype co-expressing high levels of interferon γ (IFNγ) was shown to be enhanced and stabilized. These so-called Th1-like Th17 cells are associated with inflammatory processes in AIDs [43, 68, 71-74]. Development of Th1-like Th17 cells was observed in inflamed tissue of AID-patients, e.g. suffering from arthritis, type 1 diabetes, multiple sclerosis or IBD [75-78].

3.2. NFκB regulation by IκBζ

Microbe-induced TLR signaling as well as cytokine- and antigen-recognition result in activation of the nuclear factor κB (NFκB) transcription factors which are crucial players in the regulation of inflammation, immunity, as well as proliferation, differentiation and survival of almost all cell types and tissues [32, 79]. This family of transcription factors consists of the proteins p65 (RelA), RelB, c-Rel, p105/p50 (NFκB1) and p100/p52 (NFκB2), which can bind to each other and from homo- or heterodimers [79]. Their distinct transcriptional activity is defined by the combination of subunits, the cross-talk with other transcription factors and signaling pathways [80]. NFκB dimers in the cytosol are kept in an inactive form by bound inhibitor of κB proteins (IκB). Upon stimulation of the cell *via* TLR signaling, the IκB kinase (IKK) complex is activated and phosphorylates IκB, marking it for ubiquitination and proteasomal degradation. The freed NFκB dimer can then localize to the nucleus where it binds to target gene promoters and activates or inhibits their transcription [79, 80].

Due to the numerous NFκB target genes, activating signals and involvement in many cellular functions, activation of NFκB needs to be tightly regulated. One NFκB-regulating protein is

the atypical inhibitor of the NFκB protein IκBζ, which is encoded by the *Nfkbiz* gene and is also known as molecule-possessing ankyrin repeats induced by lipopolysaccharide (MAIL) or IL-1-inducible nuclear ankyrin-repeat protein (INAP) [81]. It harbors a six ankyrin repeat domain at its carboxyl terminus which interacts with the NFκB protein to positively or negatively regulate its transcriptional activity in the nucleus [82-84]. As a primary NFκB response gene, the *Nfkbiz* gene is quickly induced upon various inflammatory signals *via* TLR- and cytokine receptor signaling [85-87]. IκBζ is required especially for the development of Th17 cells by enhancing IL-17A expression in T cells through binding to regulatory regions of the *Il-17a* gene in cooperation with RORγt and RORα [83]. *Nfkbiz*$^{-/-}$ mice were shown to be resistant to EAE [83, 88]. Besides, IκBζ is expressed in a variety of cell types and is essential for the induction of a subset of secondary response genes, e.g. *Il-6, Il-12* and *Ccl2* [85]. Recently, upregulation of the *Nfkbiz* gene in inflamed colonic tissue of patients with IBD was revealed, making it an interesting target of novel therapeutic regulation [89].

3.3. Inflammatory bowel disease

IBD, such as UC and Crohn's disease (CD), are chronic inflammatory disorders of the gastrointestinal tract with remitting-relapsing course of disease [90]. With an increasing prevalence and incidence worldwide, IBD is a serious health economic burden with an estimated 1.3 million people suffering from IBD in Europe [91]. Although their etiology remains largely unknown, multiple factors, such as genetic predisposition, environmental factors, epigenetics and gut microbiota, are thought to influence disease onset and progression [92]. UC is characterized by a continuously extending inflammation of the colonic mucosa usually starting from the rectum, whereas CD is attributed by a transmural, discontinuous inflammation of any part of the gastrointestinal tract [93]. Both diseases show similar symptoms, ranging from abdominal pain and diarrhea to extra-intestinal manifestations, malnutrition and life-threatening complications [94]. A significant reduction of life quality and an increasing incidence worldwide emphasize the need for deciphering IBD pathogenesis and new targeted treatment options [95]. Dysbiosis and a dysregulated immune response, e.g. an imbalance of effector T cells and Tregs, to the resident microbiota play a tremendous role in the pathogenesis of IBD, which is not surprising due to the proximity of microbiota and mucosal immune system [52, 96]. However, functional analysis of the microbiota is supposed to have a greater relevance in IBD pathology than compositional analysis, highlighting the impact of the microbiota on the host in terms of e,g. microbial metabolites and bacteria-immune system interactions [16].

Current treatment strategies for IBD aim at relief of symptoms as well as induction and maintenance of remission phases by applying a broad spectrum of drugs such as

corticosteroids, aminosalicylates and immunosuppressants. These treatment options are aggressive and powerful but also come with side effects, whereas milder therapeutics with fewer side effects oftentimes fail [97]. Development of supportive treatment to improve the patients' initial conditions and to decrease the risk of relapse is therefore one aim in IBD-research. Targeting the dysbiotic microbiome with antibiotics to reduce bacteria presumably related to IBD-development or overall bacterial burden only works scarcely, being even more in line with the fact of low microbial diversity as a characteristic in IBD [98]. Besides, a long-term usage of antibiotics promotes the development of antibiotic resistance in commensals, a prevalent issue to be certainly avoided [99]. Thus, promoting a homeostatic, physiological microbiota by administration of living bacteria, e.g. as probiotics or fecal microbiota transplantation, or microbial components appears as a promising, non-invasive therapeutic approach without major side effects but still needs extensive investigations on the influenced host-microbiota interactions [97, 98].

Aim of this work

Understanding the cross-talk between host immune system and gut microbiota contributes to a better comprehension of dysbiosis-driven diseases and provides new therapeutic targets and approaches for the treatment of such diseases. Our group could already show that the symbiotic *B. vulgatus* is able to reduce and prevent intestinal inflammation, whereas the pathobiont *E. coli* promotes inflammatory immune responses [14, 15, 41, 42]. DCs play a major role in this differential immune response by recognizing the bacteria and inducing the respective cells of the adaptive immune system by cytokine secretion and antigen presentation. *B. vulgatus* induces a semi-mature DC phenotype, inducing a regulatory T cell response and contributing to intestinal homeostasis. *E. coli*, in contrast, induces a fully mature DC phenotype contributing to a pro-inflammatory immune response. Both commensals thus differ in their immunogenicity. This thesis aims at further characterization of commensal immunogenicity by comparing their immunomodulatory functions and the cellular pathways they influence.

First, the probably most immunogenic MAMP of these commensals, LPS, is investigated regarding its influence on DC response. Since *B. vulgatus* exerts protective effects in mouse models of experimental colitis, its LPS is hypothesized to participate in the mediation of these beneficial effects and thus hypothesized to achieve similar effects when administered as a purified agent. Involved DC responses are compared with responses induced by the whole bacterium, so a novel therapeutic agent can be developed based on these findings (Publication a). Additionally, the commensal's effect on the ability of DCs to induce a protective or a pathologic Th17 response associated with IBD and other AIDs is investigated. For this, regulation of the transcription factor IκBζ, which is required for DC-mediated Th17 differentiation, by *B. vulgatus* and *E. coli* is analyzed (Publication b). Furthermore, the Th subset differentiation induced by these commensals under inflammatory condition is characterized in greater detail in order to gain insights on the overall pro- or anti-inflammatory Th cell proportion (Publication b). Completing the picture, influence of *B. vulgatus* and *E. coli* on the development of Bregs as a regulatory immune mechanism for tolerating commensals and down-regulating commensal-induced pro-inflammatory pathways is examined. Again, LPS is thought to be the main mediator of the differential immunogenicity mediated by B cells and shall thus be further analyzed regarding its effects on B cells (Publication c). This thesis is intended to shed light on host-microbe interactions by exploring how bacterial immunogenicity shapes gut homeostasis and inflammation. Findings may help to better understand how a dysbiotic microbiome could be modulated in order to improve the host's health condition, especially in IBD and other AIDs associated with dysbiosis.

Results and discussion

Parts of this Results and discussion section have been published in:

a. Steimle, A.*, **Michaelis, L.***, Di Lorenzo, F., Kliem, T., Münzner, T., Maerz, J. K., Schäfer, A., Lange, A., Parusel, R., Gronbach, K., Fuchs, K., Silipo, A., Oz, H. H., Pichler, B. J., Autenrieth, I. B., Molinaro, A., Frick, J.S.: Weak agonistic LPS restores intestinal immune homeostasis. Molecular Therapy 27, 1974-1991 (2019) **shared first author*

b. **Michaelis, L.**, Treß, M., Löw, H.C., Klees, J., Klameth, C., Lange, A., Grießhammer, A., Schäfer, A., Menz, S., Steimle, A., Schulze-Osthoff, K., Frick, J.S.: Gut commensal-induced IκBζ expression in dendritic cells influences the Th17 response. Frontiers in Immunology 11, 612336 (2021)

c. Maerz, J.K., Trostel, C., Lange, A., Parusel, R., **Michaelis, L.**, Schäfer, A., Yao, H., Löw, H.C., Frick, J.S.: Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses. Frontiers in Immunology 10, 3093 (2020)

a) Weak agonistic LPS restores intestinal immune homeostasis

The influence of the microbiota on the progression and outcome of IBD is demonstrated in many studies, associating the microbiota composition with certain phenotypes of remission or inflammatory periods of disease [17-20, 100]. The interplay between host and microbes influencing the immune response by shifting it towards a rather regulated, homeostatic, or a rather dysregulated, inflammatory response is thus a main focus of IBD research. Yet, not only the presence, abundance or proportion of certain microbial species account for these microbiota-mediated immunomodulatory effects. Bacterial immunogenicity, i.e. the extent and nature of immune response induction by single bacteria, is an important feature which greatly impacts the course of disease [101]. As one of the most abundant and potent immunogenic bacterial components in the intestinal lumen, LPS is able to determine the outcome of inflammation in a mouse model for experimental colitis depending on its structure and thus its endotoxicity [102]. Its overall structure is highly conserved and consists in its smooth form of an O-specific polysaccharide (O-chain), a core oligosaccharide (core OS) and lipid A, which anchors LPS to the outer leaflet of the gram-negative cell wall [103]. It represents an important MAMP which is recognized *via* the MD2/TLR4 complex by host cells (see 2.1). Yet, strain-specific differences in the chemical structure of LPS, especially in the lipid A composition, determine its immunogenicity *via* different binding modes to the MD2/TLR4 complex [104]. Its immunogenic effects can range from strong agonistic activity, causing activation of the innate immune system, to antagonistic activity which even blocks immune cell responses [105, 106]. In the study described here, LPS was isolated from the model symbiont *B. vulgatus* mpk and the model pathobiont *E. coli* mpk by purification from cell extracts with extensive enzymatic treatment (RNase, DNase, proteinase K), exhaustive dialysis, ultracentrifugation and gel-filtration chromatography. Purity was verified through several SDS-PAGE with silver staining or Coomassie Brilliant Blue staining.

<u>B. vulgatus *and isolated LPS of* B. vulgatus *actively ameliorate established inflammatory reactions in a mouse model for experimental colitis*</u>

The mouse commensal *B. vulgatus* has already been shown to exhibit anti-inflammatory properties in various different mouse models for colitis when administered before onset of disease [14, 15, 42]. This led to the question whether this commensal was not only able to prevent the onset of microbiota-mediated inflammation but also can reduce already established inflammation. In the T cell transfer model of colitis, transplantation of naïve $CD3^+CD4^+CD25^-CD62L^+CD45Rb^{hi}$ T cells into recombination-activating gene 1-deficient (*Rag1*$^{-/-}$) mice lacking functional T cells and B cells leads to development of an immune-mediated intestinal inflammation [107]. This is based on an initial lack of Tregs and quick

induction of inflammatory Th1 and Th17 cells and is highly dependent on microbiota composition [108-110]. As hypothesized, when live *B. vulgatus* was administered in a concentration of 5 x 10^8 bacteria per mL drinking water to *Rag1*$^{-/-}$ mice starting 4 weeks after T cell transfer, a considerable reduction of intestinal inflammation was observed (Publication a, Fig. 1). This was indicated by non-invasive *in vivo* positron emission tomography (PET) analysis of [^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG)-injected mice, a method suitable for the detection of ongoing inflammatory processes in living animals. Regions with higher [^{18}F]-FDG-uptake, besides organs such as bladder and heart, represent sites of inflammation. Control groups of untreated *Rag1*$^{-/-}$ mice and T cell transplanted *Rag1*$^{-/-}$ mice without *B. vulgatus* administration showed no ongoing inflammatory processes and severe signs of intestinal inflammation, respectively. Since LPS is regarded as one of the most potent surface molecules and DC-maturation-inducing component of gram-negative bacteria, T cell transplanted *Rag1*$^{-/-}$ mice with established colonic inflammation were also treated with isolated LPS of *B. vulgatus* (160 µg/mL drinking water). As seen in Publication a Fig. 4, isolated LPS of *B. vulgatus*, administered 4 weeks after T cell transfer exerted similar inflammation-reducing properties as live *B. vulgatus*, indicated by decreased histological colitis scores (HCS), reduced Th17 response and high amounts of intestinal aldehyde dehydrogenase (ALDH) mRNA suggesting increased Treg-inducing potential. Thus, restoring intestinal homeostasis by application of purified *B. vulgatus* LPS as the only active compound suggests it as a novel therapeutic agent for the treatment of IBD.

LPS of B. vulgatus *induces semi-mature CD11c*$^+$ *cells*

The underlying cellular and molecular mechanisms involved in the anti-inflammatory effect of *B. vulgatus* LPS were determined in the following experiments by investigating DC-responses. Suppression of intestinal inflammation by *B. vulgatus* mpk was already associated with the induction of semi-mature CD11c$^+$ cells [15, 102]. This immune-regulating effect could also be demonstrated for various other *Bacteroides* species, such as *B. dorei*, *B. vulgatus* ATCC8486, *B. fragilis* and *B. thetaiotaomicron*: Stimulation of WT bone marrow-derived dendritic cells (BMDCs) with these strains resulted in similar cytokine secretion profiles and a semi-mature DC phenotype, which was hypo-responsive to further stimulation (Publication a, Fig. 2). Comparison of genes of lipid A biosynthesis showed high similarities between the strains, with some exceptions for *B. thetaiotaomicron* strains (Publication a, Table 1). On the other hand, comparison of the lipid A core synthesis genes between *B. vulgatus* mpk and *E. coli* MG1655 showed only low genetic similarities, indicating that *E. coli* produces a different LPS (Publication a, Table 2). This lipid A homology of *Bacteroides* strains thus suggested an association with the ability to induce the semi-mature DC phenotype. In general, Lipid A is considered to be responsible for mediating the strength

of its biological activity and induced cell responses, therefore determining bacterial immunogenicity [111, 112]. Stimulation of WT BMDCs with purified LPS resulted in cell responses regarding cytokine secretion and maturations status similar to BMDCs stimulated with the respective bacteria from which the LPS originated (Publication a, Fig. 3). Furthermore, *B. vulgatus* LPS was also able to induce hypo-responsiveness of BMDCs, concerning the expression of T cell activating molecules. By inhibiting TLR4-signaling with the selective TLR4 inhibitor TAK242 in BMDCs stimulated with *B. vulgatus*, with its isolated LPS as well as with *E. coli*, cytokine secretion and BMDC maturation was abolished for all used stimuli. Induction of semi-maturation by *B. vulgatus* was thus concluded to be mainly mediated by its LPS, which was sufficient to induce the hypo-responsive phenotype. Moreover, in contrast to an immune response-blocking antagonistic LPS, *B. vulgatus* LPS was proposed to act as a weak agonist.

Weak agonistic LPS is not a competitive inhibitor of strong agonistic LPS and induces DC semi-maturation only in the absence of strong agonistic LPS

Colitis-diminishing effects of purified *B. vulgatus* LPS in *Rag1*$^{-/-}$ mice described above were hypothesized to be caused by a competitive inhibition at the MD2/TLR4 receptor complex-binding site. Blocking the binding of a strong agonistic LPS, such as *E. coli* LPS, by occupying the MD2/TLR4 receptor complex with *B. vulgatus* LPS could thus prevent full DC maturation and inflammatory responses. Both commensal LPS variants were shown to express similar binding affinities to the MD2/TLR4 receptor complex (Publication a, Fig. 5A). However, experiments with human embryonic kidney (HEK) cells overexpressing mouse TLR4 (mTLR4) and pre-incubated with *B. vulgatus* LPS showed that further incubation with *E. coli* LPS could remove about 50% of already bound *B. vulgatus* LPS (Publication a, Fig. 5D). For quantifying the amount of LPS that was still bound to the receptor, LPS was conjugated with a biotin tag. Additional incubation with phycoerythrin (PE)-coupled streptavidin enabled visualization of biotinylated LPS amounts *via* flow cytometry. When this experiment was performed the other way around (pre-incubation with biotinylated *E. coli* LPS and subsequent incubation with *B. vulgatus* LPS), *B. vulgatus* LPS was able to remove only about 20% of already bound *E. coli* LPS (Publication a, Fig. 5C). In line, simultaneous stimulation of BMDCs with *B. vulgatus* LPS and *E. coli* LPS led to full DC maturation, indicated by a high amount of secreted pro-inflammatory cytokines, tumor necrosis factor (TNF) and IL-6, as well as high MHC II surface expression (Publication a, Fig. 6). *B. vulgatus* LPS, which is able to induce the semi-mature DC phenotype when it is the sole stimulus, was thus not able to anticipate *E. coli* LPS-induced maturation effects. Taken together, *B. vulgatus* LPS was demonstrated not to act as a competitive inhibitor of strong agonistic

E. coli LPS, thereby it is not able to prevent from *E. coli* LPS-mediated strong BMDC activation.

This seemed to contradict the *in vivo* findings mentioned above describing inflammation-reducing properties of *B. vulgatus* LPS when administered to mice with established colitis. It was thus hypothesized that the tolerant, semi-mature DC phenotype was induced by *B. vulgatus* LPS in absence of other agonistic LPS. Heat-inactivated fecal samples from *Rag1*$^{-/-}$ mice with established severe colitis ("DYSM" samples) were applied as a dysbiotic microbiota composition as it is developed during intestinal inflammation. DYSM samples contained various endogenous LPS competing with *B. vulgatus* LPS for MD2/TLR4 binding, as well as other immuno-stimulatory components. Indeed, BMDCs primed with *B. vulgatus* LPS and challenged with DYSM samples showed decreased secretion of pro-inflammatory cytokines when compared to mock-primed and DYSM-challenged BMDCs (Publication a, Fig. 7B). However, simultaneous stimulation with *B. vulgatus* LPS and DYSM resulted in fully mature BMDCs, an even stronger pro-inflammatory cytokine secretion compared to stimulation with *B. vulgatus* LPS or DYSM alone. This dose-response effect could also be observed when BMDCs were stimulated with high concentrations of *B. vulgatus* LPS (Publication a, Fig. 6), making the concentration of *B. vulgatus* LPS a decisive factor for the induction of semi-mature DCs. In the *in vivo* experiment, a daily concentration of 1 mg *B. vulgatus* LPS per mouse was appropriate for reduction of inflammation, indicating that this concentration was sufficient to exceed the amount of endogenous LPS while still being in a semi-maturation-inducing concentration range.

B. vulgatus *LPS as potential therapeutic agent?*

Taking the weak agonistic activities into account, *B. vulgatus* LPS was proposed to induce weak but still detectable intracellular signaling and NFκB activation beneath the threshold required for a pro-inflammatory response, thereby, providing a simple anti-inflammatory cell response. Additionally, *B. vulgatus* LPS induced semi-mature DCs which contribute to maintenance of intestinal homeostasis. Being an effective ligand for the MD2/TLR4 receptor complex while at the same time exerting only weak agonistic activity was considered to be attributed to the chemical structure of *B. vulgatus* LPS. Supportingly, a recent study by Di Lorenzo *et al.* reported on the full structure of *B. vulgatus* LPS, revealing hypoacylated and mono-phosphorylated lipid A with a galactofuranose-containing core OS and an O-antigen built up of mannose and rhamnose [113]. The authors assigned these structural characteristics to a commensal LPS, assuming that the weak-agonistic activity of *B. vulgatus* LPS is mediated by the combination of these chemical features.

Bacteroides LPS in general has been reported to contribute to the preservation of intestinal immune homeostasis by other groups, supporting the idea that commensals adapt to the host by inducing tolerance against harmless gut microbes [114]. This study enlarges the picture by revealing that commensal-derived LPS not only promotes intestinal immune homeostasis but is also able to restore it in mice with established severe colitis. An indirect LPS-mediated shift of microbiota composition from dysbiotic to homeostatic cannot be excluded, though. The findings presented in this study may serve as the base for developing new therapeutic strategies for the treatment of immune-mediated intestinal inflammation, i.e. IBD. Instead of systematically suppressing the patient's immune response, as it is performed in standard IBD therapy, LPS (derivative)-based treatment would act locally at the site of inflammation. This way, unwanted side-effects would be avoided.

b) Gut commensal-induced IκBζ expression in dendritic cells influences the Th17 response

In the previously described study, a protective effect exerted by *B. vulgatus* LPS was demonstrated in a mouse model for immune-mediated colitis. By inducing semi-mature DCs, *B. vulgatus* LPS contributes to the symbiotic properties of *B. vulgatus* and thus promotes intestinal immune homeostasis. In contrast, pathobiotic *E. coli* rather induces fully mature DCs *via* its LPS. These fully mature DCs are able to induce a pro-inflammatory T cell response due to the secreted cytokines and expressed T cell activation molecules, i.e. MHC II, CD40, CD80, CD86, and contribute to inflammation. However, the composition of *B. vulgatus*- or *E. coli*-induced $CD4^+$ Th cell subsets has not been described yet. The applied T cell transfer mouse model of colitis is known to be driven by an initial lack of Tregs and induction of inflammatory Th1 and Th17 cells [108, 109]. Due to the dichotomous nature of Th17 cell responses, being either protective against extracellular infections or contributing to development of autoimmune inflammation, DC-mediated Th17 cell responses induced by the commensal *B. vulgatus* and *E. coli* were further characterized in the study presented in this chapter.

Another transcription factor required for Th17 development besides RORγt is the atypical inhibitor of NFκB (IκB) protein IκBζ, which is encoded by the *Nfkbiz* gene. IκBζ is expressed by a variety of cell types and is essential for the induction of a subset of secondary response genes, e.g. *Il-6, Il-12, Il-17* and *Ccl2* [83, 85, 87, 115]. *Nfkbiz*$^{-/-}$mice are resistant to EAE, a model for Th17-mediated autoimmune diseases resembling a multiple-sclerosis-like phenotype [83, 88]. The necessity of IκBζ for Th17 induction was thus demonstrated. Yet, the effect on the induction of protective or pathologic nature of Th17 cells was not shown so far. Since mice deficient for IκBζ develop a severe inflammatory phenotype, showing Sjögren's Syndrome-like symptoms, and hepatocytes lacking IκBζ showed defective proliferation due to impaired TLR4 signaling [86, 116], a regulated expression IκBζ is supposed to be required for a balanced immune response. How gut commensals contribute to regulation of IκBζ expression, especially in DCs, was evaluated in this study.

Expression of IκBζ promotes intestinal homeostasis in a mouse model of acute colitis but is dependent on gut commensal immunogenicity and TLR4 signaling

Since Th17 cells play a major role in the pathology of IBD, the role of IκBζ in IBD was worth to be investigated. An altered function of IκBζ was suggested to contribute to IBD since an upregulation of the *Nfkbiz* gene expression was detected in inflamed intestinal tissue of UC patients [89]. However, applying the mouse model of dextran sodium sulfate (DSS)-induced

acute colitis, expression of IκBζ was demonstrated to promote intestinal homeostasis. This could be shown by a significantly higher susceptibility to DSS-induced acute colitis in mice deficient for IκBζ compared to WT mice. Deficiency for IκBζ resulted in a significantly increased weight loss and increased disease activity index (DAI), as well as clear signs of severe colitis shown by histopathological examination of colon sections (Publication b, Fig.1). Concluding, a certain degree of IκBζ expression plays an important role in maintaining intestinal immune homeostasis but also needs tight regulation in order to not induce an excessive pro-inflammatory immune response. One important element having a large impact on intestinal homeostasis or development of inflammatory diseases is the microbiota. This was already shown in previous studies by our group, with DCs acting as one of the decisive players in commensal-mediated immune responses [13, 15, 41, 117, 118]. Due to the close link between gut commensals and host immunity, the influence of gut commensals on IκBζ expression in cells of the intestinal tissue was analyzed. In DCs, IκBζ activity is of particular importance since it regulates the ability of DCs to induce and manifest a Th17 response. Furthermore, intestinal epithelial cells are in direct contact with the microbiota and can mediate an immune response induced by immunogenic bacteria.

Indeed, BMDCs and cells of a mouse small intestinal epithelial cell line (mICcl2 cells) stimulated with the previously described model commensals *B. vulgatus* and *E. coli* express distinct levels of IκBζ, measured in gene expression and intracellular protein levels, corresponding to their immunogenicity (Publication b, Fig. 2A, B, D and E). *B. vulgatus* only induced low *Nfkbiz* gene expression and IκBζ protein levels, comparable to levels observed in unstimulated controls. These semi-mature BMDCs also secreted low amounts of cytokines IL-1β, IL-6 and IL-23 which are required for the differentiation of Th17 cells (Publication b, Fig. 2C). In contrast, *E. coli*-stimulation resulted in high *Nfkbiz* gene expression and IκBζ protein levels with a maximum of expression 2 hours after stimulation (Publication b, Fig. 2A and B). These fully mature DCs were able to induce differentiation of Th17 cells, indicated by high levels of secreted IL-1β, IL-6 and IL-23. However, *E. coli*-stimulation also induced an increased secretion of anti-inflammatory IL-10 by BMDCs into the cell culture supernatant compared to *B. vulgatus*-stimulated BMDCs, as described and discussed in greater detail in chapter regarding Publication c. An IκBζ-dependent production of IL-6 and IL-10 upon a strong stimulus could be observed, indicated by a significant reduction of IL-6 and IL-10 secretion by *Nfkbiz*$^{-/-}$ BMDCs stimulated with strong immunogenic *E. coli* compared to WT BMDCs. However, *B. vulgatus*-stimulation did not lead to different cytokine levels dependent on the presence of IκBζ. This result supports previously reported findings of IL-6 and IL-10 production being dependent on IκBζ [86, 119] and complements them with the variance of bacterial immunogenicity for respective cytokine production by BMDCs. IκBζ activity was not shown to be responsible for IL-23 secretion and only indirectly responsible for IL-1β secretion

since it upregulates transcription of the gene encoding NOD-like receptor protein 3 (NLRP3), a component of inflammasomes responsible for the cleavage of inactive pro-IL1β into active IL-1β [120]. The fact that intestinal epithelial cells responded with IκBζ expression to a comparable extend as BMDCs showed that bacteria display similar immunogenicity on different cell types of the gut barrier, facilitating a uniform and coordinated immune response by these different cell types.

As described above in Publication a, immunogenicity of *B. vulgatus* and *E. coli* is mainly mediated by their characteristic LPS and affects both maturation status and cytokine secretion of BMDCs. Likewise, the induction of IκBζ seemed to be mainly dependent on TLR4 signaling. This was demonstrated by a significant reduction of *Nfkbiz* gene expression and resultant IκBζ protein levels in *E. coli*-stimulated BMDCs deficient for TLR4, or both TLR2 and TLR4, but not in BMDCs deficient for only TLR2 (Publication b, Fig. 3A and B). Due to the low immunogenicity of *B. vulgatus*, no differences in already very low IκBζ expression were observed between WT BMDCs and BMDCs lacking either of the mentioned TLRs. Secretion of Th17-relevant cytokines upon *E. coli* stimulation were also induced mainly by TLR4 signaling, supporting previous results (Publication b, Fig. 3C). Stimulation with isolated LPS from *B. vulgatus* and *E. coli* confirmed the hypothesis of LPS being the main MAMP responsible for IκBζ induction, since IκBζ levels of LPS-stimulated BMDCs were comparable to IκBζ levels measured in BMDCs stimulated with the respective bacterium (Publication b, Fig. 3E). Interestingly, a synergistic effect of *E. coli*-induced TLR2 and TLR4 signaling in terms of IκBζ expression and cytokine secretion could be observed when comparing BMDCs with deficiencies for both TLRs with BMDCs deficient for only one of these TLRs. This result supported previous findings describing a marked increase in pro-inflammatory cytokine secretion by mouse peritoneal macrophages upon co-stimulation with TLR2- and TLR4-ligands compared to stimulation with single ligands [121]. A possible role of IκBζ in TLR-ligand-induced BMDC maturation was suggested by an observed correlation between high MHC II expression and IκBζ levels in stimulated BMDCs, which was decreased upon deficiency for TLR2 and/or TLR4 (Publication b, Fig. 3D). Taken together, bacterial immunogenicity exerted by a characteristic LPS regulated IκBζ expression in DCs and thus contributed to drive an inflammation-promoting or tolerogenic DC phenotype.

The unique composition of the cytokine milieu in response to various commensals differently polarizes T cells

Depending on the combination and amount of secreted cytokines, DCs are able to induce different $CD4^+$ Th cell subsets, e.g. Tregs, Th1, Th2 or Th17 cells. In an *in vitro* assay, polarization of naïve $CD4^+$ T cells with the non-manipulated cytokine milieu created by

B. vulgatus- or *E. coli*-stimulated BMDCs did not lead to significant differences in differentiation, although bacteria exert different immunogenicity (Publication b, Fig. 4). This could be explained by a balanced immune response, i.e. also inducing production of counter-regulating IL-10 besides pro-inflammatory cytokines in amounts appropriate for the strength of the stimulus. This is further discussed in the chapter regarding Publication c. Nonetheless, a slightly but not significantly increased survival of T cells could be observed upon differentiation with cytokine mixes originating from bacteria-stimulated BMDCs. Secreted cytokines such as IL-6 serve as T cell survival factor and are secreted in higher amounts by BMDCs upon contact to bacterial antigens [122]. Neutralization of IL-10 in the cytokine mixes by adding an anti-IL-10 antibody created an imbalanced cytokine milieu, mimicking an inflammatory environment. Under these circumstances, naïve $CD4^+$ T cells increasingly differentiated into pro-inflammatory Th1, Th2 and Th17 subsets but also into Tregs. Differentiation with the cytokine mix secreted by *E. coli*-stimulated BMDCs led to significantly increased numbers of Th2 cells when IL-10 was neutralized, suggesting a Th2-inhibiting action by IL-10 under strong stimuli (Publication b, Fig. 4B, top right panel). Overshooting Th2 responses are associated with allergic reactions, hence a strategy for inducing IL-10-secreting DCs by strong stimuli, such as bacteria or bacterial components, is of large therapeutic interest [123]. An increased differentiation into Th17 cell subsets, especially into those co-expressing IL-17 and Foxp3 or IFNγ, could be observed upon neutralization of IL-10 in the cytokine mix secreted by *B. vulgatus*-stimulated BMDCs (Publication b, Fig. 4B, bottom panels). However, detected percentage of those subsets was very low, questioning the biological relevance of this *in vitro* finding. The cytokine mix secreted by unstimulated, immature BMDCs induced comparatively high numbers of Tregs which were not influenced by neutralization of IL-10 (Publication b, Fig. 4B, center panel). Since immature BMDCs are known to promote T cell anergy and generate Tregs [37], a Treg-promoting effect independent of antigen-presentation processes and IL-10 was suggested here worth to be further investigated in future studies.

E. coli *promotes a pro-inflammatory $CD4^+$ T cell response in the mouse model of T cell transfer colitis*

The chronic intestinal inflammation induced by the transfer of naïve $CD4^+$ T cells into *Rag1*$^{-/-}$ mice is largely dependent on the microbiota [110]. Continuous and excessive administration of a symbiont, i.e. *B. vulgatus*, or a pathobiont, i.e. *E. coli*, to SPF *Rag1*$^{-/-}$ mice was thus hypothesized to influence the total immunogenicity of the microbiota. Therefore, DC and T cell responses driving intestinal inflammation upon T cell transfer one week after start of bacterial administration were expected to be influenced by these commensals. In this experiment, no significant difference in body weight loss between the different experimental

groups could be observed (Publication b, Fig. 5). Moreover, analysis of serum cytokines showed no significant difference in composition or concentration, indicating no effect of administered bacteria on systemic inflammation. Nonetheless, *Nfkbiz* gene expression in colonic tissue was found to be higher in *E. coli*-administered mice compared to expression in *B. vulgatus*-administered mice, suggesting a more pronounced Th17 response to a microbiota rich in *E. coli*. This supported the *in vitro* findings described above, showing increased *Nfkbiz* gene expression and resultant IκBζ protein levels in *E. coli*-stimulated BMDCs and intestinal epithelial cells. The colonic barrier is weakened in IBD, enabling a close contact of commensals with the epithelial layer [124]. This increased contact represents an antigen-overload and can lead to an inappropriate and dysregulated response of $CD4^+$ T cells, finally resulting in the differentiation of pro-inflammatory T cell phenotypes and, eventually, in chronic inflammation if no regulation is induced.

Flow cytometric characterization of immune cells in cLP and mLN revealed that total numbers of cLP DCs were significantly higher in *B. vulgatus*-administered mice compared to the control group, but of immature status indicated by low MHC II expression, and intermediate IκBζ protein levels (Publication b, Fig. 6A and B). In *E. coli*-administered mice, total numbers of cLP DCs were only slightly but insignificantly higher than in the control group and also of immature status and low IκBζ protein levels. In mLN, high numbers of highly mature DCs were observed in bacteria-administered mice, most likely originating from the intestine and migrated to the mLN where they activate and present antigens to naïve $CD4^+$ T cells. However, those DCs also expressed only low amounts of IκBζ and seemed to contradict the earlier observed positive correlation of DC maturation and IκBζ levels. It is conceivable that cLP and mLN DCs represented cells in different stages of maturation and differentiation. Accumulation of IκBζ in the cell leads to a negative feedback loop, suppressing NFκB-induced gene transcription due to the inhibitory activity of IκBζ. It was speculated that IκBζ expression levels were already diminished in analyzed cLP and mLN DCs as a result of its self-limitation.

Flow cytometric analysis of cLP $CD4^+$ T cells revealed that absolute T cell numbers were comparable between all experimental groups. However, *E. coli*-administration led to rather high numbers of pro-inflammatory $CD4^+$ Th cell phenotypes with significantly higher numbers of Th1 cells compared to the control group and *B. vulgatus*-administered group and significantly higher numbers of Th2 cells compared to the control group. In *B. vulgatus*-administered mice, $CD4^+$ T cells in cLP expressed rather protective phenotypes, with clearly higher numbers of Tregs compared to control and *E. coli*-administered mice (Publication b, Fig. 6C, top panels). No significant differences were observed in the total numbers of IL-17^+Th17 cells in all experimental groups. Nonetheless, *B. vulgatus*-administration led to

significantly higher numbers of anti-Th17 Tregs co-expressing IL-17 and Foxp3, and slightly higher numbers of protective IL-10-producing Th17 cells than *E. coli*-administration or no bacterial treatment. Nonetheless, numbers of pro-inflammatory IFNγ and IL-17 co-expressing Th1-like Th17 cells were not significantly different to the other experimental groups (Publication b, Fig 6C, bottom panels). Taken together, contributing to a dysbiotic microbiota by increasing the prevalence of *E. coli* by administration *via* the drinking water resulted in a higher prevalence of pro-inflammatory $CD4^+$ Th cell subsets with low numbers of protective/anti-inflammatory subsets. This shifted Th-subset balance was not able to dampen inflammatory responses. Intestinal inflammation was thus promoted by *E. coli*, similar to what was observed in T cell-transplanted *Rag1*$^{-/-}$ mice with *Enterobacteriaceae*-rich microbiota [102]. In contrast, increasing the prevalence of *B. vulgatus* in the intestine led to a potent regulation of inflammation, indicated by high numbers of Tregs and other anti-inflammatory $CD4^+$ Th cell subsets, re-establishing the Th1/Th2/Th17/Treg balance supporting homeostasis. This finding supported and complemented previously described anti-inflammatory properties of *B. vulgatus* and its isolated LPS by adding details of commensal-mediated T cell differentiation, see Results and discussion section of Publication a.

Increased abundance of B. vulgatus *in microbiota dampens the secretion of pro-inflammatory cytokines by BMDCs*

As described above, *B. vulgatus*-administration in the mouse model of T cell transfer colitis resulted in an increased differentiation of regulatory/anti-inflammatory $CD4^+$ Th cell subsets whereas higher numbers of *E. coli* in the gut promote differentiation of pro-inflammatory $CD4^+$ T cell subset in an immune-compromised host. However, a differential activation of DCs in cLP or mLN could not be clearly observed in these mice. Thus, the influence of the microbiota prior and after T cell transfer and bacterial administration in established colitis on WT BMDC responses was analyzed to link *in vivo* findings to previously observed *in vitro* results. Stimulation of WT BMDCs with heat-inactivated fecal samples of control, *B. vulgatus*- or *E. coli*-administered mice resulted in IκBζ expression relationships comparable with single bacterial stimulation: Increased abundance of *E. coli* in the microbiota induced significantly higher levels of IκBζ protein than all the other microbiota, whereas a *B. vulgatus*-rich microbiota did not induce a significant increase in IκBζ levels (Publication b, Fig. 7B and C). In contrast to previous *in vitro* findings, a significant increase in secretion of pro-inflammatory cytokines was not observed in *E. coli*-rich microbiota-stimulated samples compared to those stimulated with the heat-inactivated microbiota of control mice. Rather, BMDC stimulation with feces from *B. vulgatus*-administered mice decreased the secretion of pro-inflammatory cytokines required for Th17 induction. This could be observed by comparing the cytokine levels of BMDCs stimulated with the feces of T cell transplanted control mice and *E. coli*-

administered mice (Publication b, Fig. 7D). A slight but insignificant decrease of IL-10 secretion was observed in BMDCs stimulated with the microbiota from T cell transplanted mice compared to stimulation with the naïve endogenous microbiota of Rag1$^{-/-}$ mice prior to T cell transfer, indicating fewer immune-regulatory properties of the microbiota present in established inflammation.

Taken together, a modulation of the host's immune response by administration of commensal bacteria (or their components) could define the outcome of a Th17-driven disease at least in part *via* regulation of IκBζ in DCs.

c) Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses

Besides DCs and T cells, another cell type playing a critical role in the onset and course of inflammatory processes are B cells. Due to their production of autoantibodies, studies focusing on AIDs such as multiple sclerosis and IBD mainly represent B cells as drivers of inflammation and disease [125-127]. However, B cells can also exert anti-inflammatory effects, as demonstrated in mouse models for acute colitis and EAE showing a more severe phenotype in the absence of B cells [128-132]. The observed immune tolerance-promoting effect was primarily accredited to a specific B cell subpopulation – the Bregs [133]. Until now, Bregs cannot be identified by specific markers but can be defined by their immune-regulatory and anti-inflammatory capabilities: suppression of Th1 cells, inhibition of autoimmune pathogenesis and maintenance of immune homeostasis [134]. The most important feature of Bregs is the secretion of anti-inflammatory IL-10, a feature shared with protective and regulatory T cell subsets, Tregs and IL-10^+ IL-17^+ anti-Th17 Tregs, which are mentioned above in the chapter concerning Publication b [134]. The function of B cells can be manipulated by microbial influences, e.g. by pathogenic bacteria, viruses and parasites as part of their survival strategies [135]. Depending on the microbiota composition, commensal bacteria were shown to induce homeostasis-promoting B cells and modulate intestinal inflammation by interaction with involved immune cells [62, 136]. As already prevalently mentioned in this thesis, the immunogenicity of commensal bacteria determines and guides host immune responses. In the previous sections, the inflammation-promoting potential of *E. coli* and the homeostasis-promoting effect of *B. vulgatus* were discussed, especially under conditions prevailing in an immune-compromised, B cell-deficient host, the *Rag1*$^{-/-}$ mouse. Here, the B cell modulatory abilities of *B. vulgatus* and *E. coli* were analyzed to complement the image of immunomodulation by these commensals of different immunogenicity.

Strong immunogenic E. coli *induces activation and differentiation of regulatory B cell phenotypes* via *TLRs*

In previous findings mentioned in Publication a and b, a TLR-dependent immunomodulation of DCs by the model commensals *B. vulgatus* and *E. coli* was observed with LPS being the main MAMP for inducing cell responses. Similarly, B cells express TLRs for MAMP recognition and, thus, a TLR-dependent B cell activation was hypothesized. Activation, survival and proliferation of naïve CD19^+ B cells isolated from WT mice as well as mice deficient for both TLR2 and TLR4 were analyzed daily *via* flow cytometry upon stimulation with *B. vulgatus* and *E. coli* for 72 hours. Indeed, *E. coli* stimulation induced survival and proliferation of WT B cells but not of B cells deficient for TLR2 and TLR4. In unstimulated

control WT B cells, WT B cells stimulated with low immunogenic *B. vulgatus* and all B cells deficient for both TLR2 and TLR4, no proliferation or survival could be observed, indicated by decreasing numbers of viable B cells and a lack of proliferating cells after up to 72 hours (Publication c, Fig. 1A and B). Comparable with DC responses, *E. coli*-stimulation induced strong TLR-dependent upregulation of maturation markers MHC II, CD80, CD86 and IgM in WT B cells but not in TLR2/TLR4-deficient B cells, whereas *B. vulgatus* induced only moderate levels of these markers in WT B cells (Publication c, Fig. 1C).

Since *E. coli*-stimulated DCs not only secrete high levels of pro-inflammatory cytokines but also high levels of anti-inflammatory IL-10 depending on TLR signaling (compare Publication b, Fig. 2C and Fig. 3C), induction of a counter-regulative immune mechanism was hypothesized. With this mechanism, strong immunogenic commensals might contribute to the maintenance of intestinal immune homeostasis. In line, *E. coli* stimulation of B cells resulted in an increased differentiation of the best characterized Breg subsets, the B10 cells ($CD19^+CD5^+CD1d^+IL\text{-}10^+$), T2-MZP cells ($CD19^+CD21^{hi}CD23^{hi}CD24^{hi}$) and Tim-$1^+$ ($CD19^+Tim\text{-}1^+$) cells compared to a stimulation with PBS (control) or *B. vulgatus* (Publication c, Fig. 2B). An increased secretion of IL-10 by B cells into the cell culture medium was observed upon *E. coli*-stimulation compared to control or *B. vulgatus*-stimulated B cells, whereas secretion of pro-inflammatory TNFα was equally low in all treatment conditions (Publication c, Fig. 2C). Expression of suppressor molecules on the B cell surface, that are not directly linked to specific Breg subsets, were significantly elevated in *E. coli*-stimulated samples but not in the other stimulatory conditions (Publication c, Fig. 2D). These *in vitro* findings are in accordance with a study by Hudak *et al*. which demonstrated that a large number of $CD19^+$ B cells in the cLP can act as APCs by directly recognizing the MAMP peptidoglycan, taking it up and contributing to intestinal immune regulation [137]. However, this recognition was not enabled by cellular protrusions as it is characteristic for DCs [138]. Taken together, these findings support the hypothesis that *E. coli* induces a counter-regulative immune response mediated by B cells in order to balance its strong immunogenicity.

E. coli-*stimulated B cells inhibit DC and T cell activation and induce Treg differentiation*

The regulatory properties of *E. coli*-induced Bregs were demonstrated by a co-culture of B cells with immature DCs. In contrast to developing a fully mature DC phenotype upon *E. coli*-stimulation, as it was observed in a DC mono-culture, co-culturing DCs with naïve B cells suppressed DC maturation upon *E. coli*-stimulation (Publication c, Fig. 3). This effect was demonstrated to be independent of direct cell-cell contact since it was also observed when a Transwell membrane was separating the two cell types. Accordingly, secretion of

pro-inflammatory TNFα by *E. coli*-stimulated DC was significantly reduced when they were cultured together with B cells compared to the mono-culture conditions, independent of direct or indirect contact to B cells (Publication c, Fig. 3C, left panel). This finding suggested that a soluble factor able to cross the Transwell membrane was responsible for the maturation-suppressing effect. Supportingly, adding recombinant IL-10 to DC mono-culture could reduce *E. coli*-induced DC maturation, indicated by lower maturation marker expression on the cell surface, as well as significantly decreased TNFα-secretion compared to sole *E. coli*-stimulation (Publication c, Fig. 3B and C). IL-10 was secreted by B cells as a main regulatory factor and found to be equally distributed in cell culture supernatants of Transwell-separated upper and bottom chamber in significantly higher concentrations compared to DC mono-culture (Publication c, Fig. 3C, right panel). Neutralization of IL-10 in *E. coli*-stimulated B cell/DC co-culture resulted in impeding the B cell mediated DC maturation-inhibiting effect, indicated by restored full DC maturation and increased levels of secreted TNFα (Publication c, Fig. 3B and C, yellow bars).

These findings led to the assumption that *E. coli*-induced Bregs were able to largely influence DC antigen-presentation to T cells by suppressing DC maturation. Since antigen-pulsed B cells stimulate $CD4^+$ T cells for proliferation and differentiation into Th2 cells with higher efficiency than antigen-pulsed $CD11c^+$ DCs [128], T cell activation by commensal-primed B cells was analyzed. In agreement with DC co-culture results, T cell activation and proliferation by *E. coli*-stimulated B cells as professional APCs was inhibited in B cell/T cell co-cultures in a B cell dose-dependent manner, with a significant reduction already observable at a T cell/B cell ratio of 1:1 (Publication c, Fig. 4). Furthermore, *E. coli*-stimulated B cells promoted the differentiation of naïve T cells into Tregs and prevented Th17 differentiation when present in a T cell/B cell ratio of 1:5 (Publication c, Fig. 4C). With this influence on T cell differentiation, B cells direct the induction of Th2 cells [139-141]. In contrast, the incubation of *B. vulgatus*-primed B cells with naïve T cells did not result in reduced T cell proliferation at a T cell/B cell ratio of 1:1, only at a higher B cell concentration with a T cell/B cell ratio of 1:5 (Publication c, Fig. 4B). However, next to polarization of Tregs and Th2 cells, *B. vulgatus*-primed B cells induced Th1 cells and Th17 cells, resulting in a rather pro-inflammatory Th1/Th2/Th17/Treg balance.

Induction of Bregs by E. coli *contributes to counter-regulation of DSS-induced inflammation*

The results described above suggest that *E. coli*-induced Bregs were able to directly or indirectly regulate the action of other immune cells. In a host with functional B cells, this induction of regulatory immune cell phenotypes by *E. coli* was hypothesized to down-regulate inflammatory processes and to re-establish intestinal immune homeostasis. In the mouse

model of DSS-induced acute colitis performed in gnotobiotic WT C57BL/6 mice, colonization with *E. coli* seven days prior to induction of colitis, but not with *B. vulgatus*, could significantly reduce signs of disease compared to the germ-free control group (Publication c, Fig. 5B, C and D). This was indicated by reduced weight loss, DAI and HCS in the *E. coli*-associated mice compared to the germ-free control group. Flow cytometric enumeration and characterization of immune cells in different organs demonstrated that colonization with *E. coli* resulted in reduced proliferation and infiltration of $CD11b^+Gr1^+$ neutrophils in spleen and cLP as well as increased numbers of $CD4^-CD19^+IL\text{-}10^+$ Bregs in spleen and mLN compared to the germ-free control group and colonization with *B. vulgatus* (Publication c, Fig. 6A). This *E. coli*-dependent increased Breg induction could already be observed prior to inflammation (Publication c, Supplementary Fig. 5), and negatively correlated with the HCS (Publication c, Fig. 6B). Supportingly, other studies also reported the decreased abundance of Bregs in human and mouse intestinal inflammation [142-144].

In contrast, colonization with weak immunogenic *B. vulgatus* resulted in only weak amelioration of inflammation, shown by only slightly decreased weight loss, DAI, HCS, clearly reduced infiltration of $CD11b^+Gr1^+$ neutrophils in cLP and only low induction of Bregs compared to the germ-free control group (Publication c, Fig 5). Regarding the polarization of $CD4^+$ T cells, it was shown that colonization with *E. coli* led to higher percentages of Tregs in spleen and mLN, as well as decreased percentages of Th1 and Th17 cells in mLN compared to the germ-free control group (Publication c, Fig. 6C). Colonization with *B. vulgatus* did not lead to such pronounced differentiation of Tregs. However, differentiation of Th2 cells was not dependent on colonization status since no difference was observed between the experimental groups (Publication c, Fig. 6C).

Taken together, the strong immunogenicity of *E. coli* enhanced induction of Bregs and Tregs, thereby contributing to a powerful regulation and even suppression of inflammation in this *in vivo* model and also in other AIDs [145]. One has to keep in mind, that this anti-inflammatory B cell-mediated effect was observed in WT mice expressing functional T cells and B cells. On the contrary, the mouse model of T cell induced colitis mentioned in the chapters describing Publication a and b represents an immune-compromised host since *Rag1*$^{-/-}$ mice lack functioning B and T cells which are essential for an adequately working adaptive immune system. Other mouse models used for studying IBD mechanisms include *Il2*$^{-/-}$ mice or *Il10*$^{-/-}$ mice, exhibiting a disturbed proliferation and induction of Bregs or dysfunctional Bregs, respectively [14, 102, 110]. *E. coli* is able to exert observed inflammation-reducing effects only in a host with a fully equipped immune system without any genetic predispositions, presumably with functional Bregs. As a pathobiont, it does not actively use specific mechanisms to harm the host under certain conditions but induces a strong immune

cell response in need of regulation. However, in the immune-compromised hosts, counter-regulative pathways are restricted and cannot compensate for these extensive immune responses induced by *E. coli*. Hence, the strong immunogenic commensal rather promotes inflammatory conditions in these hosts. This characteristic has also been observed in the bacterium *Helicobacter hepaticus* which colonizes the lower intestine in the mouse and can activate the innate immune system without causing disease since it induces an anti-inflammatory, regulatory macrophage response [146, 147]. In mice with a malfunctioning immune system, it induces colitis due to an aberrant Treg function [148-150]. In contrast, the weak immunogenic commensal *B. vulgatus* successfully promotes the regulatory immune response only under healthy conditions or in an immune-compromised host with developed colitis, and not under inflammatory conditions in the gnotobiotic WT mice used in this study. The low immunogenicity was assumed to be responsible for the ineffective regulatory response besides the stunted immune system and gut barrier found in germfree mice.

Although Bregs are only present in minor cell frequency in the tissue, they were assumed to play a major role in ameliorating inflammatory immune responses. Here, a T cell-independent activation of B cells supposedly occurred by direct binding of MAMPs with B cell-associated TLRs. Supporting findings of previous chapters, LPS was found to be the main trigger for B cell proliferation and Breg differentiation. Nevertheless, the *in vivo* encounter of naïve B cells is subject to limitations including intestinal barrier functions and translocation of microbial structures to secondary lymphoid organs such as lymph nodes or spleen. Furthermore, the degree of commensal immunogenicity correlated with the extent of Breg induction and B cell survival and had a large effect on other immune cells and the overall immune response as well as maintenance of immune homeostasis.

Conclusion and outlook

Findings presented in this thesis could shed light on new aspects of host-commensal interactions and their resulting effects. It was demonstrated that commensal-induced immune responses shaping gut homeostasis and inflammation are mediated *via* DCs and B cells depending on the bacterial immunogenicity. As main agent determining the degree of immunogenicity, LPS alone was sufficient to induce observed immune responses which can be further characterized by the composition and ratio of induced Th subsets. In DCs, the transcriptional regulator IκBζ was shown to be differentially regulated by commensals, leading to the DCs' ability to induce protective or pathologic Th17 responses by creating a dedicated cytokine milieu for $CD4^+$ Th cell differentiation. These immune responses can be of antithetic nature, with the strong immunogenic *E. coli* inducing a DC- and T cell-mediated inflammation-promoting response but inducing a regulative B cell response. In contrast, *B. vulgatus* expressing only low immunogenicity induces tolerogenic semi-mature DCs and regulatory T cell phenotypes but does not induce B cell-mediated regulation of inflammation. The function of the specific bacteria diverges depending on the environmental setting provided by the host. Critical for this divergence is the state of the host's immune competence. Under immune homeostatic conditions, as it prevails in health, the host tolerates even strong immunogenic commensals by executing a counter-regulative immune response induced by the commensals themselves. Low immunogenic symbionts contribute to the balanced condition by acting immune educating *via* induction of regulatory T and B cells. In predisposed or diseased hosts, the counter-regulative immune response is not sufficient to compensate inflammatory processes which are promoted by strong immunogenic commensals.

Concerning the large role of the microbiota in AIDs, these new insights can be utilized for development of novel treatment options for immune-mediated diseases. AIDs are oftentimes associated with a dysbiotic microbiota, which features an increased abundance of strong immunogenic pathobionts and thus increased numbers of immunogenic antigens and interactions with the mucosal immune system. With findings presented in this thesis, novel microbiota-based therapeutic strategies for the treatment of T cell-mediated inflammatory diseases, especially IBD, can be developed and optimized. As a treatment strategy, the immune-regulative role of symbionts, which are abundant in decreased numbers under dysbiotic conditions, should thus be reinforced by enriching the mucosal environment with specified commensals or their immunogenic components. Isolated *B. vulgatus* LPS was demonstrated to ameliorate intestinal inflammation in the mouse. Additionally, considering the novel insights on immunogenicity-dependent regulation of IκBζ in DCs as well as

induction of Bregs by commensals, the basis for a novel potent treatment option supporting conventional IBD therapies was created within this thesis.

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Steimle, A., Gronbach, K., Beifuss, B., Schäfer, A., Harmening, R., Bender, A., Maerz, J.K., Lange, A., **Michaelis, L.**, Maurer, A., Menz, S., Mccoy, K., Autenrieth, I.B., Kalbacher, H., Frick, J.S.: Symbiotic gut commensal bacteria act as host cathepsin S activity regulators. Journal of Autoimmunity 75, 82-95 (2016)

Steimle, A.*, **Michaelis, L.***, Di Lorenzo, F., Kliem, T., Münzner, T., Maerz, J. K., Schäfer, A., Lange, A., Parusel, R., Gronbach, K., Fuchs, K., Silipo, A., Oz, H. H., Pichler, B. J., Autenrieth, I. B., Molinaro, A., Frick, J.S.: Weak agonistic LPS restores intestinal immune homeostasis. Molecular Therapy 27, 1974-1991 (2019)
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Steimle, A., Menz, S., Bender, A., Ball, B., Weber, A.N.R., Hagemann, T., Lange, A., Maerz, J.K., Parusel, R., **Michaelis, L.**, Schäfer, A., Yao, H., Löw, H.C., Beier, S., Tesfazgi Mebrhatu, M., Gronbach, K., Wagner, S., Voehringer, D., Schaller, M., Fehrenbacher, B., Autenrieth, I.B., Oelschlaeger, T.A., Frick, J.S.: Flagellin hypervariable region determines symbiotic properties of commensal Escherichia coli strains. PLoS Biology 17, e3000334 (2019)

Maerz, J.K., Trostel, C., Lange, A., Parusel, R., **Michaelis, L.**, Schäfer, A., Yao, H., Löw, H.C., Frick, J.S.: Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses. Frontiers in Immunology 10, 3093 (2020)

Michaelis, L., Treß, M., Löw, H.C., Klees, J., Klameth, C., Lange, A., Grießhammer, A., Schäfer, A., Menz, S., Steimle, A., Schulze-Osthoff, K., Frick, J.S.: Gut commensal-induced IκBζ expression in dendritic cells influences the Th17 response. Frontiers in Immunology 11, 612336 (2021)

List of poster presentations

“Regulation of host cathepsin B activity is essential for maintenance of intestinal homeostasis”

9th Seeon Conference “Microbiome, Probiotics and Host”, Seeon, Germany, 2016

“Inflammasome activation by *E. coli* Nissle 1917”

10th Seeon Conference “Microbiome, Probiotics and Host”, Seeon, Germany, 2017

“Gut commensal-induced IκBζ expression in dendritic cells influences the Th17 response”

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Appendix: Publications

Publication a, p.49-81:

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**shared first author*

Molecular Therapy
Original Article

Weak Agonistic LPS Restores Intestinal Immune Homeostasis

Alex Steimle,[1,4] Lena Michaelis,[1,4] Flaviana Di Lorenzo,[2] Thorsten Kliem,[1] Tobias Münzner,[1] Jan Kevin Maerz,[1] Andrea Schäfer,[1] Anna Lange,[1] Raphael Parusel,[1] Kerstin Gronbach,[1] Kerstin Fuchs,[3] Alba Silipo,[2] Hasan Halit Öz,[1] Bernd J. Pichler,[3] Ingo B. Autenrieth,[1] Antonio Molinaro,[2] and Julia-Stefanie Frick[1]

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Generated by gram-negative bacteria, lipopolysaccharides (LPSs) are one of the most abundant and potent immunomodulatory substances present in the intestinal lumen. Interaction of agonistic LPS with the host myeloid-differentiation-2/Toll-like receptor 4 (MD-2/TLR4) receptor complex results in nuclear factor κB (NF-κB) activation, followed by the robust induction of pro-inflammatory immune responses. Here we have isolated LPS from a common gut commensal, *Bacteroides vulgatus* mpk (BVMPK), which provides only weak agonistic activity. This weak agonistic activity leads to the amelioration of inflammatory immune responses in a mouse model for experimental colitis, and it was in sharp contrast to strong agonists and antagonists. In this context, the administration of BVMPK LPS into mice with severe intestinal inflammation re-established intestinal immune homeostasis within only 2 weeks, resulting in the clearance of all symptoms of inflammation. These inflammation-reducing properties of weak agonistic LPS are grounded in the induction of a special type of endotoxin tolerance via the MD-2/TLR4 receptor complex axis in intestinal lamina propria CD11c$^+$ cells. Thus, weak agonistic LPS represents a promising agent to treat diseases involving pathological overactivation of the intestinal immune system, e.g., in inflammatory bowel diseases.

INTRODUCTION

Inflammatory bowel diseases (IBDs) are characterized by chronic relapsing intestinal inflammation, with Crohn's disease (CD) and ulcerative colitis (UC) being the most frequent and clinically relevant forms of this disease complex.[1] The etiology of IBD is considered to be multifactorial, with genetics,[2] environmental factors,[3] and intestinal microbiota composition[4] contributing to the pathology. Therapy of IBD in humans is currently focused on symptomatic treatment, often by means of immunosuppression.[5] Furthermore, IBD patients require intensive medical intervention, rendering IBD not only a major health care but also a growing economic challenge.[6] This underlies the need for novel, low-priced, and innovative therapeutic approaches.

It is widely accepted that the composition of the intestinal microbiota contributes to the progression and outcome of IBD. However, not only the presence, abundance, or proportion of certain live microbial species account for such microbiota-mediated effects. Indeed, the structure, and consequently the endotoxicity, of lipopolysaccharides (LPSs) from gram-negative bacteria determines the outcome of inflammation in a mouse model for experimental colitis.[7] Furthermore, the composition and variety of the intestinal LPSome strongly impact its inflammation-promoting or homeostasis-preserving properties.[8] This makes LPS an interesting potential drug to target dysregulated intestinal immune responses.

LPS, in its smooth form, consists of three distinct domains: (1) an O-specific polysaccharide (O-chain); (2) a core oligosaccharide (core OS); and (3) the lipid A, which anchors LPS to the outer leaflet of the bacterial cell wall.[9] With its highly conserved overall structure and composition, LPS represents an important microbe-associated molecular pattern (MAMP), playing an essential role for the recognition of bacterial invasion by the host innate immune system[10,11] through recognition by the host myeloid-differentiation-2/Toll-like receptor 4 (MD-2/TLR4) receptor complex. Importantly, the chemical structure of LPS, primarily the detailed composition of lipid A, drastically influences its biological activity, ranging from strong activation of the innate immune system in the case of agonistic LPS to a complete block of immune responses in the case of antagonists.[10,12] This different behavior is reflected in diverse binding modes of such lipid A structures to the MD-2/TLR4 receptor complex.[13]

Given the high amount of endogenous LPS in the gastrointestinal lumen, it is important for the mammalian host to avoid overstimulation of immune cells. Two crucial mechanisms contribute to achieving this aim: (1) a tight intestinal epithelial barrier protecting underlying immune cells in the lamina propria (LP) from luminal LPS, and (2) the induction of endotoxin tolerance.

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Endotoxin tolerance denotes a phenotype of antigen-presenting cells that are hyporesponsive toward a second LPS (endotoxin) stimulus through receptor desensitization as the result of a first LPS stimulus.[14,15] Although being a well-documented phenomenon, the molecular mechanisms underlying endotoxin tolerance still remain incompletely understood. Endotoxin tolerance has been observed both *in vitro* and *in vivo* in animal models as well as in humans.[16] The hyporesponsiveness of endotoxin-tolerant cells is reflected in their inability to express and secrete pro-inflammatory cytokines, such as interleukin (IL)-12, IL-6, and IL-1β, in response to a second LPS stimulus.[14] However, the detailed pattern of tolerant genes is cell type dependent.[16] Noteworthy, not only cytokine expression is drastically reduced during endotoxin tolerance but also surface expression of T cell-activating molecules, such as major histocompatibility complex (MHC) class II and CD40.[17,18]

Endotoxin tolerance is established while LPS-stimulated antigen-presenting cells undergo maturation characterized by increased expression of pro-inflammatory cytokines and MHC class II surface expression. So-called semi-mature cells, however, exhibit tolerance toward a secondary LPS stimulus, but they fail to express pro-inflammatory cytokines in response to the first, semi-maturation-inducing stimulus.[19] For example, the well-documented mouse gut commensal *Bacteroides vulgatus* mpk (BVMPK) induces semi-mature $CD11c^+$ cells in the intestinal LP, which is thought to contribute to immunomodulating properties of this bacterial strain, resulting in the prevention of colitis induction in several mouse models for experimental colitis.[20–22] BVMPK belongs to the bacterial phylum *Bacteroidetes*, which represents the most abundant gram-negative phylum in the mammalian gut.[23] Notably, not only BVMPK but also other *Bacteroides* species, such as *Bacteroides fragilis* (BF), provide beneficial and host immune system-modulating properties.[24] In this context, BF is of particular interest since its advantageous properties were demonstrated to be rooted in a structural component: the zwitterionic polysaccharide A (PSA).

Here we demonstrate that BVMPK drastically reduced already established intestinal inflammation in mice, thus leading to a complete healing of damaged intestinal tissue. Intriguingly, this effect was mirrored by using a structural component other than PSA: LPS. BVMPK LPS was found to exhibit weak agonistic activity for interaction with the host MD-2/TLR4 receptor complex, and the administration of purified BVMPK LPS re-established intestinal immune homeostasis in a mouse model for experimental colitis. The weak agonistic properties of this LPS are thought to be responsible for its active amelioration of inflammation through the induction of semi-mature LP $CD11c^+$ cells. Hence, weak agonistic LPS might be a novel and effective therapeutic agent for the treatment of intestinal inflammatory disorders such as IBD.

RESULTS

BVMPK Actively Ameliorates Established Inflammatory Reactions in a Mouse Model for Experimental Colitis, as Confirmed by Non-invasive *In Vivo* PET Imaging

We have already demonstrated that mouse gut-associated commensal BVMPK exhibits anti-inflammatory properties in various different mouse models for experimental colitis.[20–22] These anti-inflammatory properties appeared when BVMPK was administered before the onset of disease, resulting in the prevention of microbiota-mediated intestinal inflammation. This prompted us to verify whether this commensal was also capable of reducing inflammatory reactions in an already inflamed environment. Since we aimed to induce long-term chronic inflammatory conditions, we chose a T cell transplantation model[25] using *Rag1*-deficient mice, which fail to express functional T and B cells.[26] Upon transplantation of naive $CD4^+$ T cells, these $Rag1^{-/-}$ mice develop a chronic form of intestinal inflammation, which is highly dependent on the microbiota composition.[27]

Therefore, we transplanted $Rag1^{-/-}$ mice harboring a highly dysbiotic microbiota (DYSM)[7] with 5×10^5 naive wild-type (WT) $CD4^+CD62L^+CD45Rb^{hi}$ T cells. The composition of the microbiota is described in the Materials and Methods. At 4 weeks after T cell transplantation, mice were administered 5×10^8 viable BVMPK/mL drinking water until the end of the experiment. BVMPK-containing drinking water was renewed every 2 days, and anaerobic BVMPK remained viable under these conditions due to its aerotolerant properties (data not shown). Untreated $Rag1^{-/-}$ mice were used as negative controls, and T cell-transplanted mice that were not administered BVMPK were used as positive controls (Figure 1A).

Since we aimed to real-time monitor the progression of intestinal inflammation in each individual, we injected all mice with [^{18}F]-fluorodeoxyglucose ([^{18}F]FDG), and we performed non-invasive *in vivo* positron emission tomography (PET). [^{18}F]FDG is suitable for the detection of ongoing inflammatory processes in living animals, since sites of inflammation are depicted as regions with higher [^{18}F]FDG uptake in PET scans compared to non-inflamed surrounding environments.[28] However, some organs such as heart and bladder generally provide a basic tracer uptake signal independent of their inflammatory status. We monitored the colonic [^{18}F]FDG uptake over time from all mice used for this experiment. Therefore, each mouse was injected with [^{18}F]FDG, and PET imaging was performed at the day of T cell transplantation (week 0) as well as 4, 6, and 8 weeks after T cell transplantation (Figure 1B). The obtained data were corrected for decay, due to the half-life time of [^{18}F], and normalized to the injected activity per animal.

As demonstrated in the upper panel of Figure 1B, non-transplanted $Rag1^{-/-}$ mice provided low [^{18}F]FDG uptake during the whole observation period, indicating no ongoing inflammatory processes in the colon. In line with previous publications,[7,22] $Rag1^{-/-}$ mice harboring a DYSM and transplanted with naive T cells exhibited severe colonic inflammation, as illustrated by a high colonic [^{18}F]FDG uptake at weeks 4 and 6 (Figure 1B, middle panel). Since the inflammation in these animals exceeded an ethically justifiable level, these animals had to be sacrificed at week 6. As a third group, T cell-transplanted $Rag1^{-/-}$ mice, displaying severe signs of intestinal inflammation at week 4 (Figure 1B, lower panel), were administered viable BVMPK via drinking water from week 4 to week 8 after T cell transplantation. In these mice, we observed a considerable reduction in colonic [^{18}F]

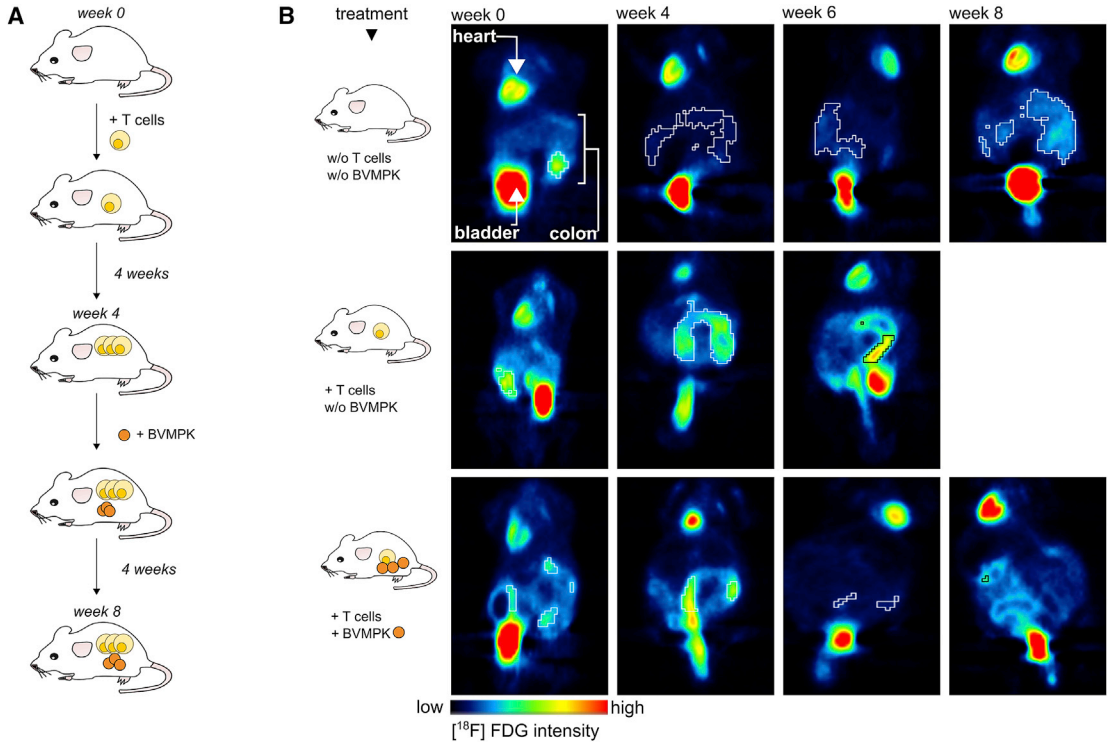

Figure 1. Administration of Live *B. vulgatus* mpk Reduces Intestinal Inflammation in *Rag1*$^{-/-}$ Mice with Established Colonic Inflammation, as Confirmed by Non-invasive *In Vivo* PET Imaging

(A) Experimental setup: *Rag1*$^{-/-}$ mice harboring a highly dysbiotic microbiota were transplanted with CD4^{+} T cells to induce intestinal inflammation as described. 4 weeks after T cell transplantation, mice started receiving live *B. vulgatus* mpk (BVMPK) in the drinking water at a concentration of 5×10^{8} mL^{-1}. BVMPK treatment was continued for 4 more weeks. 8 weeks after T cell transplantation, mice were sacrificed and analyzed. As controls, one group was transplanted without the following BVMPK administration and one group was not T cell transplanted. (B) High-resolution non-invasive small animal *in vivo* PET imaging. 8.3 ± 1.3 MBq [^{18}F]FDG was injected into the tail vein of each mouse. Static PET scans were performed at the day of T cell transplantation and repeated 4, 6, and 8 weeks after T cell transplantation. Data were corrected for decay and normalized to the injected activity. PET images of one representative individual per group are depicted (n = 8–10 mice per group). Organs providing high-intensity signals (heart, bladder, and inflamed colon) are labeled in the upper left panel.

FDG uptake already after 2 weeks. Another PET scan at week 8 revealed comparably low [^{18}F]FDG uptake, indicating that the inflammatory processes, visible at week 4, were cleared by BVMPK administration. The PET results were confirmed by a reduced histological score and a reduced IL-17 expression in BVMPK-fed T cell-transplaned *Rag1*$^{-/-}$ mice, as compared to only T cell-transplaned *Rag1*$^{-/-}$ mice (Figures 4B–4D).

This clearly demonstrated that the anti-inflammatory properties of the commensal BVMPK did not only prevent the induction of inflammation in T cell-transplanted *Rag1*$^{-/-}$ mice, as published previously,[22] but also actively ameliorated already established colonic inflammation, alleviating symptoms of colitis in this mouse model.

Induction of Hyporesponsive CD11c^{+} Cells Is a Common Feature of Various *Bacteroides* Species

We have already demonstrated that BVMPK-mediated suppression of intestinal inflammation strongly correlates with the induction of a hyporesponsive or so-called semi-mature phenotype of LP CD11c^{+} cells.[7,22] We assumed that such hyporesponsive LP CD11c^{+} cells were responsible for the observed inflammation-preventing and -reducing effects in *in vivo* models for experimental colitis. Since *Bacteroidetes* represents the major gram-negative phylum in the human intestine,[29,30] we aimed to examine the semi-maturation-inducing properties of *Bacteroides* species other than BVMPK, namely, *B. dorei* (BD), *B. vulgatus* ATCC8482 (BV8482), BF, and *B. thetaiotaomicron* (BTIO).

It was already demonstrated that BVMPK induced semi-maturation *in vitro* in bone marrow-derived dendritic cells (BMDCs). This phenotype is characterized by low but measurable secretion of IL-6 with the simultaneous absence of secretion of other pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-12, and IL-1β, as well as the absence of anti-inflammatory IL-10.[22,31] Furthermore, semi-mature BMDCs (smBMDCs) only provide slightly increased surface expression of MHC class II, CD40, CD80, and CD86.[22,31] In general, LP CD11c^{+} cells strongly resemble BMDCs, as shown by comparable expressions of CD45, CD11b, and CD103 while being CD3neg, Ly6Gneg, Ly6Gneg, CD45Rneg, and CD64neg (Figure S1). Therefore, we used BMDCs to assess whether *Bacteroides* strains

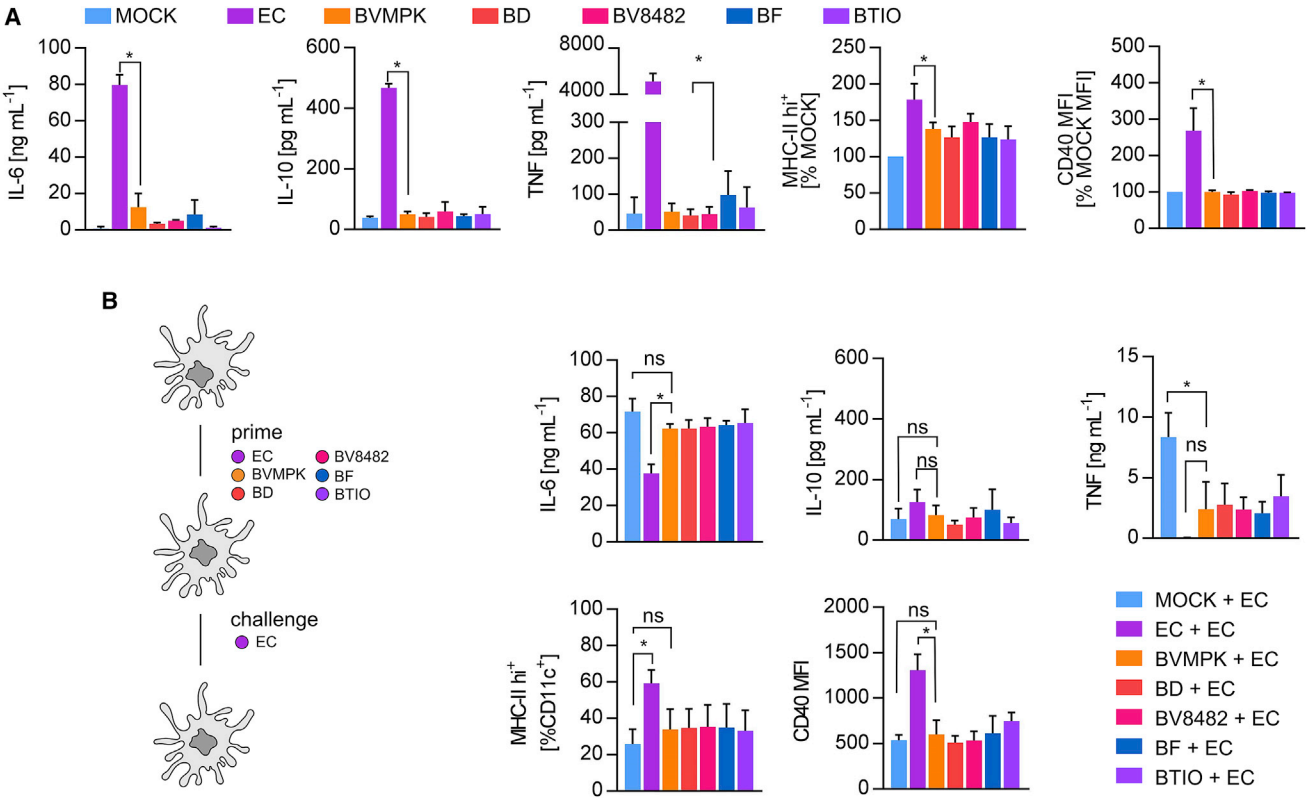

Figure 2. Induction of Hyporesponsive CD11c$^+$ Cells Is a Common Feature of Various *Bacteroides* Species

(A) Stimulation of CD11c$^+$ bone marrow-derived dendritic cells (BMDCs) generated from WT C57BL/6 mice (n = 3 mice) with PBS (mock), *E. coli* mpk (EC), *B. vulgatus mpk* (BVMPK), *B. dorei* (BD), *B. vulgatus* ATCC8482 (BV8482), *B. fragilis* (BF), and *B. thetaiotaomicron* (BTIO) for 16 h at an MOI of 1. Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and CD40 were detected by flow cytometry, and the population of MHC class II hi$^+$ cells and CD40 MFI, respectively, were normalized to the mock control of BMDCs generated from the same individual. (B) WT BMDCs (n = 3 mice) were stimulated with PBS (mock), EC, BVMPK, BD, BV8482, BF, and BTIO at an MOI of 1 for 24 h (prime). Cell culture medium was removed and exchanged for fresh medium before stimulation (challenge) with EC for an additional 16 h. Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and CD40 were detected by flow cytometry. Statistical analysis was performed using one-way ANOVA. p values <0.05 were considered to be statistically significant and are indicated with an asterisk (*). Columns and error bars represent mean ± SD.

other than BVMPK induce the effects of hyporesponsiveness in CD11c$^+$ cells.

BMDCs were generated from the bone marrow of WT C57BL/6N mice, and they were stimulated for 16 h with PBS (mock) as a negative control as well as with BVMPK, BD, BF, BV8482, and BTIO. *E. coli* mpk (EC) was used as a positive control to induce fully mature BMDCs.[22] All bacteria were added to BMDCs at an MOI of 1. As demonstrated by the low secretion of IL-6, the absence of IL-10 and TNF, as well as by only slightly increased MHC class II and CD40 surface expressions, all tested *Bacteroides* strains induced comparable effects in BMDCs, which were comparable to BVMPK-induced effects, therefore suggesting the induction of semi-maturation by all tested *Bacteroides* strains (Figures 2A and S2). As expected, EC stimulation led to the strong secretions of IL-6, IL-10, and TNF as well as increased MHC class II and CD40 surface levels.

A characteristic feature of BVMPK-induced semi-mature (sm) CD11c$^+$ cells is hyporesponsiveness toward further bacterial stimuli in terms of surface expressions of MHC class II, CD40, CD80, and CD86 as well as secretions of cytokines, such as TNF, but not IL-6. Therefore, we primed BMDCs with BVMPK, BD, BV8482, BF, BTIO, or EC for 24 h and challenged them with EC. Importantly, medium was changed between prime and challenge to determine whether primed cells were still capable of secreting pro-inflammatory cytokines in response to EC challenge. As seen in Figure 2B, IL-6 secretion was not reduced in *Bacteroides*-primed and EC-challenged BMDCs compared to unprimed EC-challenged BMDCs. IL-10 secretion was generally low among all different stimulus combinations. However, priming of BMDCs with all tested *Bacteroides* strains resulted in the tolerance of TNF expression after EC challenge in contrast to unprimed BMDCs. In line with this, priming of BMDCs with all tested *Bacteroides* strains resulted in (significantly) lower MHC class II and CD40 surface expressions after EC challenge compared to EC-primed BMDCs. However, these results point at the induction of semi-maturation and, therefore, tolerance toward TNF and surface T cell-activating surface molecule expression by all tested *Bacteroides* species. Thus, the induction of smBMDCs is not a unique property of BVMPK.

Table 1. Comparison of the Lipid A Core Synthesis Genes between BVMPK and BV8482, BD, and BTIO

BVMPK	BV8482		BD		BTIO	
ID	ID	SIM (%)	ID	SIM (%)	ID	SIM (%)
BvMPK_3283	BVU_0099	100	HMPREF1064_02220	99	BT4205	82
BvMPK_3832	BVU_0098	99	HMPREF1064_02221	99	BT4206	86
BvMPK_3821	BVU_0097	99	HMPREF1064_02222	99	BT4207	69
BvMPK_4264	BVU_0525	100	HMPREF1064_04085	99	BT3697	84
BvMPK_1137	BVU_1917	99	HMPREF1064_04942	97	BT4004	74
BvMPK_0444	BVU_1603	99	HMPREF1064_03036	96	BT1880	100
BvMPK_1465	BVU_1476	99	HMPREF1064_03097	98	BT2747	66
BvMPK_0774	BVU_1062	98	HMPREF1064_01726	97	BT2152	34
BvMPK_3353	BVU_3834	99	HMPREF1064_04014	96	not identified	
BvMPK_0983	BVU_1238	99	HMPREF1064_01498	93	not identified	
BvMPK_2934	BVU_3293	99	HMPREF1064_03350	98	BT1854	48

Genetic similarities (SIMs) were calculated by alignment of the genes from BV8482, BD, and BTIO compared to BVMPK. IDs refer to the respective gene identifiers.

Bacteroides Species Share a Conserved Lipid A Synthesis Core

Since stimulation with heat-killed BVMPK, BD, BF, BV8482, and BTIO also resulted in BMDC semi-maturation as induced by viable bacteria (see also Figure S3), we supposed the crucial semi-maturation-inducing bacterial factor to be a structural component that is shared by all these strains. *Bacteroides* belong to gram-negative bacteria and harbor LPS in the outer membrane of the bacterial cell wall. LPS is known to be one of the most potent surface molecules and dendritic cell (DC) maturation-inducing components of gram-negative bacteria. It consists of lipid A, a core oligosaccharide, and an O-antigen composed of polysaccharides of various lengths. Jacobson et al.[32] have recently reported on the differences in the poly- and oligosaccharide portions among BF, BV8482, BD, and BTIO. Since all these strains induced semi-maturation in our experiments (Figure 2), we hypothesized that the induction of semi-maturation occurs independently of LPS carbohydrate moieties. Therefore, we focused on the lipid A part. Lipid A of BD and BF was reported to be similar in structure and composition, being mono-phosphorylated and harboring only 5 acyl chains.[33,34]

To identify candidate genes for *Bacteroides* lipid A biosynthesis, we performed BLASTP searches against genomes of BV8482, BD, and BTIO using BVMPK genes as reference. We found lipid A biosynthesis genes of all investigated *Bacteroides* spp. to be homologous, since, with some exceptions for BTIO, the similarities among the sequences exceeded 90% (Table 1). In the next step, we compared the biosynthesis genes for BVMPK lipid A with the Raetz pathway of EC (Table 2). We could clearly show that *Bacteroides* lipid A synthesis genes differ significantly from the EC lipid A synthesis genes. The similarities between the genes were only between 25% and 42%, and the EC genome was found to lack the BVMPK_0774 and BVMPK_3353 (Table 2). These differences probably result in different lipid A compositions. The observed gene homology among the lipid A cores of BVMPK, BD, BTIO, and BV8482 is in line with our hypothesis that immunogenic effects on dendritic cells by all tested *Bacteroides* spp. are mediated by their typical LPS. Correspondingly, EC, which produces a different type of LPS, induced clearly different effects. Overall, this suggests that the structure of *Bacteroides* spp. LPS is a crucial determinant for the induction of semi-maturation in $CD11c^+$ cells. Work is ongoing to establish the chemical structure of LPS from BVMPK (LPSBV) and its structure-activity relationship.

Isolated LPS of BVMPK Induces Hyporesponsive Semi-mature $CD11c^+$ Cells

To verify the hypothesis that *Bacteroides* spp. LPS is crucial for the induction of hyporesponsive $CD11c^+$ cells, we isolated LPSBV as well as from EC (LPSEC), which contains a strongly agonistic *bis*-phosphorylated and hexaacylated lipid A. We generated BMDCs from WT C57BL/6 mice, and we stimulated these cells with either viable BVMPK or EC at an MOI of 1. Additionally, BMDCs were stimulated with purified LPSBV or LPSEC, both at concentrations of 50 $ng^{-1}/10^6$ BMDCs for a total of 16 h. As shown in Figure 3A, stimulation of BMDCs with LPS resulted in the same BMDC phenotype as stimulation with the respective bacteria from which the LPS was isolated. Both, BVMPK and LPSBV stimulation led to low expressions of MHC class II, CD40, CD80, and CD86 (Figure 3A) as well as to a low secretion of IL-6 and an absence of TNF (Figure 3A). On the contrary, stimulation of BMDCs with either EC or LPSEC led to significant increases in the surface expressions of MHC class II and T cell co-stimulatory molecules CD40, CD80, and CD86 as well as to increased secretions of IL-6 and TNF (Figure 3A).

To assess hyporesponsiveness toward a secondary stimulus, we stimulated BMDCs for 24 h with PBS (mock), BVMPK, LPSBV, EC, and LPSEC followed by a challenge with either EC or PBS as a negative control. Importantly, medium was changed between prime and challenge to determine whether primed cells were still capable of secreting pro-inflammatory cytokines in response to EC challenge. To determine the change in the surface expressions of T cell co-stimulatory molecules in response to EC challenge, we determined the proportion

Table 2. Comparison of the Lipid A Core Synthesis Genes between BVMPK and EC MG1655

BVMPK	EC MG1655	
ID	ID	SIM
BvMPK_3283	944849	42
BvMPK_3832	944816	36
BvMPK_3821	944882	33
BvMPK_4264	949053	27
BvMPK_1137	944838	31
BvMPK_0444	945526	25
BvMPK_1465	949048	26
BvMPK_0774	not identified	
BvMPK_3353	not identified	
BvMPK_0983	945863	35
BvMPK_2934	945450	27

Genetic similarities (SIMs) were calculated by alignment of the genes from *Escherchia coli* K12 MG1655 (EC MG1655) compared to BVMPK. IDs refer to the respective gene identifiers.

of MHC class IIhi$^+$, CD40$^+$, CD80$^+$, and CD86$^+$ cells in primed and EC-challenged BMDCs, and we compared them with PBS-challenged controls that were primed with the same stimulus. Thus, high bars indicate high responsiveness and low bars indicate tolerance (Figure 3B). As demonstrated in Figure 3B, both, BVMPK- and LPSBV-primed EC-challenged BMDCs provided significantly lower secretion of TNF. Furthermore, increases in CD80 and CD86 surface expressions in LPSBV-primed and EC-challenged BMDCs were significantly lower compared to PBS-primed and EC-challenged BMDCs, indicating the induction of hyporesponsiveness of LPSBV-primed cells concerning the surface expressions of T cell-activating molecules. As expected, priming with EC and LPSEC leads to full DC maturation.

To verify that LPSBV- and BVMPK-induced semi-maturating effects on BMDCs are rooted in LPS-dependent TLR4 signaling, we pre-incubated BMDCs for 1 h with the competitive TLR4 antagonist TAK242 (Figure S4) before stimulation with LPSBV, BVMPK, and EC for 16 h (Figure 3C). TAK242 pre-incubation abolished cytokine secretion and modulation of MHC class II and CD40 surface expressions for all the used stimuli. Therefore, we concluded that (1) BVMPK induces BMDC semi-maturation mainly via its LPS, and (2) purified LPSBV is sufficient to induce hyporesponsive semi-mature BMDCs.

TAK242 is described to be a selective TLR4 antagonist,[35,36] and our control experiments confirmed that TAK242 led to an inhibition of TLR4, but not TLR2, signaling. However, we cannot exclude that TAK242 may also influence other signaling pathways.

LPS-mediated induction of TLR4 signaling results in the activation of nuclear factor κB (NF-κB) and, therefore, in the expression of pro- and anti-inflammatory cytokines. Since BVMPK and EC as well as LPSBV induce a different cytokine secretion pattern via TLR4 signaling, we compared the NF-κB-activating potential of BVMPK and EC, as well as of LPSBV and LPSEC. For this purpose, we used HEK cells expressing the mouse MD-2/TLR4 receptor complex (mTLR4-HEK) (Figure 3D), and we stimulated them for 16 h. The resulting IL-8 secretion was detected as an indirect measure of MD-2/TLR4-mediated NF-κB activation, since IL-8 secretion is a direct consequence of TLR4-mediated NF-κB activation.[37] Stimulation of mTLR4-HEK cells with either viable BVMPK or EC at an MOI of 1 resulted in significantly higher IL-8 secretion from EC-stimulated cells compared to BVMPK-stimulated cells (Figure 3D, upper panel). Stimulation of mTLR4-HEK cells with isolated LPSBV or LPSEC resulted in a concentration-dependent increase in IL-8 secretion when LPSBV was used. LPSEC stimulation resulted in saturated IL-8 secretion, even at concentrations as low as 1 ng mL^{-1} (Figure 3D, lower panel). The increased IL-8 secretion as a result of NF-κB activation[37] indicated significantly stronger activation of LPSEC compared to LPSBV in mTLR4-HEK cells. Additionally, we measured the phosphorylation of S534 of the NF-κB subunit p65 (p-p65) via flow cytometry as a measure of NF-κB transactivation activity[38,39] in mouse BMDCs, in response to either LPSBV or viable BVMPK and EC, respectively. Stimulation with BVMPK and LPSBV of BMDCs for 30 min resulted in a significantly lower p-p65 induction compared to EC stimulation (Figure 3E).

Furthermore, we determined intracellular aldehyde dehydrogenase (ALDH) activity in mouse BMDCs stimulated with viable BVMPK or EC for 16 h (Figures 3F and S5). ALDH triggers the induction of inflammation-ameliorating Foxp3$^+$ regulatory T cells (Tregs) by metabolizing vitamin A-derived retinol into retinoic acid (RA).[40] RA has been shown to be a crucial mediator for the induction of Tregs, and ALDH$^+$ intestinal dendritic cells are considered to be important mediators for immune homeostasis.[19,41,42] BVMPK-stimulated BMDCs showed a significantly higher ALDH activity compared to EC-stimulated BMDCs, indicating a stronger Treg induction potential.

LPS is known to be a strong agonist for the MD-2/TLR4 receptor complex. However, it was reported that certain LPS structures also induce TLR2-mediated signaling, i.e., *Helicobacter pylori* LPS,[43] while the TLR2-activating capacity of *Porphyromonas gingivalis* LPS remains controversial and is probably an experimental artifact due to a lipoprotein contamination.[44] Therefore, these reports raise the question for a contribution of TLR2 signaling to the LPSBV-mediated immunogenic effects on CD11c$^+$ cells as well as for a potential contamination of the used LPSBV preparations, which might, in consequence, induce TLR2 receptor activation. Concerning potential and yet unrecognized contaminations, not only lipoproteins but also the presence of capsular polysaccharides has to be considered. During the purification process, an additional ultracentrifugation step was performed to eliminate the presence of capsular polysaccharides from the LPSBV preparations. Nevertheless, we checked whether solubilized LPSBV preparations induce TLR2 signaling, since both

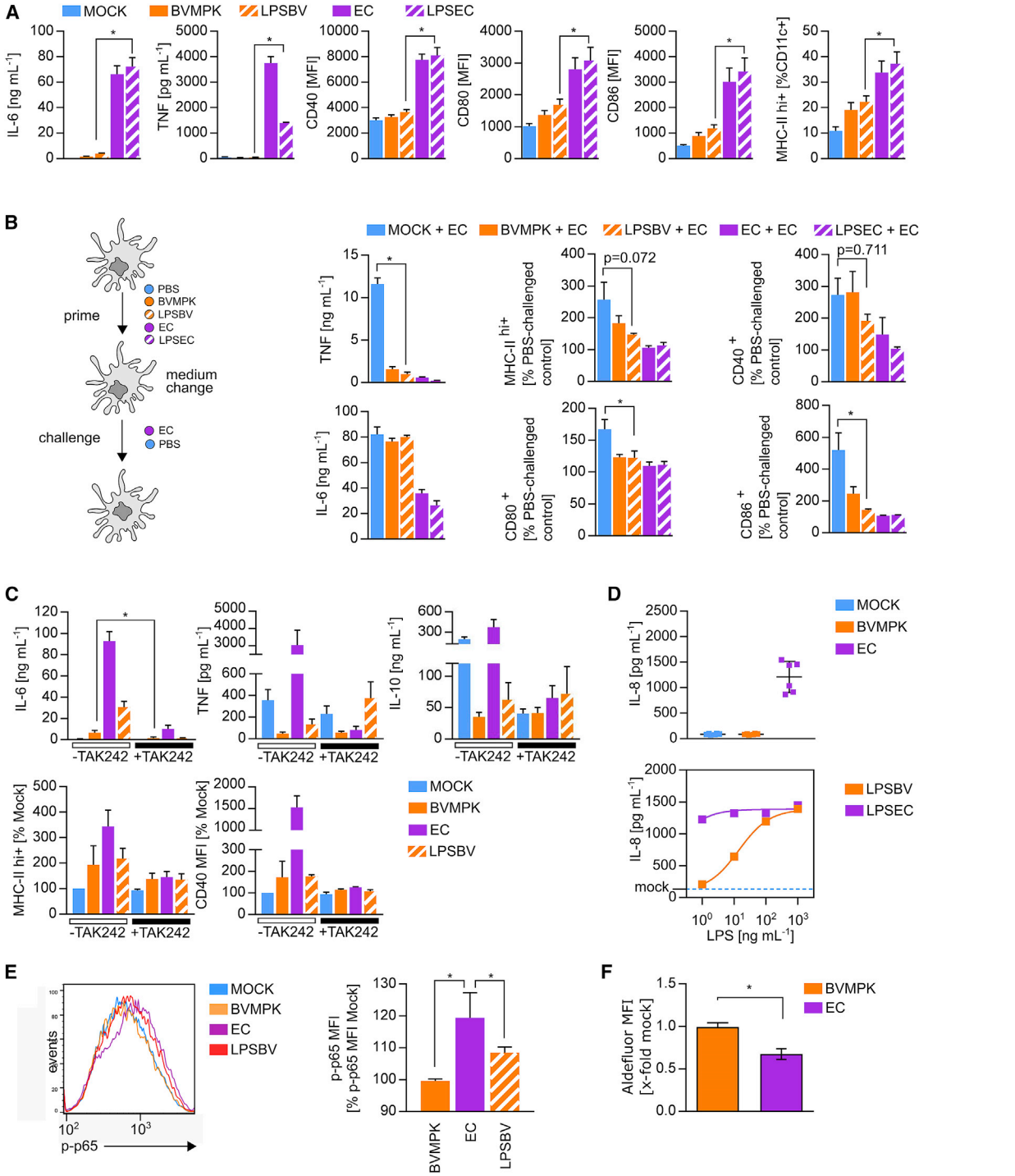

Figure 3. Isolated LPS of BVMPK Induces Hyporesponsive Semi-mature CD11c$^+$ Cells

(A) Stimulation of CD11c$^+$ bone marrow-derived dendritic cells (BMDCs) generated from WT C57BL/6 mice (n = 4 mice) with PBS (mock), *E. coli* mpk (EC), *B. vulgatus mpk* (BVMPK), *E. coli* mpk LPS (LPSEC), and *B. vulgatus* mpk LPS (LPSBV). Cytokine secretion was detected by ELISA. Surface expressions of CD40, CD80, CD86, and MHC class II were detected by flow cytometry. (B) WT BMDCs (n = 4 mice) were stimulated with PBS (mock), EC, LPSEC, BVMPK, and LPSBV for 24 h (prime). Cell culture medium was removed and exchanged for fresh medium before stimulation (challenge) with EC for an additional 16 h. Cytokine secretion was detected by ELISA. Surface expressions of MHC class II, CD40, CD80, and CD86 were detected by flow cytometry, and the population of MHC class II hi$^+$ cells and CD40 MFI, CD80 MFI, and CD86 MFI were normalized to the PBS-challenged control of BMDCs generated from the same individual with the same priming stimulus. (C) Stimulation of CD11c$^+$ bone marrow-derived

(legend continued on next page)

potential contaminants, capsular polysaccharides[45–48] and lipoproteins[49–51] from gram-negative bacteria, would result in TLR2 receptor activation (Figure S6). We detected minor differences in IL-6 secretion of LPSBV-stimulated WT BMDCs compared to TLR2-deficient BMDCs, indicating a slight activation of TLR2 by LPSBV (Figure S6A). However, using *TLR2*$^{-/-}$ BMDCs, we could exclude the contribution of TLR2-mediated signaling to the induction of hyporesponsive semi-mature BMDCs (Figure S6B). Therefore, we conclude that LPSBV-induced MD-2/TLR4 receptor signaling, but not TLR2 signaling, is crucial for the induction of BMDC semi-maturation.

Taken together, these results indicate a significantly weaker MD-2/TLR4 receptor activation of LPSBV compared to LPSEC, resulting in reduced NF-κB transactivation capacity. However, and in contrast to being a MD-2/TLR4 antagonist, LPSBV actively induced hyporesponsive semi-maturation in BMDCs without inducing the expression of pro-inflammatory cytokines. Thus, we suppose LPSBV to be rather weak agonistic than antagonistic.

Administration of Purified LPSBV Reduces Established Intestinal Inflammation in a Mouse Model of Experimental Colitis

Since (1) weak agonistic LPSBV provided the same semi-maturation-inducing capacities as viable BVMPK in BMDCs and (2) BVMPK-mediated prevention of intestinal inflammation in *Rag1*$^{-/-}$ mice correlated with the induction of semi-mature LP CD11c^{+} cells, we were interested to know if the administration of LPSBV resulted in the same inflammation-reducing effects in T cell-transplanted *Rag1*$^{-/-}$ mice. Therefore, we used *Rag1*$^{-/-}$ mice harboring a highly DYSM, and we induced colonic inflammation through the transplantation of 5×10^5 naive WT CD4^{+}CD62L^{+}CD45RB^{hi} T cells. At week 4, transplanted mice showed clear signs of colonic inflammation, such as bloody feces and diarrhea, which is in line with the PET imaging that was performed to monitor intestinal inflammation (Figure 1B). At this point, we started to administer viable BVMPK (5×10^8 bacteria/mL drinking water) as well as LPSBV at a concentration of 160 μg mL^{-1} in the drinking water for 4 additional weeks (Figure 4A). Mice with a C57BL/6 genetic background, such as *Rag1*$^{-/-}$, were reported to consume around 6 mL drinking water each day,[52] suggesting the daily uptake of LPSBV to be around 1 mg/mouse.

Figure 4B illustrates representative H&E-stained colonic sections from each group taken at the end of the experiment. As expected, non-treated T cell-transplanted animals showed severe signs of colonic inflammation (Figure 4B, left panel). However, the LPSBV- and BVMPK-treated animals exhibited significantly lower intestinal inflammation (Figure 4B, middle and right panels) compared to non-treated mice (Figure 4C). CD3^{+}CD4^{+} T cells isolated from the colonic LP (cLP) of live BVMPK-treated *Rag1*$^{-/-}$ mice expressed significantly lower amounts of IL-17 (Figure 4D), thus providing a clearly reduced Th17 response,[53] which crucially contributes to the induction of colitis in this mouse model.

Furthermore, qRT-PCRs from colonic scrapings revealed that live BVMPK-treated animals expressed significantly higher amounts of ALDH mRNA (Figure 4E), which supports our *in vitro* findings of a higher Treg-inducing potential of antigen-presenting cells that encountered BVMPK (Figure 3F).

LPSBV-treated T cell-transplanted *Rag1*$^{-/-}$ mice also provided significantly lower proportions of IL-17-expressing CD3^{+}CD4^{+} cLP T cells as well as higher *Aldh* mRNA expression in colonic scrapings. These data indicated that both live BVMPK and isolated LPSBV are equally able to ameliorate established inflammatory processes in the large intestine, by downregulating the Th17 immune response promoting cytokines and by favoring Treg-inducing environmental conditions.

Weak Agonistic LPSBV Is Not a Competitive Inhibitor of Strong Agonistic LPS

The results obtained from *in vivo* experiments using *Rag1*$^{-/-}$ mice raised the question of whether LPSBV acts as a competitive inhibitor at the MD-2/TLR4 receptor complex-binding site, therefore preventing agonistic LPS from binding and thus inducing complete CD11c^{+} cell maturation characterized by pro-inflammatory immune responses. First, we aimed to determine and compare binding constants of LPSBV and the prototype agonistic LPSEC to the mouse MD-2/TLR4 receptor complex. Therefore, we established an optical titration setting to trigger *quasi* dissociation constants (K_D) of both LPSBV and LPSEC, using biotinylated LPSBV (bioLPSBV). Prior to that, bioLPSBV was confirmed to provide the identical activation of the mouse MD-2/TLR4 receptor complex (Figure S7), therefore suggesting that biotinylation did not affect LPSBV-binding behavior.

We are aware of the fact that we could not determine real K_D values, since we did not know the exact molarity of the used LPS solutions. The assembly of amphiphilic LPS monomers into micelles, vesicles,

dendritic cells (BMDCs) generated from WT C57BL/6 mice (n = 5 mice) with PBS (mock), BVMPK, EC, and LPSBV either with or without pre-incubation with TLR4 receptor antagonist TAK242 at 1 h prior to bacterial stimulation. Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and CD40 were detected by flow cytometry, and the population of MHC class II hi^{+} cells and CD40 MFI, respectively, were normalized to the untreated mock control of BMDCs generated from the same individual. (D) Stimulation of mouse MD-2/TLR4 receptor complex expressing HEK cells (mTLR4-HEK) with PBS (mock), EC, and BVMPK at an MOI of 1 (n = 2 experimental replications with 3 technical replicates, upper panel) or LPSEC and LPSBV at various concentrations (n = 3 technical replicates, lower panel). The resulting IL-8 secretion as a measure of NF-κB activation was detected by ELISA, and it is indicated by a blue line for mock samples in the lower panel. (E) Stimulation of BMDCs (n = 4 mice) with PBS (mock), EC, BVMPK, and LPSBV for 30 min. The resulting phosphorylation of intracellular p65, phosphorylated at S534 (p-p65), was detected by flow cytometry. The resulting MFI was normalized to the mock control of BMDCs generated from the same individual. (F) Stimulation of WT BMDCs (n = 5 mice) with PBS (mock), BVMPK, and EC at an MOI of 1 for 16 h. ALDH levels in BMDCs were then detected using an Aldefluor ALDH activity assay. Statistical analysis was performed using one-way ANOVA (A, B, and E) or Student's t test (C and F). p values <0.05 were considered to be statistically significant and are indicated with an asterisk (*). Columns and error bars represent mean ± SD.

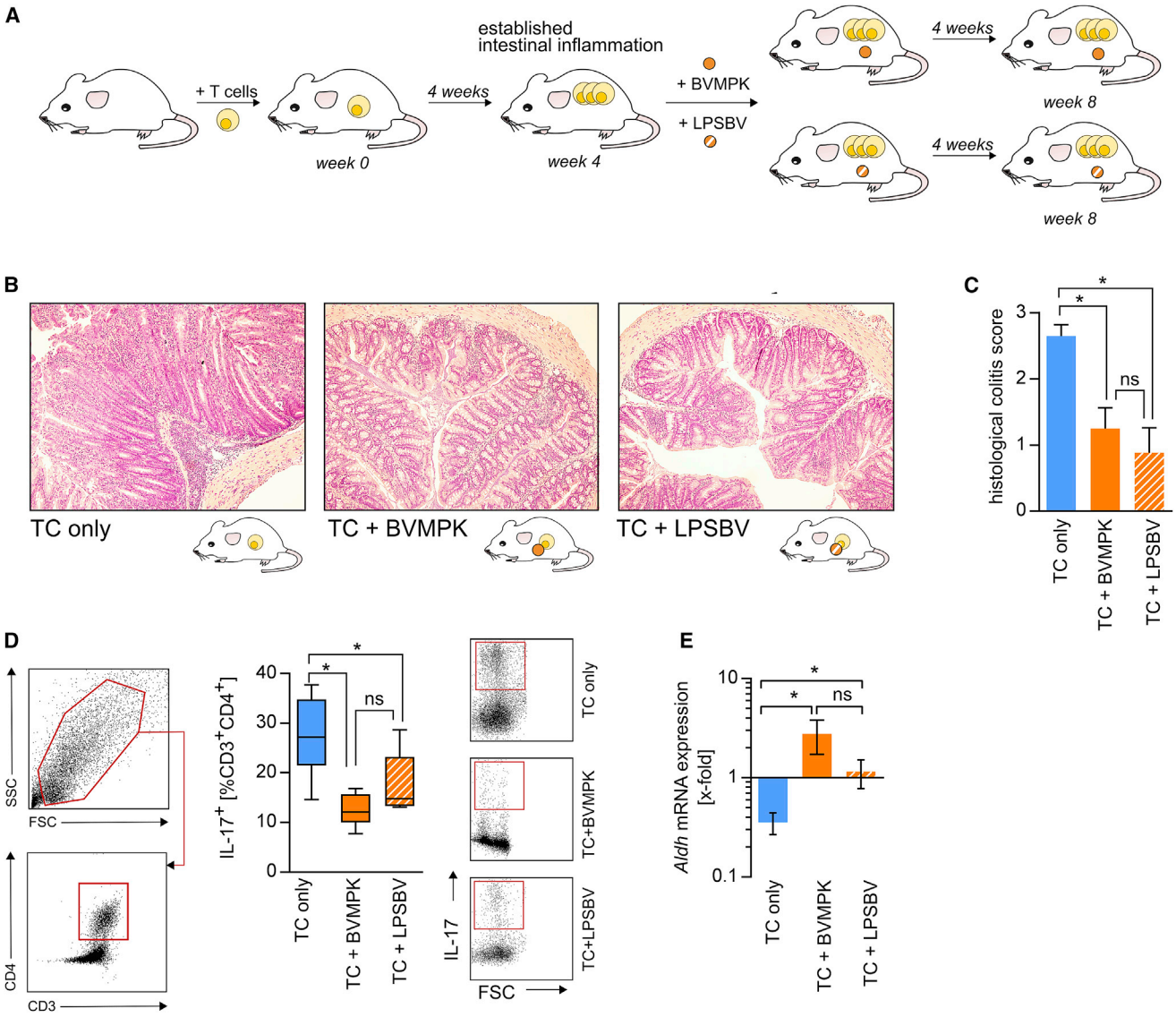

Figure 4. Isolated *B. vulgatus* mpk LPS Provides the Same Capability to Reduce Intestinal Inflammation in *Rag1*$^{-/-}$ Mice with Established Colonic Inflammation as Live *B. vulgatus* mpk

(A) Experimental setup: *Rag1*$^{-/-}$ mice harboring a highly dysbiotic microbiota were transplanted with naive CD4$^+$ T cells to induce intestinal inflammation as described. 4 weeks after T cell transplantation, mice were not treated (TC only, n = 5), treated with live *B. vulgatus* mpk (TC + BVMPK, n = 8) by administration of 5 × 10^8 bacteria mL^{-1} in the drinking water, or treated with isolated *B. vulgatus* mpk LPS (TC + LPSBV, n = 8) in the drinking water at a concentration of 160 μg mL^{-1}. Mice were sacrificed and analyzed 8 weeks after T cell transplantation. (B) Representative H&E-stained colonic sections at week 8. (C) Histological colitis score at week 8, ranging from 0 to a maximum of 3. (D) Left panel: gating strategy to determine the CD3$^+$CD4$^+$ T cell population in the colonic lamina propria (cLP) at week 8. Right panel: proportion of IL-17$^+$ cells among the population of cLP CD3$^+$CD4$^+$ T cells, determined by flow cytometry. (E) Relative *Aldh2* mRNA expression in colonic scrapings at week 8, determined by qRT-PCR. Data were normalized to *Aldh2* mRNA expression in colonic scrapings of non-inflamed non-T cell-transplanted *Rag1*$^{-/-}$ mice. Statistical analysis was performed using Kruskal-Wallis test (C) or one-way ANOVA (D and E). p values <0.05 were considered to be statistically significant and are indicated with an asterisk (*). Columns and error bars represent mean ± SD (C and E). Boxplots depict the mean as well as the 25th and 75th percentiles, and whiskers depict the highest and lowest values (D).

or even more complicated structures is highly dependent on the buffer and ionic strength, and it is, therefore, hardly predictable. This exacerbated the determination of the molarity of LPS monomers, which effectively have access to the receptor, rendering them active ligands thus contributing to K_D values. However, assuming that (1) bioLPSBV and LPSEC provide a comparable monomeric molecular weight, (2) bioLPSBV and LPSEC behave in a similar chemical manner under the experimental conditions, and (3) all experiments were carried out incubating both LPSs at the same time, we can speculate that a qualitative comparison using K_D values in the unit g L^{-1}

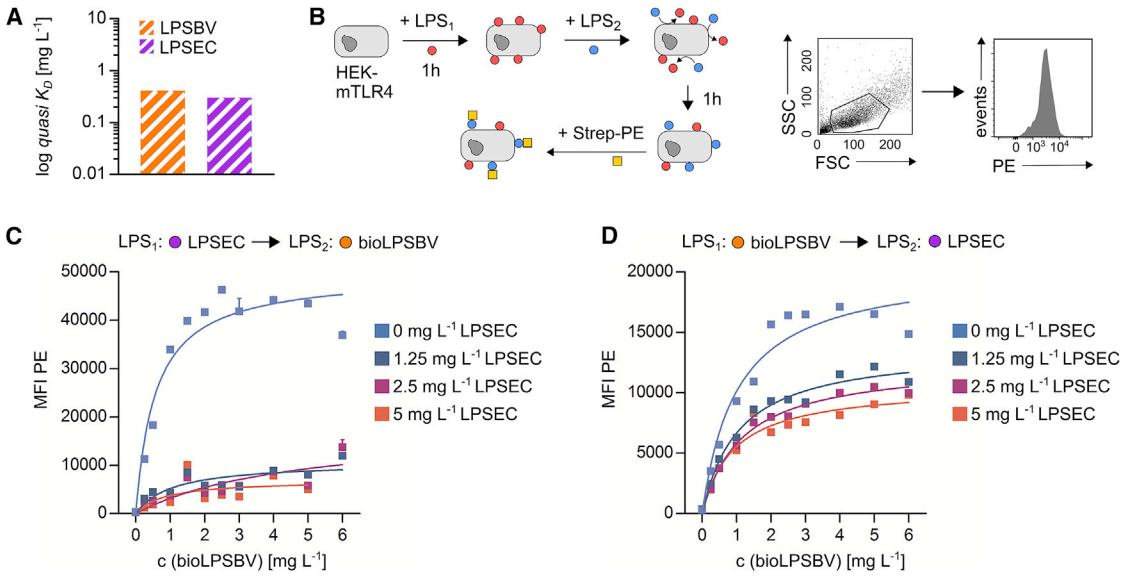

Figure 5. Weak Agonistic LPSBV Is Not a Competitive Inhibitor of Strong Agonistic LPS

(A) Determination of the dissociation constant (g L^{-1}, quasi K_D) of LPSBV and LPSEC using an optical titration-based approach. (B) Left panel: experimental setting. 1×10^5 HEK cells expressing murine CD14, MD-2, and TLR4 (mTLR4-HEK) were incubated with a certain concentration of either LPSEC ranging from 0 to 5 mg L^{-1} or biotinylated LPSBV (bioLPSBV) ranging from 0 to 6 mg L^{-1} for 1 h. After 1 h, the opposite LPS (either LPSBV or LPSEC) was added for an additional 1 h. PE-coupled streptavidin (Strep-PE) was added for 30 min, and the resulting PE fluorescence associated with mTLR4-HEK cells was detected by flow cytometry. Right panel: gating strategy to determine PE fluorescence of intact mTLR4-HEK cells is shown. (C) Binding curves of 4 distinct concentrations of LPSEC added first to mTLR4-HEK (n = 3 technical replicates) plotted against varying concentrations of subsequently added bioLPSBV. (D) Binding curves of 7 distinct concentrations of bioLPSBV added first to mTLR4-HEK (n = 3 technical replicates) plotted against varying concentrations of subsequently added LPSEC. Squares with error bars represent mean ± SD (C and D).

instead of mol L^{-1} is qualifiable for a comparison of their binding affinity. We finally determined quasi K_D to be 0.412 g L^{-1} for LPSBV and 0.304 g L^{-1} for LPSEC (Figure 5A, see also Figure S8 for more details on optical titration). These data allowed us to conclude that LPSBV and LPSEC provide similar binding affinity to the mouse MD-2/TLR4 receptor complex.

Next, we tested the capability of each of these two distinct LPSs to remove already bound LPS from the receptor complex. Thus, we incubated mTLR4-HEK cells with different concentrations of either bioLPSBV or LPSEC for 1 h, and, subsequently, we added the opposite LPS for 1 h and in several concentrations. Subsequent additional incubation with phycoerythrin (PE)-coupled streptavidin (Strep-PE) allowed for flow cytometry-based visualization of mTLR4-HEK cell-bound bioLPSBV (Figure 5B). Pre-incubation with LPSEC followed by subsequent incubation with bioLPSBV resulted in a low detected PE fluorescence, fairly independent of the employed LPSEC and bioLPSBV concentrations (Figure 5C). This indicated that, once the murine MD-2/TLR4 receptor complex was bound by LPSEC, bioLPSBV was not able to remove LPSEC from the receptor-binding site. Pre-incubation with LPSEC, even at low concentrations of 1.25 mg L^{-1}, resulted in a decrease of the PE signal to about 20% of the PE signal that arose when cells were not pre-incubated with LPSEC (Figure 5C). Contrariwise, pre-incubation of mTLR4-HEK cells with bioLPSBV followed by subsequent incubation with LPSEC resulted in a strong reduction of the PE signal, which is exclusively derived from bound bioLPSBV, of about 50%, even when low concentrations of LPSEC were added (Figure 5D). Since we assumed that the detected PE fluorescence was directly proportional to the amount of bound bioLPSBV, it can be stated that bioLPSBV was able to remove about 20% of already bound LPSEC (Figure 5C), while LPSEC was able to remove about 50% of already bound bioLPSBV (Figure 5D).

We have already demonstrated that LPSBV is able to induce tolerant semi-mature BMDCs *in vitro* (Figure 3) and reduce established intestinal inflammation in T cell-transplanted *Rag1*$^{-/-}$ mice harboring a complex microbiota (Figure 4). So far, all experiments investigating smBMDCs *in vitro* were performed in the absence of other LPS structures for the first 16 h of the semi-maturation process. Physiological conditions in the colonic lumen provide the presence of different commensal LPSs in large amounts at the same time. Therefore, we stimulated BMDCs with weak agonistic LPSBV and prototype agonistic LPSEC at the same time and at different concentrations for 16 h (Figure 6A) to investigate if LPSBV-induced semi-maturation even occurs in the presence of a strong MD-2/TLR4 receptor complex agonist. Thus, we checked for the secretion of pro-inflammatory cytokines and the surface expressions of MHC class II and TLR4.

We detected a concentration-dependent effect of LPSBV stimulation on BMDCs in the absence of LPSEC (light blue lines in Figure 6B). LPSBV concentrations of up to 100 ng mL^{-1} (which equals 50 ng/10^6 BMDCs) led to a semi-mature BMDC phenotype, as

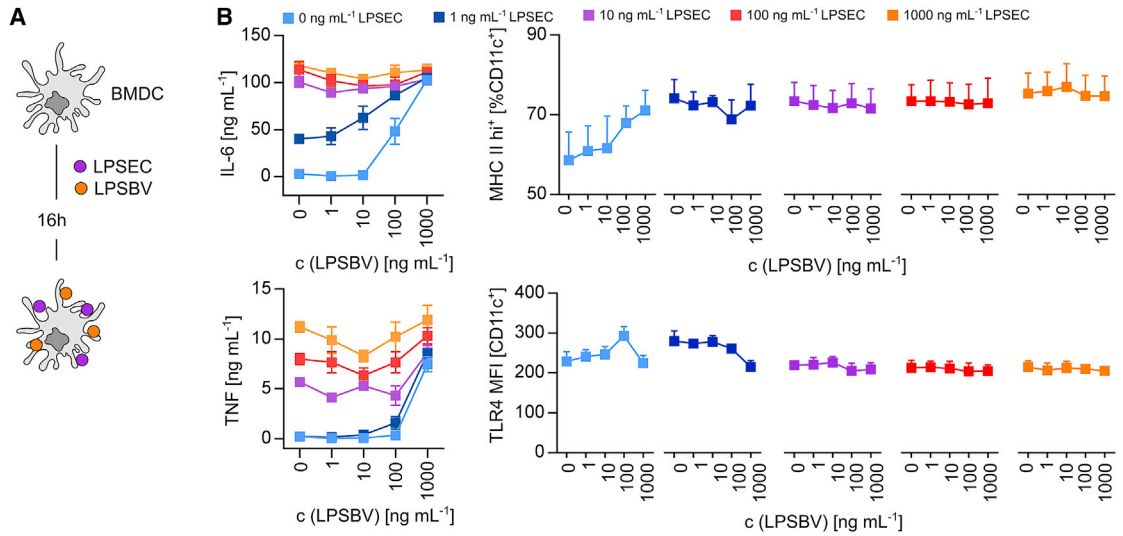

Figure 6. LPSEC-Induced BMDC Maturation Can Not Be Anticipated by the Simultaneous Presence of LPSBV *In Vitro*

(A) Experimental setting for (B): simultaneous stimulation of WT BMDCs (n = 3 mice) with LPSEC and LPSBV with varying concentrations of both LPSs. (B) Detection of cytokine secretion and surface expressions of MHC class II and TLR4 of the experimental approach depicted in (A). Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and TLR4 were detected by flow cytometry. Squares with error bars represent mean ± SD.

previously demonstrated in Figure 3. Using 1,000 ng mL^{-1} or 500 ng pure LPSBV/10^6 cells resulted in a strong activation of BMDCs, as indicated by high TNF, IL-6, and MHC class II surface expressions (Figure 6B). This underlined and confirmed the observation that LPSBV is not a MD-2/TLR4 receptor complex antagonist but rather a weak agonist, with the overall concentration determining its final endotoxicity. The addition of agonistic LPSEC to LPSBV-stimulated BMDCs led to strong increases in secretion rates of pro-inflammatory cytokines (Figure 6B) as well as in the expression of MHC class II (Figure 6B) for all the used LPSEC concentrations. Therefore, the simultaneous presence of LPSBV could not anticipate LPSEC-induced maturation effects. Surprisingly, TLR4 expression on the cell surface remained relatively constant among all differently stimulated BMDCs (Figure 6B), indicating that no significant TLR4 endocytosis is detectable upon binding of these two LPS structures to the MD-2/TLR4 receptor complex, which was in opposition to what was previously reported for other intestinal commensally derived LPSs.[54]

So far, these results indicated that the simultaneous encounter of BMDCs with weak agonistic LPSBV and strong agonistic LPSEC prevented LPSBV-induced semi-maturation. However, LPSBV administration into mice harboring a complex microbiota reduced intestinal inflammation. Since LPSBV-induced semi-maturation of $CD11c^+$ cells might account for the observed inflammation-reducing effects of LPSBV *in vivo*, we were interested to know how competition between LPSBV and endogenous LPS as well as other microbial components from a complex mouse microbiota affects the phenotype of $CD11c^+$ cells. Therefore, we generated BMDCs from WT C57BL/6 mice, and we stimulated them with isolated LPSBV as described before. Simultaneously, we added autoclaved feces that were taken from *Rag1*$^{-/-}$ mice 4 weeks after T cell transplantation, which provided severe intestinal inflammation (Figure 1B). These feces, therefore, represent a DYSM composition that arises during intestinal inflammation. The DYSM harbors various endogenous LPSs competing with LPSBV for binding to the MD-2/TLR4 receptor complex on LP $CD11c^+$ cells as well as various other immunomodulating components. Additionally, the DYSM represents the microbiota composition LPSBV faced when it was administered to inflamed *Rag1*$^{-/-}$ mice and exhibited inflammation-ameliorating properties. DYSM stimulation of BMDCs resulted in slight, but not statistically significant, increases in the secretions of pro-inflammatory cytokines and T cell co-stimulatory molecules (Figure 7A). This indicated that the DYSM impacted $CD11c^+$ cell maturation, albeit not as strongly as EC with agonistic LPS (Figure S9), at least at the chosen DYSM concentration. Interestingly, simultaneous stimulation of BMDCs with DYSM and LPSBV induced significantly stronger expressions of IL-6 and TNF as well as increased surface expressions of MHC class II and CD40 than either of the stimuli administered alone.

Since simultaneous stimulation of BMDCs with DYSM and LPSBV resulted in fully mature BMDCs, we pre-incubated BMDCs with LPSBV (prime) for 24 h before adding the DYSM (challenge). As seen in Figure 7B, pre-incubation of LPSBV resulted in an induction of tolerance of IL-6 and TNF expressions, preventing the secretion of pro-inflammatory cytokines in response to DYSM challenge. However, surface expressions of MHC class II and CD40 were not altered in LPSBV-primed and DYSM-challenged BMDCs compared to unprimed and DYSM-challenged BMDCs.

These experiments demonstrate that the simultaneous stimulation of $CD11c^+$ BMDCs cells with LPSBV and components of an endogenous DYSM did not lead to semi-maturation. Therefore, we concluded that

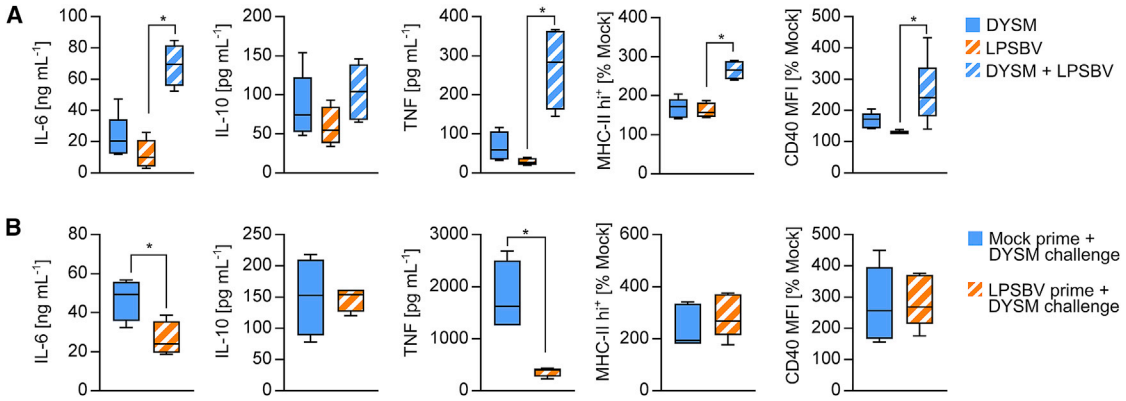

Figure 7. LPSBV-Mediated BMDC Tolerance Is Induced in the Absence of Agonistic LPS and Protects from Pro-inflammatory Response to a Dysbiotic Microbiota Composition

(A) Feces from *Rag1*$^{-/-}$ mice providing intestinal inflammation (DYSM) was autoclaved and prepared as described in the Materials and Methods. WT BMDCs (n = 4–5 mice) were stimulated with DYSM, LPSBV (100 ng mL^{-1}), or both simultaneously (DYSM + LPSBV). Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and CD40 were detected by flow cytometry and normalized to the mock control of BMDCs generated from the same individual. (B) WT BMDCs were stimulated (prime) with either PBS (mock) or LPSBV for 24 h. Medium was changed and cells were stimulated afterward (challenge) with DYSM. Cytokine secretion was detected by ELISA. Surface expressions of MHC class II and CD40 were detected by flow cytometry and normalized to an unchallenged mock control of BMDCs generated from the same individual. Statistical analysis was performed using one-way ANOVA (A) or Student's t test (B). p values <0.05 were considered to be statistically significant and are indicated with an asterisk (*). Boxplots depict the mean as well as the 25th and 75th percentiles, and whiskers depict the highest and lowest values.

LPSBV-mediated induction of smBDMCs required the absence of agonistic LPS for the initial phase of semi-maturation induction.

DISCUSSION

BVMPK belongs to the gram-negative bacterial phylum *Bacteroidetes*, representing one of the two most prominent phyla in the mammalian gut.[29,30] However, the proportion of *Bacteroidetes* in the intestinal microbiota is dependent on the inflammatory status of the gut. In ulcerative colitis patients, the proportion of *Bacteroides* spp. is markedly decreased,[55] supporting the idea of *Bacteroides* spp. being important beneficial players in the intestinal microbiota. Additionally, we have already shown that the administration of BVMPK prevents disease induction in different mouse models for experimental colitis,[20–22] mainly by the induction of hyporesponsive semi-mature CD11c^{+} cells (smDCs) in the cLP. These smDCs are responsible for the prevention of pro-inflammatory immune responses.[20–22,31] In this study, we report that the administration of BVMPK drastically reduces established and ongoing pathological inflammatory processes in the intestine of a mouse model for experimental colitis using *Rag1*$^{-/-}$ mice. These immune response-regulating properties were mediated by LPSBV, as the healing effects could be obtained using purified LPSBV only. We therefore propose that LPSBV might be a novel therapeutic agent for the treatment of chronic gut inflammatory disorders, by restoring physiological intestinal immune homeostasis.

BVMPK is not the only *Bacteroides* strain that exhibits such beneficial immunomodulatory properties. BF was reported to protect against intestinal inflammation in a mouse model for experimental colitis[56] as well as against CNS demyelination and inflammation during experimental autoimmune encephalomyelitis.[48] Interestingly, these properties were mediated by one of its capsular PSAs through interaction with host TLR2.[48,56,57] So far, we have no information on the chemical structure of BVMPK capsular polysaccharides, but the close relation between BF and BVMPK and the convincing immune system-regulating properties of BF PSA prompted us to check for a contribution of TLR2 signaling to the observed immunological effects in CD11c^{+} cells in response to LPSBV stimulation. This is of particular interest since such a TLR2-mediated signaling might be due to contamination of the used LPSBV preparations with capsular polysaccharides[45–48,56,57] and lipoproteins[49–51] or due to the LPS itself.[43,44,58] Although a slight TLR2 activation was detected, LPSBV preparations crucially mediated the induction of CD11c^{+} cell semi-maturation via the MD-2/TLR4 receptor complex.

In this context, we demonstrated that the immunomodulatory properties of LPSBV are clearly distinguishable from that of strong TLR4 agonists such as, e.g., *E. coli*-derived prototype LPS, or antagonists, which block any TLR4-mediated signaling. Since LPSBV did not induce the expression of pro-inflammatory cytokines but actively induced hyporesponsiveness toward subsequent LPS stimuli in CD11c^{+} cells, thereby fairly merging the properties of TLR4 antagonists and agonists, we propose LPSBV to be a weak agonist concerning its interaction with the MD-2/TLR4 receptor complex.

To elucidate how endogenous LPSs from a complex mouse microbiota affect the phenotype of CD11c^{+} cells, we autoclaved the feces of inflamed *Rag1*$^{-/-}$ mice (DYSM). Hence, the DYSM represents the endogenous LPS composition as well as other microbial compounds LPSBV faced when it was administered to inflamed *Rag1*$^{-/-}$ mice and exhibited inflammation-ameliorating properties. Interestingly, simultaneous stimulation of BMDCs with DYSM and LPSBV induced significantly stronger expressions of IL-6 and TNF as well

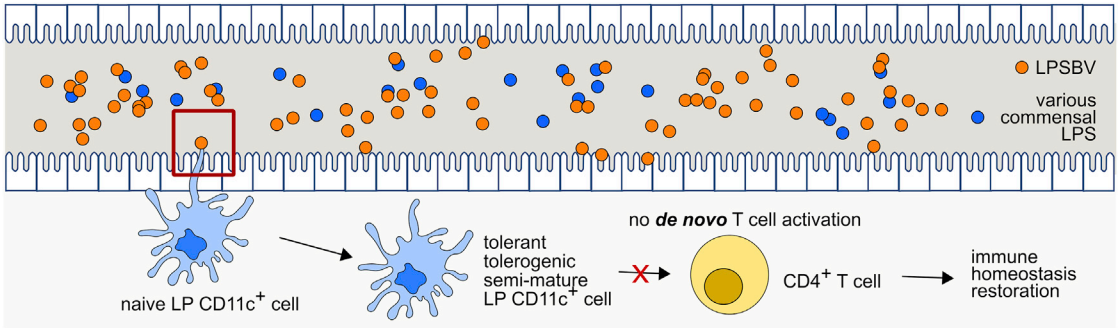

Figure 8. Proposed Mechanism of How Weakly Agonistic LPSBV Influences MD-2/TLR4 Receptor Complex Activation and Prevents the Initiation of a CD4$^+$ T Cell-Mediated Immune Response via Modulation of the Intestinal CD11c$^+$ Cells

Therapeutic administration of LPS from symbiotic *B. vulgatus* mpk ends up in large amounts in the intestine, exceeding the amount of present LPS from other intestinal commensal bacteria. Excess LPSBV primes naive CD11c$^+$ cells into a tolerant, tolerogenic, and semi-mature phenotype that fails to activate CD4$^+$ T cells. This prevention of a *de novo* activation of CD4$^+$ T cells leads to a phase-out of ongoing inflammatory processes, while *de novo* induction of an immune response is prevented. However, our data indicate that this only happens if LPSBV is the only TLR4 ligand that CD11c$^+$ cells encounter when they are still naive and immature. LPSBV needs a certain period of time to induce CD11c$^+$ cell tolerance. Simultaneous encounter with agonistic LPS does not lead to CD11c$^+$ cell tolerance, and it does not, therefore, promote abrogation of inflammatory processes.

as increased surface expressions of MHC class II and CD40 than either of the stimuli administered alone. This finding is most likely due to the fact that the induction of cytokines as well as the expression of cell surface markers follows a dose-response effect. It can be seen in Figure 6B that the stimulation of BMDCs with high concentrations of LPSBV results in the induction of TNF and IL-6 secretions. Adding LPSBV and the DYSM sample simultaneously results in the stimulation of the cells with high LPS levels, hence inducing the secretions of proinflammatory cytokines and expressions of BMDC activation surface markers. The anti-inflammatory potential of LPSBV is nicely demonstrated in Figure 7B where BMDCs were primed with either PBS (mock) or LPSBV and challenged with DYSM. In this experiment, it can be clearly seen that priming the cells with LPSBV led to reduced secretions of TNF and IL-6 upon a challenge with DYSM.

Referring to the therapeutic effects of LPSBV in efficiently reducing intestinal inflammation, we conclude that the concentration of LPSBV located in the intestinal lumen is a decisive factor and must exceed the number of endogenous agonistic LPSs in order to induce tolerant semi-mature CD11c$^+$ in the LP. Such exceeding LPSBV concentrations are thought to enhance the probability that intestinal DCs only encounter weak agonistic LPSBV by enhancing the ratio between weak agonistic to endogenous agonistic LPSs. This seems to be indispensable since we demonstrated that a simultaneous encounter with a strong agonistic LPS prevented LPSBV-induced DC semi-maturation and induced pro-inflammatory responses instead. We could further demonstrate that prototype agonistic LPSEC was able to remove already bound LPSBV from the MD-2/TLR4 receptor complex more efficiently than vice versa.

In a scenario of exceeding LPSBV concentrations in the gastrointestinal lumen, this effect is thought to be irrelevant. As demonstrated, the endotoxicity of LPSBV is dependent on its concentration, and a very high LPSBV concentration leads to complete DC maturation underlying its weak agonistic properties, clearly distinguishing it from an antagonist. Nevertheless, we demonstrated that the administration of a daily dose of about 1 mg LPSBV/mouse via the drinking water led to the observed inflammation-reducing effects in *Rag1*$^{-/-}$ mice with colitis, indicating that such a concentration is sufficient to exceed the amount of endogenous LPS in the gastrointestinal tract while simultaneously being in a semi-maturation-inducing concentration range. Once CD11c$^+$ cell semi-maturation is induced, this phenotype cannot be overcome, and it is, therefore, thought to result in the prevention of a *de novo* T cell activation in the intestine. Therefore, after a phase-out of ongoing inflammatory processes, newly induced semi-mature DCs through LPSBV administration would prevent a continuous T cell activation (Figure 8) and, therefore, promote healing of damaged colonic tissue.

In general, LPS is a potent MD-2/TLR4 receptor complex agonist leading to strong intracellular signaling in target cells, resulting in the transcription of genes associated with pro-inflammatory immune responses.[13] LPS-induced strength of this intracellular signaling is widely considered to be mostly mediated by its lipid A portion.[59,60]

Lipid A structures of various *Bacteroides* spp. were already reported to harbor 4–5 acyl chains and only 0–1 phosphate group.[8] Since we revealed strong genetic similarities between the lipid A synthesis core of BVMPK and other *Bacteroides* spp., we suggest LPSBV also to be hypo-acylated and hypo-phosphorylated compared to LPSEC. Hexa-acylated and *bis*-phosphorylated *E. coli* lipid A is considered to be the most potent activator of the MD-2/TLR4 receptor complex-mediated signaling, since it was demonstrated that five of the six acyl chains are buried inside the MD-2-binding cavity while the sixth acyl chain points out to the MD-2 surface-mediating hydrophobic interactions with the TLR4 ectodomain, which are necessary for TLR4 activation.[12,34] This might partly explain the lower endotoxicity of hypo-acylated lipid A structures lacking this sixth acyl chain and

the weak agonistic activity of hypo-acylated LPSBV. Furthermore, both 1- and 4'- phosphates on the lipid A diglucosamine backbone were demonstrated to be important moieties for MD-2/TLR4 receptor complex activation.[13] Since *Bacteroidales* possessed only one phosphate at position 1 of the reducing glucosamine,[8] this may also contribute to its weakly agonistic effects, as a missing 4'-phosphate was demonstrated to result in a 100-fold reduction in endotoxic activity.[61]

We suppose the weak agonistic features of LPSBV to be responsible for the observed healing effects in mice with intestinal inflammation. In this context, weak agonistic LPS is thought to induce a weaker, but still detectable, intracellular signaling and NF-κB activation, providing a basic anti-inflammatory intracellular transcription program without exceeding a pro-inflammatory threshold. Furthermore, LPSBV leads to the active induction of hyporesponsive $CD11c^+$ cells. These properties clearly distinguish LPSBV from strong agonistic LPSEC, which induces endotoxin tolerance but also strong pro-inflammatory signaling. However, weak agonistic LPS is also different from antagonistic LPS, which does not promote pro-inflammatory reactions but also does not promote tolerance induction in TLR4-expressing cells. The property of LPSBV of being an effective ligand for the MD-2/TLR4 receptor complex and, at the same time, a weak agonist must, of course, be attributed to its chemical structure. We think that this is a chemical paradigm of the commensal intestinal microbiota LPS, contributing to the adaption of microbes to the host. In line with this, other groups reported on the contribution of *Bacteroides* LPS to the preservation of intestinal homeostasis.[8] However, our study represents the first successful attempt to actively restore intestinal immune homeostasis in mice providing severe intestinal inflammation by using commensally derived LPS.

However, the immunogenic effects of weak agonistic LPS seem to be situation dependent. In another study, Vatanen et al.[33] revealed a higher incidence of type 1 diabetes (T1D) in children who were less exposed to strong agonistic LPS in early childhood, accompanied by higher proportions of microbes harboring hypo-acylated and hypo-phosphorylated LPS. In line with this, continuous intraperitoneal administration of *E. coli* LPS starting shortly after birth delayed the onset of T1D in non-obese diabetic (NOD)/Shilt mice while the administration of hypo-acylated and hypo-phosphorylated BD LPS failed to do so.[33] This study supported the hygiene hypothesis that assumes that early exposure to highly immunogenic microorganisms in early childhood benefits immune system development and protects the host from allergic and autoimmune diseases. At first sight, these observations seem to be contradictory to our results. Though, in our study, we aimed to actively re-establish immune homeostasis from severe intestinal inflammation in adult animals. This approach is different from the aim to protect from spontaneous disease onset through microbiota modulation in infants. Additionally, other groups consider hypo-phosphorylated and hypo-acylated *Bacteroides* LPS to be antagonistic. However, we have clearly demonstrated that these LPS structures rather act as weak agonists.

Our results and those of other groups raise the question of the potential use of weak agonistic LPS as a suitable therapeutical tool to restore homeostatic conditions not only in experimental mouse models but also in IBD patients. Therapy of IBD patients is, to date, restricted to a general suppression of the patient's immune response, often associated with undesirable side effects. LPS (derivative)-based treatment might avoid this problem by acting only locally at the site of inflammation, the intestine.

Although our results strongly indicate a direct LPS-mediated modulation of $CD11c^+$ cells in the intestinal LP to be the key driver for the observed healing effects, we cannot completely rule out that the re-establishment of intestinal homeostasis only represents a secondary effect in response to a potential LPSBV-induced microbiota shift from dysbiotic to homeostatic.

Nevertheless, we aim to promote purified LPSBV as an alternative for the treatment of intestinal inflammatory disorders or IBD, providing evidence that this compound demonstrated its beneficial effects as not being an antagonist but rather a weak agonist. Concluding, we contribute this study to IBD therapy-related research, offering a completely new approach that avoids the disadvantages of current state-of-the-art IBD therapies.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River Laboratories, and C57BL/6J-*Rag1*^{tm1Mom} (*Rag1*$^{-/-}$) mice were obtained from our own breeding. All animals were kept and bred under specific pathogen-free (SPF) conditions in individually ventilated cages (IVCs), receiving standard chow and regular drinking water. For the isolation of bone marrow from C57BL/6 mice, only female mice aged 6–12 weeks were used. *Rag1*$^{-/-}$ mice were used as a mouse model for T cell transplantation-dependent experimental chronic colitis. Animal experiments were reviewed and approved by the responsible institutional review committee and the local authorities (Regierungspräsidium Tübingen, Permit H6/10, Anzeigen 01.12.11, 09.01.15, 14.06.17).

T Cell-Mediated Induction of Chronic Colitis in *Rag1*$^{-/-}$ Mice

C57BL/6J-*Rag1*^{tm1Mom} (*Rag1*$^{-/-}$) mice were transplanted with 5×10^5 splenic $CD4^+CD62L^+CD45RB^{hi}$C WT T cells at 8–10 weeks of age. *Rag1*$^{-/-}$ mice harbored a so-called DYSM, which efficiently triggers the induction of pathological intestinal inflammation upon transplantation of naive T cells, as reported previously.[7,22] This microbiota was absent of Norovirus, Rotavirus, and *Helicobacter hepaticus*. Furthermore, detailed next-generation sequencing-based analysis of DYSM composition revealed a significantly increased relative abundance of the bacterial phyla *Proteobacteria*, *Verrucomicrobia*, and *Firmicutes*, while the relative abundance of *Bacteroidetes* was drastically reduced compared to a symbiotic microbiota composition, as published previously by our group.[7] During the experiments, *Rag1*$^{-/-}$ mice were kept under SPF conditions in IVCs and analyzed 8 weeks after T cell transplantation, as indicated in the Results.

Radiopharmaceuticals

[^{18}F]fluoride was produced by using ^{18}O (p,n) ^{18}F nuclear reaction on the PETtrace cyclotron (General Electric Medical Systems, GEMS, Uppsala, Sweden). [^{18}F]FDG synthesis was performed as described elsewhere.[62] After the synthesis, specific activity was calculated and revealed to be >50 GBq/mmol with a radiochemical purity of >99%.

In Vivo PET Imaging

High-resolution PET imaging was performed using two identical small animal Inveon microPET scanner (Siemens Medical Solutions, Knoxville, USA) with a spatial resolution of 1.4 mm in the reconstructed images (field of view [FOV]: transaxial, 10 cm; axial, 12.7 cm).[63] By applying iterative ordered subset expectation maximization (OSEM) 2D algorithm for reconstruction, list mode data were processed. Mice were anesthetized with 1.5% isoflurane (Abbott, Wiesbaden, Germany) vaporized with O_2 (1.5 L/min) and injected intravenously (i.v.) into the tail vein with 8.3 ± 1.3 MBq [^{18}F]FDG. After tracer injection, animals were kept anesthetized for 60 min, in an anesthesia box, placed on a heating pad to maintain body temperature of animals during tracer uptake time. Shortly before the end of the uptake time, mice were placed in the FOV of the PET scanner on a warmed (37°C) scanner bed. Static (10-min) PET scans were performed on weeks 0, 4, 6, and 8 after T cell application. Data were corrected for decay, normalized to the injected activity, and analyzed using Pmod Software (PMOD Technologies, Zurich, Switzerland) by drawing regions of interest over the intestine.

Bacteria

The bacteria used for stimulation of the mouse BMDCs were EC and BVMPK, which were described in detail previously.[20–22,31,64] Additionally, we used BD CL02T12C06, BTIO VPI-5482, BV8482 ATCC 8482, and BF ATCC 25285, DSM 2151. The EC strain was grown in Luria-Bertani (LB) medium under aerobic conditions at 37°C. All *Bacteroides* strains were grown in brain-heart-infusion (BHI) medium and anaerobic conditions at 37°C.

Comparison of *Bacteroides* spp. Lipid A Synthesis Genes

Lipid A synthesis genes were identified in BVMPK (GenBank: NZ_CP013020.1) using the *B. vulgatus* ATCC8482 type strain genome sequence (GenBank: NC_009614.1). The amino acid sequences of all lipid A synthesis enzymes of BVMPK were subsequently compared to BD CL02T12C06 (GenBank: NZ_AGXJ00000000.1), BTIO VPI-5482 (GenBank: NC_004663.1), BF NCTC 9343 (GenBank: NC_003228.3), and *E. coli* K12 MG1655 (GenBank: NC_000913.3), using the standard protein BLAST (blastp suite).

Isolation of LPSBV and LPSEC

The lyophilized bacterial pellet was washed several times with distilled water, ethanol, and acetone, followed by several ultracentrifugation steps (45,000 rpm at 4°C) in order to remove cell, growth broth, and capsular contaminants.

Cells were extracted by hot phenol-water extraction.[65] Water and phenol phases were both exhaustively dialyzed and lyophilized. After inspection by SDS-PAGE, an enzymatic treatment to remove proteins and nucleic acids was executed, followed by a dialysis step. The SDS-PAGE executed on both purified water and phenol phases highlighted the presence of LPS only in the water phase from which it was further purified.

Biotinylation of LPSBV

10 mg LPSBV was biotinylated with EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Thermo Scientific), according to the manufacturer's protocol, using PBS as a solvent. To remove PBS, an exhaustive dialysis against distilled water was performed. The biotinylated LPS_{BV} ($bioLPS_{BV}$) was then collected and lyophilized. For *in vitro* experiments, lyophilized $bioLPS_{BV}$ was dissolved in distilled water in concentrations not higher than 1 mg mL^{-1}.

Cultivation of BMDCs

Bone marrow cells were isolated from C57BL/6 WT, TLR2-deficient, TLR4-deficient, and TLR2 × TLR4 double-deficient mice and cultivated as described previously.[66] At day 7 after isolation, the resulting CD11c-positive dendritic cells were used for stimulation experiments.

Stimulation of BMDCs

2×10^6 BMDCs were stimulated with PBS, BVMPK, or EC at an MOI of 1 or the respective isolated LPS at concentrations as indicated in the Results. Cells were stimulated for a maximum of 24 h. If viable bacteria were used for stimulation, gentamycin was added at a final concentration of 1 μg mL^{-1} to avoid bacterial overgrowth under aerobic conditions. If a second challenge was used, cells were stimulated with bacteria or LPS preparations for 24 h. Cell culture medium was changed before challenging the cells with a second stimulus for a maximum of additional 16 h. PBS was used as a mock stimulation control. For stimulation with a DYSM composition, feces were collected from living SPF *Rag1*$^{-/-}$ animals exhibiting severe intestinal inflammation, 4 weeks after T cell transplantation. Feces were autoclaved for 15 min at 121°C and weighed, and sterile PBS was added to obtain a final concentration of 50 mg mL^{-1}. Homogemnized feces were then filtered through a 100-μm sieve. The filtered suspension was diluted by a factor of 2.5, and 5 μL was added to 1 mL cell culture medium containing 10^6 BMDCs. The competitive TLR4 antagonist TAK242 was added at a final concentration of 10 μM at 1 h prior to stimulation with LPS, $Pam2CSK_4$, flagellin from *S.* Typhimurium (FLA-ST), or viable bacteria.

Stimulation of HEK Cells

2×10^5 HEK cells expressing murine CD14, MD-2, and TLR4 (mTLR4-HEK) in 1 mL medium were stimulated with 1–1,000 ng mL^{-1} isolated LPS or bacteria (MOI 1) for the time points indicated in the Results.

Detection of Bound Biotinylated LPSBV

After incubation with biotinylated LPSBV, mTLR4-HEK cells were scraped off, washed once in PBS + 1% fetal calf serum (FCS), and

incubated with Strep-PE for 30 min, followed by another washing step. Cell-attached Strep-PE was detected by flow cytometry. All experiments to be compared were carried out in one experimental setting to guarantee for comparability of the detected MFI (median fluorescence intensity) values of the PE fluorescence.

Cytokine Analysis by ELISA

For the analysis of secreted cytokines (IL-6, IL-10, and TNF), ELISA-based detection kits were purchased from BD Biosciences and used according to the manufacturer's instructions.

Flow Cytometry Analysis

Multi-color flow cytometrical (FCM) analyses were performed on a FACS Calibur or FACS LSRII (BD Biosciences). All fluorochrome-coupled antibodies were purchased from BD Biosciences if not stated otherwise. Data were analyzed using the FlowJo software (Tree Star, USA).

ALDH Activity Assay

Intracellular ALDH activity in BMDCs was assayed using the Aldefluor kit (STEMCELL Technologies), according to the manufacturer's instructions. Samples treated with the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) served for the determination of baseline fluorescence and defining ALDH-positive cells (Figure S5).

Purification of RNA and Quantitative Real-Time PCR

Purification of RNA from colonic scrapings was performed using QIAGEN's RNeasy Mini Kit, according to the manufacturer's instructions. Additional DNA digestion was conducted by using 4 U rDNase I and 40 U rRNasin for an RNA solution of 0.1 μg μL^{-1}. After 30 min of incubation at room temperature (RT), DNase was inactivated using Ambion DNase inactivation reagent, which was later removed by centrifugation for 1 min at 10,000 × *g*. SybrGreen-based qRT-PCR was performed on a Roche LightCycler480 using QIAGEN SybrGreen RT-PCR Kit. Primer annealing occurred at 60°C. 10–100 ng DNA-digested RNA was used for qRT-PCR. Relative mRNA expression in BMDCs stimulated with bacteria to unstimulated BMDCs was determined, with β-actin as the housekeeping gene, using the ΔΔCp method that included the specific amplification efficiency of every used primer pair and each PCR run.

Primer Sequences

Primers used for qRT-PCR were as follows: Aldh1a2 forward, 5′-AAGACACGAGCCCATTGGAG-3′; reverse, 5′-GGAAAGCCAGCCTCCTTGAT-3′; and β-actin forward, 5′-CCCTGTGCTGCTCACCGA-3′; reverse, 5′-ACAGTGTGGGTGACCCCGTC-3′.

Isolation of LP DCs and T Cells and the Adoptive Transfer of T Cells

Isolation of LP cells was performed as reported previously.[67] For adoptive transfer, splenic $CD4^+$ T cells from C57BL/6 mice were purified using a magnetic-activated cell sorting (MACS)-based negative selection kit (Miltenyi), according to the manufacturer's instructions. The isolated cells were stained for CD3ε, CD4, CD62, and CD45RB for reanalysis; purity was generally >90% with >80% being $CD3\varepsilon^+CD4^+CD62L^+CD45RB^{hi}$. 5×10^5 $CD4^+$ T cells were injected intraperitoneally (i.p.) into $Rag1^{-/-}$ mice, as described previously.[68]

Histology

Colonic tissues were fixed in neutral buffered 4% formalin. Formalin-fixed tissues were embedded in paraffin and cut into 2-μm sections. They were stained with H&E (Merck) for histological scoring. Scoring was conducted in a blinded fashion on a validated scale of 0–3, with 0 representing no inflammation and 3 representing severe inflammation characterized by infiltration with inflammatory cells, crypt hyperplasia, loss of goblet cells, and distortion of architecture.[69]

Antibodies

The following antibodies were used for flow cytometry analysis of intracellular and surface proteins: anti-mouse CD11c-allophycocyanin (APC) (clone H3; Becton Dickinson), anti-mouse MHC class II-fluorescein isothiocyanate (FITC) (clone 2G9, Becton Dickinson), anti-mouse MHC class II-BV510 (clone 2G9, Becton Dickinson), anti-mouse CD40-FITC (clone 3/23, Becton Dickinson), anti-mouse CD40-BV421 (clone 3/23, Becton Dickinson), anti-mouse CD80-FITC (clone B7-1 (16-10A1), Becton Dickinson), anti-mouse CD86-FITC (clone GL1, Becton Dickinson), anti-mouse p65-PE (pS534) (clone 96H1, Becton Dickinson), anti-mouse Ly6G/Ly6C-FITC (clone GR-1/RB-68C5, Becton Dickinson), anti-mouse CD45R-FITC (clone RA3-6B2, Becton Dickinson), anti-mouse CD64-FITC (clone X54-5/7.1, Becton Dickinson), anti-mouse CD45-BV421 (clone 30-F11, Becton Dickinson), anti-mouse CD11b-BV605 (clone M1/70, Becton Dickinson), anti-mouse CD103-PerCP-Cy5.5 (clone M290, Becton Dickinson), and anti-mouse TLR4-biotin (clone SA15-21, BioLegend).

Statistics

For comparisons of two groups, a parametric Student's t test was used for normally distributed values. For multiple comparison of more than two groups, one-way ANOVA was used for normally distributed values and non-parametric Kruskal-Wallis test was used elsewhere. p values are indicated in the figures; p values <0.05 were considered to be significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.07.007.

AUTHOR CONTRIBUTIONS

Conceptualization, A.S., J.-S.F., L.M., and A.M.; Methodology, A.S., F.D.L., and A.M.; Formal Analysis, A.S. and L.M.; Investigation and Data Curation, A.S., L.M., F.D.L., T.K., T.M., J.K.M., A.S., A.L., R.P., K.G., K.F., A.S., and H.H.Ö.; Resources, F.D.L., K.F., B.J.P., and A.M.; Writing – Original Draft, A.S., I.B.A., A.M., and J.-S.F.; Writing – Review & Editing, A.S., L.M., I.B.A., A.M., and J.-S.F.; Visualization, A.S. and L.M.; Supervision, I.B.A. and J.-S.F.; Project Administration, A.S., J.-S.F., and L.M.; Funding Acquisition, J.-S.F.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

Weak Agonistic LPS Restores Intestinal Immune Homeostasis

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Supplemental information

Supplemental information 1:

Comparison of surface molecule expression pattern of colonic lamina propria (cLP) CD11c+ cells and bone-marrow derived dendritic cells (BMDCs)

To verify the similarity of *ex vivo* isolated cLP CD11c+ cells with *in vitro* generated BMDCs in terms of expression of important surface markers, we stained both cell types with fluorophore-coupled antibodies for detection of CD11c, Ly6G, Ly6G, CD64, CD45R, CD45, CD103, CD11b and MHC-II (Figure S1). Viable cLP $CD11c^{+}$ single cells and viable BMDC single cells were demonstrated to provide similar expression of surface total MHC-II, CD45 and non-expression of Ly6G, Ly6C, CD64 and CD45R. We could detect slight differences in the expression of $CD103^{pos/neg}CD11b^{pos/neg}$ dendritic cell subpopulations, with BMDCs providing a slightly higher proportion of $CD103^{neg}CD11b^{pos}$ $CD11c^{+}$ cells while the $CD103^{neg}CD11b^{neg}$ subpopulation is slightly more frequent among cLP CD11c+ cells. However, these minor differences are assumed to be irrelevant for the performed experiments and conclusions.

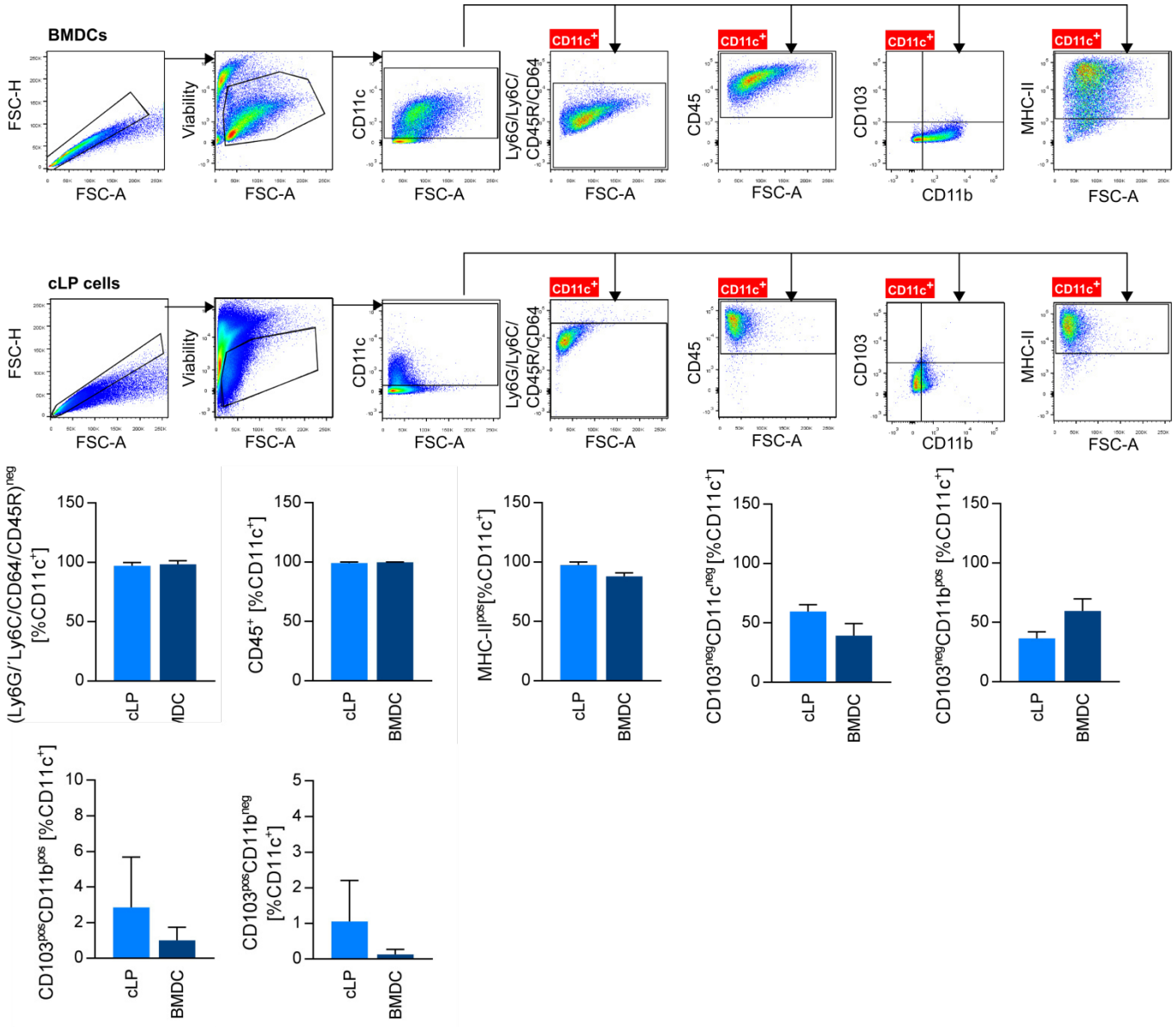

Figure S1: **Comparison between BMDCs and cLP CD11c+ cells.**

Bone-marrow derived dendritic cells (BMDCs) were generated as described in the Materials and Methods section. Colonic lamina propria (cLP) cells were isolated from $Rag1^{-/-}$ mice as described in the Materials and Methods section. Both cell types were stained with a fixable viability dye as well as with antibodies detecting CD11c, Ly6G, Ly6G, CD64, CD45R, CD45, CD103, CD11b and MHC-II. Upper panel and middle panel: Gating strategy for BMDCs (upper panel) and cLP cells (middle panel). Single cells

(FSC-A vs. FSC-H) were gated for viable cells. Viable single cells were gated for CD11c expression and $CD11c^+$ cells were analyzed for expression of Ly6G, Ly6C, CD64, CD45R, CD103, CD11b and MHC-II. Lower panel: Determination of surface expression of the respective surface molecules as a proportion of all $CD11c^+$ cells (n = 15 for each cell type). Columns and error bars represent mean ± SD

Supplemental information 2:

Gating strategy to determine CD40 surface expression as well as to define the subpopulation of MHC-II high positive cells

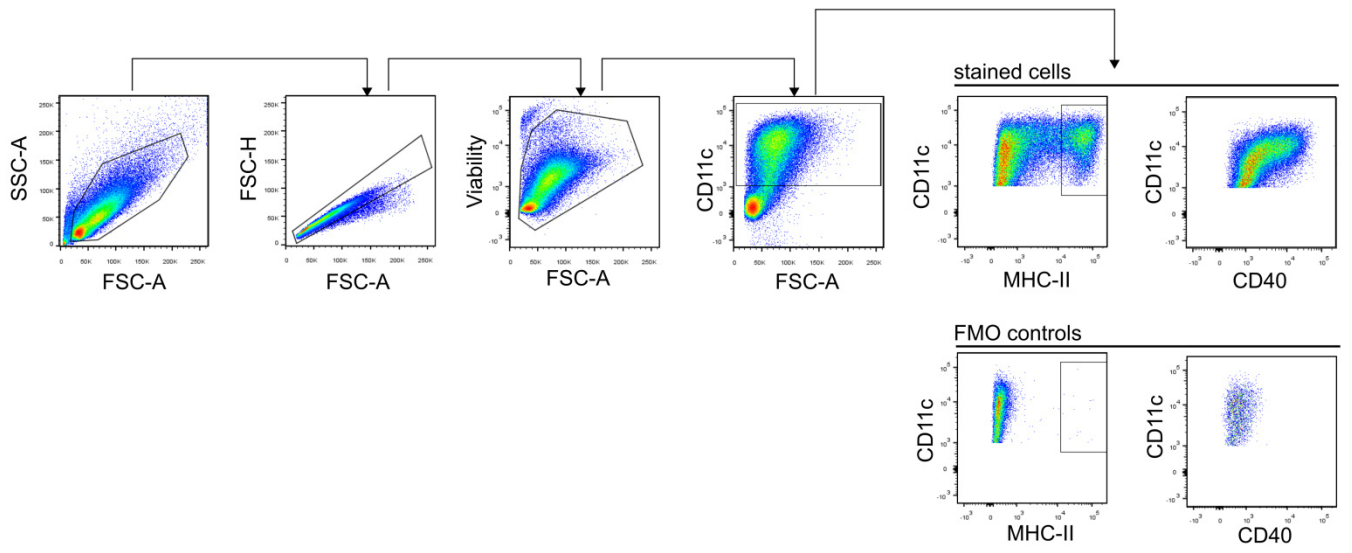

Figure S2: **BMDC gating strategy**

Gating strategy to determine CD40 surface expression as well as to define the subpopulation of MHC-II high positive cells. Viable, single $CD11c^{+}$ cells were stained for MHC-II and CD40 surface expression. Analysis of MHC-II surface expression was assessed by determination of the MHC-II high positive population, which is increased during DC maturation. CD40 expression was determined by measuring Median Fluorescence Intensity (MFI). The upper panel shows stained cells while the lower panel shows fluorescence-minus-one controls to determine $MHC\text{-}II^{neg}$ and $CD40^{neg}$ cells.

Supplemental information 3:

Heat-killed *Bacteroides* strains induce the same semi-mature phenotype in BMDCs as viable *Bacteroides* strains.

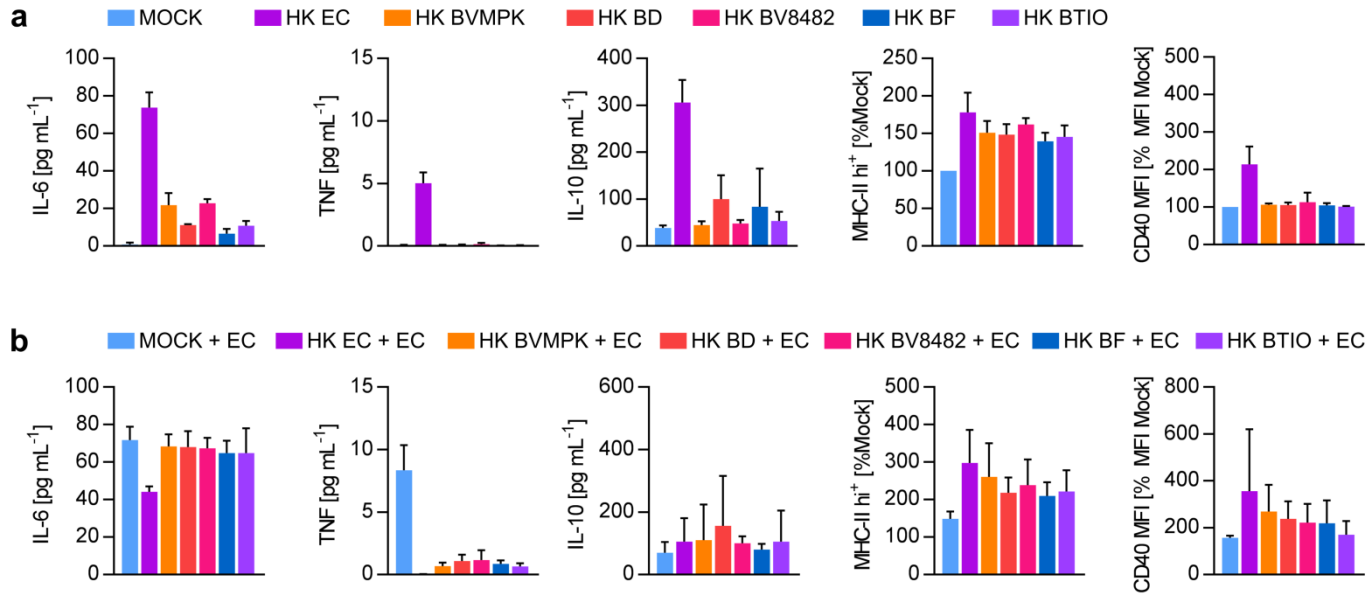

Figure S3: Heat-killed *Bacteroides* species induce the same hyporesponsive phenotype in CD11c+ cells as viable *Bacteroides* species

a) Stimulation of CD11c+ bone-marrow derived dendritic cells (BMDCs) generated from wt C57BL/6 mice (n=3) with PBS (Mock), heat-killed *E. coli* mpk (HK EC),heat-killed *B. vulgatus mpk* (HK BVMPK), heat-killed *B. dorei* (HK BD), heat-killed *B. vulgatus* ATCC8482 (HK BV8482), heat-killed *B. fragilis* (HK BF) and heat-killed *B. thetaiotaomicron* (HK BTIO) for 16 h at MOI 1. Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and the population of MHC-II hi+ cells and CD40 MFI, respectively, was normalized to the mock control of BMDCs generated from the same individual. Columns and error bars represent mean ± SD.

b) *wt* BMDCs were stimulated with PBS (mock), HK EC, HK BVMPK, HK BD, HK BV482, HK BF and HK BTIO at MOI 1 for 24h (prime). Cell culture medium removed and exchanged for fresh medium before stimulation (challenge) with viable EC for additional 16 h. Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and the population of MHC-II hi+ cells and CD40 MFI, respectively, was normalized to the mock-primed and EC-challenged control of BMDCs generated from the same individual. Columns and error bars represent mean ± SD.

Supplemental information 4:

Evaluation of TAK252 as a specific TLR4 inhibitor

To verify that TAK242 selectively inhibits TLR4 signalling, we treated BMDCs generated from wildtype mice with 10 µM TAK242 1h prior stimulation for 16 h with PBS (mock) LPS-EK as potent TLR4 ligand, FLA-ST as potent TLR5 ligand and PAM2CSK4 a potent TLR2 ligand, all at a final concentration of 100 ng/mL. Secretion of IL-6 as an indicator of TLR signalling was detected by ELISA. TAK242 significantly inhibited IL-6 secretion in cells stimulated with LPS-EK but showed no significant inhibitory activity in cells stimulated with FLA-ST or PAM2CSK4. This result supports findings by Lii *et al*., Takashima *et al.* and Matsunaga *et al* [1-3], observing no or only marginal binding of TAK242 to other TLRs compared to TLR4. We therefore consider TAK242 as a selective TLR4 inhibitor.

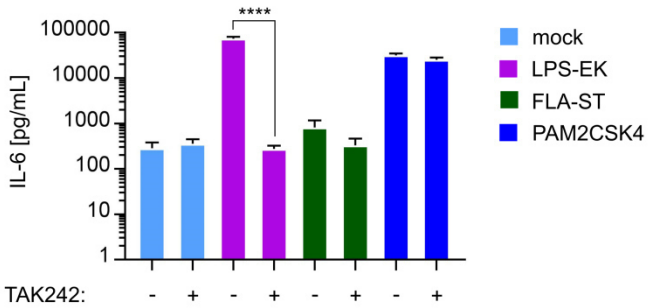

Figure S4:

TAK242 selectively inhibits TLR4 signaling

The competitive TLR4 antagonist TAK242 was added to CD11c+ bone-marrow derived dendritic cells (BMDCs) generated from wt C57BL/6 mice at a final concentration of 10 µM 1h prior to a stimulation for 16 h with with PBS (Mock) or ligands for TLR4 (LPS-EK, Invivogen), TLR5 (FLA-ST, Invivogen) and TLR2 (PAM2CSK4, Invivogen), all at a final concentration of 100 ng/mL. IL-6 secretion was detected by ELISA. Inhibitory activity of TAK242 is indicated by a significant reduction of IL-6 secretion in LPS stimulated cells but not in FLA-ST or PAM2CSK4-stimulated cells upon TAK242 treatment compared with mere TLR ligand stimulation. Columns and error bars represent mean ± SD.

Supplemental information 5:

Gating strategy to determine intracellular ALDH activity

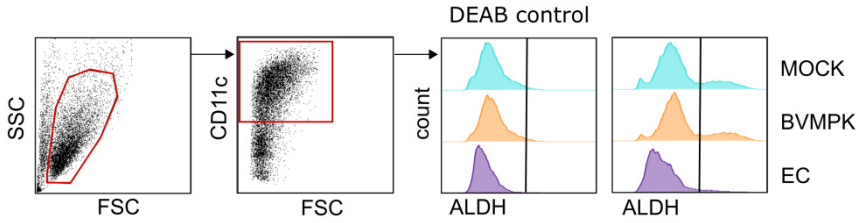

Figure S5: ALDH activity assay

Stimulation of CD11c$^+$ BMDCs generated from wt C57BL/6 mice with PBS (mock), *B. vulgatus* mpk (BVMPK) and *E. coli* mpk (EC) for 16 h. ALDH activity in stimulated cells was then analysed with the Aldefluor kit (Stemcell). Flow cytometric analysis with additional staining for CD11c was used to determine the mean fluorescence intensity (MFI) of CD11c$^+$ ALDH$^+$ cells. DEAB treated samples (left histogram panel) were used for determination of background fluorescence and defining ALDH$^+$cells , indicated by exemplary samples shown in right histogram panel.

Supplemental information 6:

LPSBV-induced semi-maturation of BMDCs requires TLR4, but not TLR2-signaling

We stimulated BMDCs generated from wildtype, TLR2-deficient, TLR4-deficient and TLR2xTLR4-double deficient mice with PBS (negative control), LPSBV (100 ng/mL) and EC (MOI1, positive control) and checked for subsequent secretion of pro-inflammatory cytokines IL-6 and TNF as well as surface expression of MHC-II and CD40. As already demonstrated in the main manuscript, stimulation of wildtype BMDCs with LPSBV resulted in intermediate secretion of IL-6, low to intermediate expression of MHC-II and CD40 as well as to absence of TNF compared to the EC positive control. IL-6 and TNF expression as well as increase in surface expression of MHC-II and CD40 was completely abolished using TLR4-deficient and TLR2xTLR4-double deficient BMDCs. Importantly, stimulation of TLR2-deficient BMDCs with LPSBV resulted in the same expression pattern of the detected cytokines and surface markers as LPSBV stimulation of wildtype BMDCs. To assess the contribution of a potential TLR2-signaling to the induction of LPSBV-induced semi-maturation in BMDCs, we primed TLR2-deficient BMDCs with PBS, LPSBV and EC and challenged them with either PBS or EC as a second stimulus as already demonstrated for wildtype BMDCs (Fig. 3). As shown in Figure S6b, hyporesponsiveness in terms of TNF and MHC-II expression was induced by LPSBV-priming, indicating that TLR2-mediated signaling is dispensable for LPSBV-induced BMDCs semi-maturation. Taken together, these results indicate that (1) either LPSBV or a potential contaminant induce a slight activation of TLR2-mediated signaling in mTLR2-HEK cells, (2) TLR4-signaling is far more important than TLR2-signaling for LPSBV-mediated effects on BMDCs in terms of cytokine expression and MHC-II/CD40 surface expression in response to stimulation with LPSBV preparations, (3) LPSBV or a potential contaminant does not activate TLR2 signaling in BMDCs and (4) LPSBV-induced MD-2/TLR4 receptor-signaling is essential for induction of semi-maturation in BMDCs.

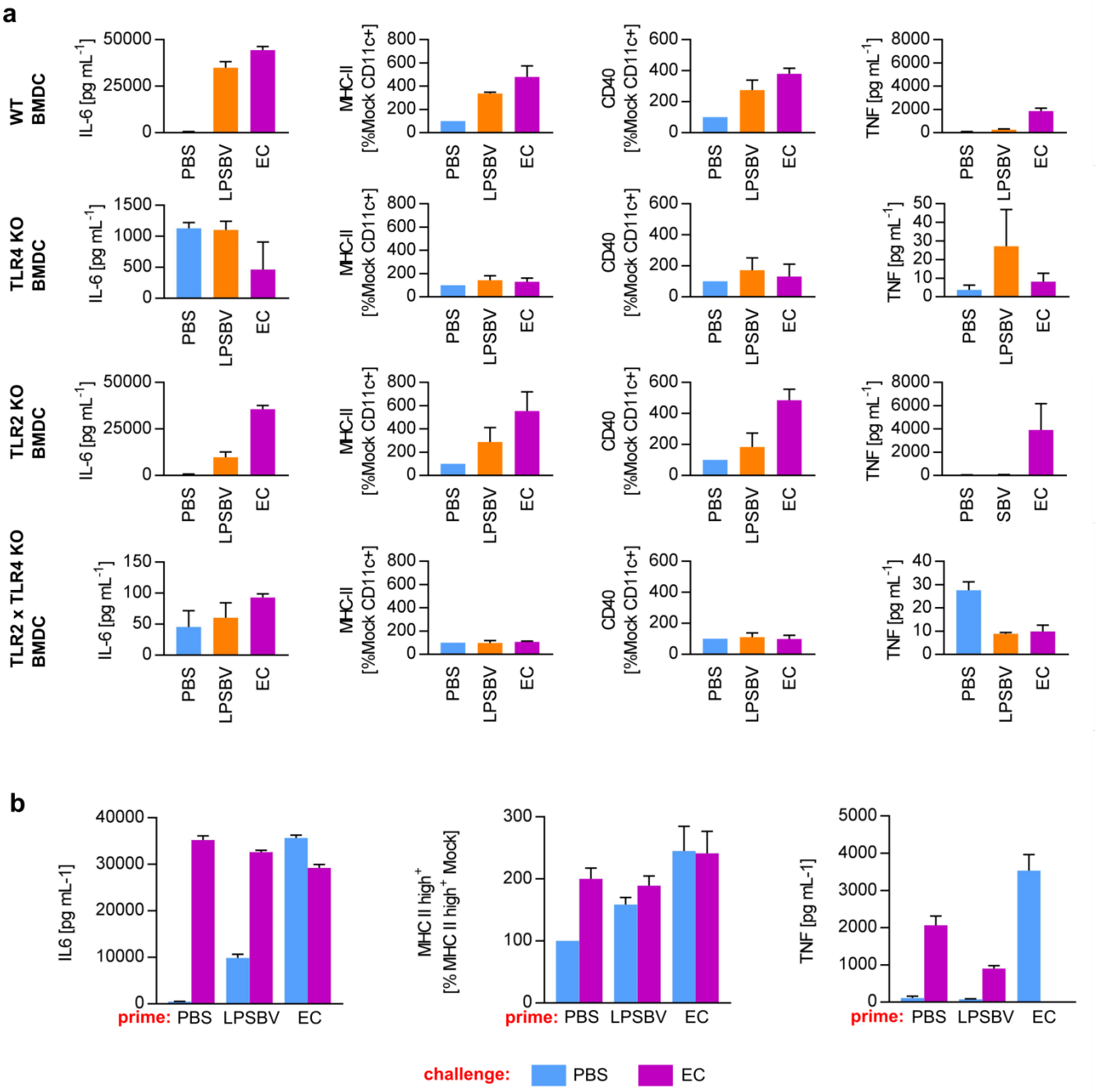

Figure S6: LPSBV-induced semi-maturation of BMDCs requires TLR4, but not TLR2-signaling

a) Stimulation of CD11c+ bone-marrow derived dendritic cells (BMDCs) generated from wt C57BL/6, TLR2-deficient, TLR4-deficient and TLR2xTLR4 double-deficient mice (n=4-8) with PBS (Mock), *E. coli* mpk (EC) and *B. vulgatus* mpk LPS (LPSBV). Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and the population of MHC-II hi+ cells and CD40 MFI, respectively, was normalized to the mock control of BMDCs generated from the same individual. Columns and error bars represent mean ± SD.

b) TLR2-deficient BMDCs were stimulated with PBS (mock), EC or LPSBV for 24h (prime). Cell culture medium was removed and exchanged for fresh medium before stimulation (challenge) with EC or PBS for additional 16 h. Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and the population of MHC-II hi+ cells and CD40 MFI, respectively, was normalized to the PBS-primed and PBS-challenged control of BMDCs generated from the same individual. Columns and error bars represent mean ± SD.

Supplemental information 7:

Biotinylation of LPSBV does not affect weak agonistic properties of LPSBV

To verify that biotinylation of LPSBV does not affect the weak agonistic properties of LPSBV, HEK cells overexpressing the mouse CD14/MD-2/TLR4-receptor complex were stimulated with LPSBV, bioLPSBV and LPSEC. We determined resulting expression of IL-8 as a measure of NFκB activation. We did not detect any differences in the NFκB activation in response to LPSBV compared to bioLPSBV (Figure 7)

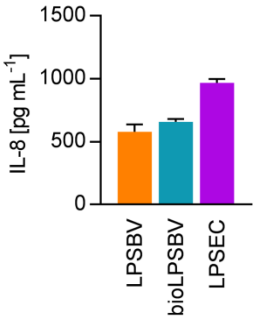

Figure S7: **Influence of LPSBV biotinylation on TLR4 receptor activation.**

mTLR4-HEK cells were stimulated with 10 ng/mL LPSBV, biotinylated LPSBV (bioLPSBV) and LPSEC. Resulting IL-8 concentration as a measure of intracellular NFκB activation was detected by ELISA. Columns and error bars represent mean ± SD.

Supplemental information 8:

Determination of quasi dissociation constants K_D of LPSBV and LPSEC

The observation that both LPS structures, LPS_{BV} and LPS_{EC}, showed different immunological effects on DCs *in vitro*, prompted us to define their binding affinity to the MD-2/TLR4 receptor complex. We established an experimental setting to determine *quasi* dissociation constants (K_D) of both LPS structures using biotinylated LPS_{BV} ($bioLPS_{BV}$). We are aware of the fact that we cannot determine real K_D-values since we do not know the exact molarity of the used LPS solutions. The assembly of amphiphilic LPS monomers into micelles, vesicles or even more complicated structures is highly dependent on the surrounding buffer and salt conditions and is therefore hardly predictable. This exacerbated the determination of the molarity of LPS monomers which effectively have access to the receptor, rendering them "active ligands" thus contributing to K_D values. However, assuming that (1) $bioLPS_{BV}$ and LPS_{EC} provide a comparable monomeric molecular weight, (2) $bioLPS_{BV}$ and LPS_{EC} behave in a similar chemical manner under the experimental conditions and (3) all experiments were carried out incubating both LPS at the same time, we can speculate that a qualitative comparison using K_D-values in the unit [g L^{-1}] instead of [mol L^{-1}] is qualifiable for a comparison of their binding affinity. In order to determine K_D values for both LPS structures, we used human embryonic kidney (HEK) cells which were stably transfected with a plasmid encoding for murine CD14, MD-2 and TLR4 (HEK-mTLR4). These latter were expressed by HEk-mTLR4 cells in excess and in equal amount by HEK-mTLR4 cells in all executed experiments, representing a fundamental prerequisite for K_D determination. LPS_{BV} was biotinylated ($bioLPS_{BV}$) as described in the experimental sections and its immunological behaviour was tested and compared to non-biotinylated LPS_{BV}. Since we could not detect any differences in the immunological behaviour (data not shown), we concluded that biotinylation did not affect the interaction with the receptor complex. We incubated HEK-mTLR4 cells with various concentrations of $bioLPS_{BV}$ and we then visualized bound $bioLPS_{BV}$ by adding PE-coupled streptavidin followed by flow cytometric analysis of the resulting PE fluorescence. As shown in Fig. S6a, non-biotinylated LPS and cells without addition of any LPS did not contribute to the PE fluorescence signal. The resulting PE-fluorescence in $bioLPS_{BV}$-treated samples was dependent on $bioLPS_{BV}$ concentration and showed a classical perpendicular hyperbola binding curve of a ligand to its respective receptor (Fig. S8a, right panel). In order to determine K_D of $bioLPS_{BV}$ and LPS_{EC} we simultaneously incubated HEK-mTLR4 cells with different concentrations of both LPS for 1 h and detected resulting PE fluorescence, which is directly proportional to the amount of bound $bioLPS_{BV}$ (Fig. S8b). In order to define the K_D value of $bioLPS_{BV}$, various concentrations of this LPS alone without addition of LPS_{EC} were used. The K_D equalled the concentration of $bioLPS_{BV}$ corresponding to the half maximal MFI PE intensity, which could be determined as 0.412 mg L^{-1}. In other words, adding 0.412 mg L^{-1} to the used 1 x 10^5 HEK-mTLR4 cells led to occupation of half of the available MD-2/TLR4 binding sites (Fig. S8c). In order to determine K_D of LPS_{EC}, several concentrations of LPS_{EC} were co-incubated with different concentrations of $bioLPS_{BV}$, resulting in 4 different binding curves representing the 4 employed LPS_{EC} concentrations (Fig. S8d). The resulting binding curves follow the equation

$$(1)\ MFI\ PE = \frac{n[bioLPS_{BV}]}{[bioLPS_{BV}] + K_D(bioLPS_{BV}) \times \left(1 + \frac{[LPS_{EC}]}{K_D(LPS_{EC})}\right)}$$

with n being the number of different binding sites (which was assumed to be 1 in our case). The binding curves shown in Fig. S8d were then plotted into a double reciprocal form resulting in 4 different straight lines whose slopes were determined using GraphPad Prism (Fig. S8e). The slopes obtained from Figure S8e were afterwards plotted against the respective LPS_{EC} concentration resulting in a straight line whose intersection with the x-axis represented the negative value of the LPS_{EC} binding constant which was determined to be 0.304 mg L^{-1}, being in the same biologically relevant range as $bioLPS_{BV}$ K_D. Therefore, based on the above described experiments, we can conclude that LPS_{BV} and LPS_{EC} provide similar binding affinity to the MD-2/TLR4 receptor complex.

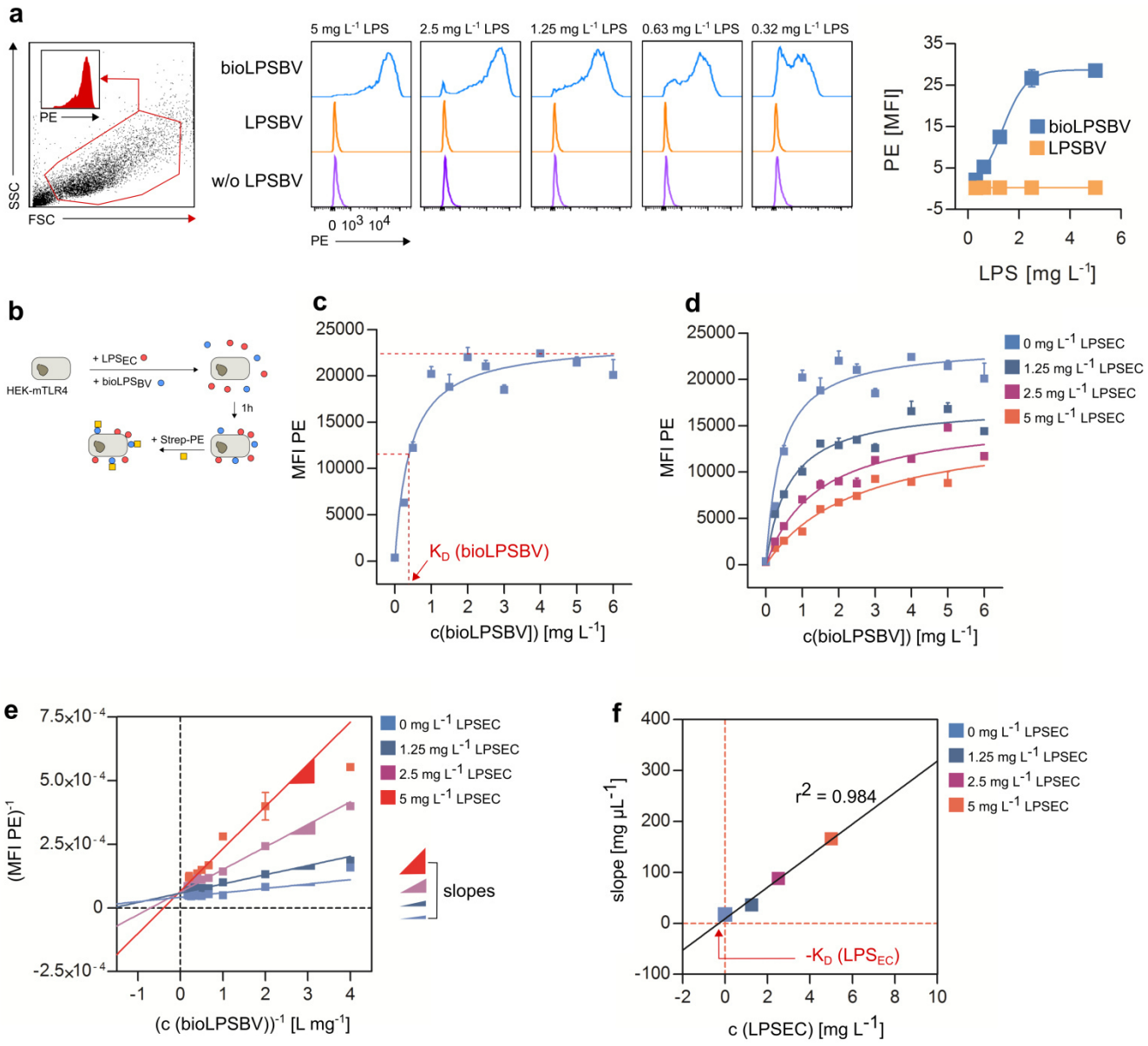

Figure S8: *B. vulgatus* mpk LPS and *E. coli* mpk LPS provide similar binding affinity to the MD-2/TLR4 receptor complex.

(a) Human embryonic kidney (HEK) cells expressing murine CD14, MD-2 and TLR4 (HEK-mTLR4) were co-incubated with *B. vulgatus* mpk LPS (LPSBV), biotinylated *B. vulgatus* mpk LPS (bioLPSBV) or PBS (w/o LPS_{BV}) at several concentrations. PE-coupled streptavidin (Strep-PE) was added to each sample in constant concentrations. Cells were washed and analysed afterwards for PE fluorescence using flow cytometry. Left panel: gating strategy to determine intact HEK-mTLR4 cells. Middle panel: representative histograms of PE fluorescence of intact HEK-mTLR4 cells that were treated as described above. Right panel: Detected mean PE fluorescence intensity (PE (MFI)) as a function of used $bioLPS_{BV}$ concentration.

(b) experimental setting for the determination of LPS binding affinity: HEK-mTLR4 cells were incubated simultaneously with various concentrations of non-biotinylated *E. coli* mpk LPS (LPSEC) or biotinylated *B. vulgatus* mpk LPS (bioLPSBV) for 1 h. Cells were washed and incubated with PE-coupled streptavidin (Strep-PE) for 30 min. PE fluorescence was detected afterwards using flow cytometry.

(c) HEK-mTLR4 cells were incubated with bioLPSBV only according to the experimental setting described in (b). By using different concentrations of bioLPSBV and the resulting detected PE fluorescence, a non-linear regression for a perpendicular hyperbola could be created. The bioLPSBV concentration corresponding to the half-maximal PE intensity equals to KD of bioLPSBV. Squares with error bars represent mean ± SD.

(d) according to (b) 1 x 10^5 HEK-mTLR4 cells were simultaneously incubated with bioLPSBV and LPSEC. For each differentially coloured perpendicular hyperbola binding curve the concentration of biotinylated *B. vulgatus* mpk LPS (bioLPSBV) was varied while the concentration of *E. coli* mpk LPS (LPSEC) was kept constant. According to (b), resulting PE fluorescence was detected by flow cytometry as demonstrated in (a). The detected PE signal is therefore directly proportional to bound bioLPSBV. Each data point represents a distinct combination of LPSEC and bioLPSBV concentrations (n=3). Squares with error bars represent mean ± SD.

(e) Data obtained from (d) were mathematically transformed into a double reciprocal form and plotted into a graph. The sigmoidal binding curves from (d) are therefore transformed into straight lines. The slopes of the straight lines were determined using GraphPad Prism. Squares with error bars represent mean ± SD.

(f) The slopes which were obtained from the double reciprocal plotting in (e) which were derived from the binding curves in (d) were plotted against the corresponding concentration of LPSEC which was kept constant in the experiment. A linearization led to a straight line whose intersection with the x-axis represents the negative value of the dissociation constant ($-K_D$) for the binding of LPS_{EC} to the murine MD-2/TLR4 receptor complex.

Supplemental information 9:

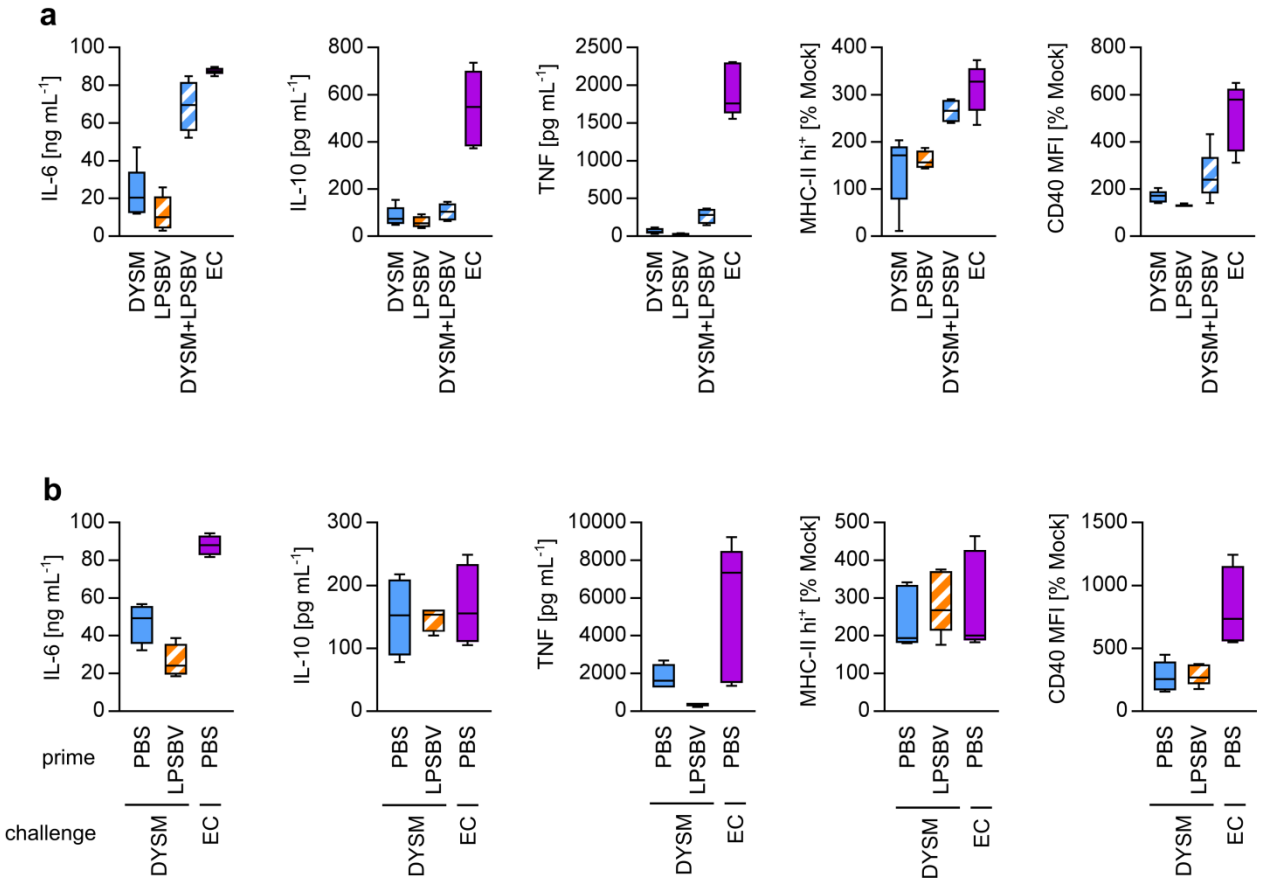

Figure S9:

a) Feces from *Rag1*$^{-/-}$ mice providing intestinal inflammation (DYSM) was autoclaved and prepared as described in Matherials and Methods. Wt BMDCs were stimulated with either DYSM, LPSBV (100 ng mL^{-1}), E. coli mpk (EC, MOI1)) or simultaneous (DYSM + LPSBV). Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and normalized to the mock control of BMDCs generated from the same individual. Box plots depict the mean as well as the 25th and 75th percentile, whiskers depict the highest and lowest values.

b) wt BMDCs were stimulated (prime) with either PBS (mock) or LPSBV for 24 h. Medium was changed and cells were stimulated afterwards (challenge) with DYSM or EC (MOI 1). Cytokine secretion was detected by ELISA. Surface expression of MHC-II and CD40 was detected by flow cytometry and normalized to an unchallenged mock control of BMDCs generated from the same individual. Box plots depict the mean as well as the 25th and 75th percentile, whiskers depict the highest and lowest values.

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Gut Commensal-Induced IκBζ Expression in Dendritic Cells Influences the Th17 Response

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Intestinal commensal bacteria can have a large impact on the state of health and disease of the host. Regulation of Th17 cell development by gut commensals is known to contribute to their dichotomous role in promoting gut homeostasis and host defense, or development of autoimmune diseases. Yet, the underlying mechanisms remain to be fully elucidated. One candidate factor contributing to Th17 differentiation, and the expression of which could be influenced by commensals is the atypical nuclear IκB protein IκBζ. IκBζ acts as a transcriptional regulator of the expression of Th17-related secondary response genes in many cell types including dendritic cells (DCs). Insights into the regulation of IκBζ in DCs could shed light on how these immune sentinel cells at the interface between commensals, innate and adaptive immune system drive an immune-tolerogenic or inflammatory Th17 cell response. In this study, the influence of two gut commensals of low (*Bacteroides vulgatus*) or high (*Escherichia coli*) immunogenicity on IκBζ expression in DCs and its downstream effects was analyzed. We observed that the amount of IκBζ expression and secretion of Th17-inducing cytokines correlated with the immunogenicity of these commensals. However, under immune-balanced conditions, *E. coli* also strongly induced an IκBζ-dependent secretion of anti-inflammatory IL-10, facilitating a counter-regulative Treg response as assessed in *in vitro* $CD4^+$ T cell polarization assays. Yet, in an *in vivo* mouse model of T cell-induced colitis, prone to inflammatory and autoimmune conditions, administration of *E. coli* promoted an expansion of rather pro-inflammatory T helper cell subsets whereas administration of *B. vulgatus* resulted in the induction of protective T helper cell subsets. These findings might contribute to the development of new therapeutic strategies for the treatment of autoimmune diseases using commensals or commensal-derived components.

Keywords: dendritic cells, Th17, intestinal commensals, inflammatory bowel disease, immunogenicity, *Escherichia coli*, *Bacteroides vulgatus*, IκBζ

INTRODUCTION

With an area around 200 times larger than the skin, the gastrointestinal mucosa is the largest immunological organ in the body (1). It faces a challenging environment and needs to maintain a careful balance between fighting intestinal intruders and tolerating commensal and nutrition-derived antigens (2). Failure of maintaining gut homeostasis promotes a shift in the microbiota composition, known as dysbiosis and characterized by a loss of bacterial diversity and/or commensals, as well as a bloom of pathobionts (3). A dysbiotic microbiota has been associated with many multifactorial autoimmune diseases such as multiple sclerosis, type 1 diabetes mellitus and inflammatory bowel diseases (IBD) (2, 4).

Dendritic cells (DCs) play a major role in the regulation of gastrointestinal mucosal immunity since they are among the first-line antigen-presenting cells at mucosal surfaces and link the innate and the adaptive immune system (5). DCs encounter a diversity of gut microbes and respond by inducing either immune tolerance to harmless commensal-derived antigens or an inflammatory response to potential pathogens. DCs recognize various surface structures on bacteria, so-called microbe-associated molecular patterns (MAMPs), *via* their patter recognition receptors (PRRs), such as Toll-like receptors (TLRs) (6, 7). Upon sampling of these antigens, DCs undergo a differentiation process resulting in e.g., semi-mature (smDCs) or mature DCs (mDCs), characterized by low or high expression of activation and maturation markers, respectively (8, 9). Under homeostatic conditions, DCs orchestrate the differentiation of naïve $CD4^+$ T cells into functionally distinct T helper cell subsets by creating an environmental cytokine milieu required for the balanced co-existence of regulatory and inflammatory $CD4^+$ T cells (10). In this role, smDCs are known to induce T cell anergy and regulatory T cells (Tregs) whereas mDCs are potent antigen presenting cells promoting $CD4^+$ and $CD8^+$ T cell responses (9).

A subset of IL-17-secreting $CD4^+$ T cells (Th17 cells) plays a dichotomous role in gut homeostasis by promoting protection against fungal and bacterial pathogens on one side, and driving inflammatory pathology and development of autoimmune diseases on the other side (11, 12). The orphan nuclear receptor RORγt is the lineage-determining "master" transcription factor directing the production of the hallmark cytokines IL-17A, IL-17F as well as IL-21 and IL-22 (12, 13). Among these, especially IL-17A plays a dominant role in driving autoimmunity (13). Due to intrinsic instability and plasticity, Th17 cells are able to transdifferentiate to more inflammatory or regulatory phenotypes in response to fluctuating physiological environments (10, 12). Differentiation of Th17 cells is dependent on interleukin 6 (IL-6) and transforming growth factor β (TGFβ), whereas their full maturation depends on IL-1β and IL-23, possibly favoring their pathological activity in the induction of autoimmunity (14, 15). Beyond their demonstrated ability to secrete all these cytokines, how DCs influence plasticity and poise protective and inflammatory responses is not fully known (14).

Besides RORγt, another transcription factor required for Th17 development is the atypical inhibitor of the nuclear factor κB (IκB) protein IκBζ which harbors six ankyrin repeats at its carboxyl terminus, and is encoded by the *Nfkbiz* gene (16, 17). Also known as MAIL or INAP, IκBζ is expressed in a variety of cell types and is essential for the induction of a subset of secondary response genes, e.g., *Il-6*, *Il-12*, *Il-17*, and *Ccl2* (16, 18–20). Transcription of the *Nfkbiz* gene is rapidly induced as primary NFκB response gene upon TLR- and cytokine-receptor signaling (18, 19, 21). The necessity of IκBζ in Th17 development was shown in *Nfkbiz*$^{-/-}$ mice which were resistant to experimental autoimmune encephalomyelitis (EAE), a model of Th17-mediated autoimmune disease with a multiple sclerosis-like phenotype (16, 22).

IκBζ expression in DCs is of particular importance for regulating Th17 development due to the steering role of DCs in states of homeostasis and inflammation. Yet, the impact of a shifting microbiota, as it is observed in states of inflammatory and autoimmune diseases, on IκBζ-dependent, DC-mediated T cell differentiation has not been characterized. In this study, we elucidate the impact of two model gut commensals on the induction of Th17 responses mediated by DCs. We could show that the induction of IκBζ-expression by commensals with low (*Bacteroides vulgatus*) and high (*Escherichia coli*) immunogenicity positively correlates with their immunogenicity in DCs. Furthermore, in an *in vivo* mouse model of IBD, enhancing abundance of these commensals influenced the differentiation of intestinal T helper cells towards rather protective and regulatory phenotypes (*B. vulgatus*) or pro-inflammatory phenotypes (*E. coli*). This effect could experimentally be traced back to the differential expression of IκBζ in DCs.

MATERIALS AND METHODS

Bacteria

Escherichia coli mpk (23) was grown overnight in Luria-Bertani (LB) medium under aerobic conditions at 37°C and subcultivated in the same medium for 2.5 h the next day prior quantification to ensure logarithmic growth phase. *Bacteroides vulgatus* mpk (23) was cultivated in liver broth for 3 days and, prior to quantification, subcultivated in Brain-Heart-Infusion (BHI) medium for 2 days and anaerobic conditions at 37°C to ensure logarithmic growth phase.

Mice

Female C57BL/6NCrl (WT) mice were purchased from Charles River Laboratories. C57BL/6J-Rag1^{tm1Mom} (*Rag1*$^{-/-}$), *TLR2*$^{-/-}$, *TLR4*$^{-/-}$ and *TLR2*$^{-/-}$x*TLR4*$^{-/-}$ mice were obtained from Jackson Laboratories. *Nfkbiz*$^{-/-}$ mice were kindly provided by Dr. M Morimatsu (24) and bred from *Nfkbiz*$^{+/-}$ breeding pairs. For isolation of bone marrow and T cells, 6–12 week old mice were used. All mice were kept and bred under specific pathogen-free (SPF) conditions.

Cultivation and Stimulation of Bone Marrow-Derived Dendritic Cells

Bone marrow cells were isolated and cultivated as described previously (25). At day 7 after isolation, $CD11c^+$ bone marrow derived-dendritic cells (BMDCs) were used for *in vitro* experiments.

1×10^6 BMDCs/ml were stimulated with PBS (mock, Thermo Fisher Scientific), *B. vulgatus* or *E. coli* at a multiplicity of infection (MOI) of 1 at 37°C. 100 ng/ml isolated LPS of *B. vulgatus* [LPS_{BV}, isolated as described in (26, 27) and (28)] or *E. coli* [LPS_{EC}, isolated as described in (26, 27) and (28)] were used for stimulation. For stimulation with a complex microbiota, fecal samples were collected from SPF *Rag1*$^{-/-}$ mice prior to administration of bacteria and induction of colitis, as well as at the end of the experiment (see T cell transfer in *Rag1*$^{-/-}$ mice). Samples were weighed, dissolved in sterile PBS to a stock concentration of 50 mg/ml, heat-inactivated for 15 min at 80°C and filtered through a 100 µm cell sieve. Fecal samples were then further diluted in sterile PBS and BMDCs were stimulated with prepared fecal samples in a concentration of 100 µg/ml. Gentamicin (1 µg/ml) was added to all samples in order to prevent bacterial overgrowth and to create equal treatment conditions. Cells were harvested after the indicated stimulation periods and processed for subsequent analyses.

Cultivation and Stimulation of mICcl2 Cells

Trans-immortalized mouse intestinal epithelial cells derived from the small intestine of a transgenic mouse were cultured as described elsewhere (29). One day prior stimulation, mICcl2 cells were seeded at a concentration of 5×10^5 cells/ml. Cells were stimulated with PBS (mock), *B. vulgatus* or *E. coli* at a MOI of 10 for 2 h and gentamicin (1 µg/ml) was added to all samples in order to prevent bacterial overgrowth and to ensure equal treatment conditions. Cells were gently detached with 0.05% trypsin-EDTA (Gibco) from culture vessels and processed for further analysis.

Isolation of Naïve T Cells

For adoptive T cell transfer and *in vitro* T cell polarization assay, splenic naïve $CD3^+CD4^+CD25^-CD45RB^+$ T cells from female WT mice were purified using a MACS-based negative selection kit (Miltenyi) according to the manufacturer's instructions.

T Cell Polarization Assay

Antigen-independent activation of naïve $CD4^+$ T cells occurred by overnight incubation with plate-bound anti-CD3 (145-2C11) antibodies (BioLegend, coated with 10 µg/ml in PBS) and 2 µg/ml anti-CD28 (37.51) (BioLegend). As polarizing factor, sterile-filtrated cell culture supernatants of 16 h-stimulated BMDCs containing stimulus-dependent cytokine concentrations were used, diluted 1:2 in T cell medium (RPMI 1640 supplemented with 10% FCS, 50 µM 2-mercaptoethanol, 25 mM HEPES, 1% non-essential amino-acids, 1% sodium pyruvate and 1% penicillin/streptomycin). In order to mimic an imbalanced cytokine milieu, neutralizing anti-IL-10 (JES5-2A5) antibodies (BioLegend, 10 µg/ml) were added to naïve T cells simultaneously with the BMDC supernatant. Cells were incubated for 4 h with GolgiStop (BD) prior to end of polarization time and processed for flow cytometry analysis.

Dextran Sodium Sulfate (DSS)-Induced Colitis in WT and *Nfkbiz*$^{-/-}$ Mice

Acute DSS colitis was induced in SPF WT and *Nfkbiz*$^{-/-}$ mice by administration of 2.5% (w/v) DSS (molecular weight 36–50 kDa, MP Biomedicals) dissolved in drinking water for 7 days. Onset of inflammation was assessed on day 0 and on days 3–7 by monitoring body weight and disease activity index (DAI) with parameters ranging from 0–3 regarding blood in stool and on anus, stool consistency, relieving posture and appearance of fur. Colon tissue was used for histopathological analysis by fixing it in 4% formalin and sections stained with Hematoxylin/Eosin (H&E).

T Cell Transfer Colitis in Rag1-/- Mice

Administration of *B. vulgatus* or *E. coli* to 10 week-old *Rag1*$^{-/-}$ mice *via* drinking water in a concentration of 2×10^8 bacteria/ml started one week prior to intraperitoneal injection of $5x10^5$ naïve T cells. Replacing drinking water with bacteria and weighing of mice occurred twice a week. Mice were kept in IVCs in order to maintain stability of the newly developed microbiota composition. Fecal samples were collected prior to administration of bacteria and at the end of the experiment. Mice were sacrificed 5 weeks after T cell transplantation for analysis. Degree of colonic inflammation was determined using colonic histological sections, stained by H&E and scored as described elsewhere (30).

Isolation of Dendritic Cells and T Cells From Colonic Lamina Propria and Mesenteric Lymph Nodes

For isolation of colonic lamina propria (cLP) cells, caecum and colon were thoroughly washed with PBS and cut into 1.5 cm pieces, followed by two incubation periods in HBSS/5% FCS/2 mM EDTA/1 mM DTT, washing in HBSS/5% FCS/1 mM HEPES for 10 min and digestion of minced pieces in RPMI/40 U/ml DNase I/0.12 mg/ml collagenase for 15 min. All steps were performed at 37°C and gentle stirring, with vortexing and filtering through a 100 µM cell strainer in between single steps. Final cell suspension was washed twice with ice-cold HBSS/5% FCS. Immune cells from mesenteric lymph nodes (mLN) were isolated by gentle disruption and passing through a nylon cell strainer (40 µm mesh) with PBS/1% FCS and a washing step with PBS/1% FCS. T cells from cLP and mLN were activated with leukocyte activation cocktail (BD Biosciences) for 4 h with subsequent processing for flow cytometry analysis.

RNA Isolation and RT-PCR

Isolation of RNA from mICcl2 cells, BMDCs and colonic tissue lysates was performed using Qiagen's RNeasy Mini Kit according to manufacturer's instructions. Additional DNA digestion was conducted by using the DNA-free DNA Removal Kit (Thermo Fisher Scientific). SybrGreen based quantitative RT-PCR was performed on a Roche LightCycler480 using Qiagen SybrGreen RT-PCR Kit. Primer annealing occurred at 60°C. 10–100 ng DNase-digested RNA was used for qRT-PCR. Relative mRNA expression in cells stimulated with bacteria to unstimulated cells was determined by using β-actin as housekeeping gene according to the ΔΔCp-method, which takes into account the specific amplification efficiency of every primer pair and each PCR run. Primer sequences: *Nfkbiz* (NCBI Gene ID: 80859) forward: GTGGGAGAACAGATCCGACG, reverse: AGTGAGTGTC

GCTGAACCAG; β-actin (NCBI Gene ID: 11461) forward: CCCTGTGCTGCTCACCGA, reverse: ACAGTGT GGGTGACCCCGTC.

Quantification of Bacteria in Fecal Samples

Plasmid standards were generated by blunt-end cloning using pJET (Thermo Fisher Scientific) and the respective specific 16s PCR fragments of *E. coli* (Primer forward: GTTAATA CCTTTGCTCATTGA, reverse: ACCAGGGTATCTAATC CTGTT (31) or *B. vulgatus* (Primer forward: AACCTGCCG TCTACTCTT, reverse: CAACTGACTTAAACATCCAT (32). The concentration of the isolated plasmids was determined and the standard concentrations were prepared in 10-fold serial dilutions in a range of 100,000–10 copies. Bacterial DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions. DNA concentration was measured using Qubit dsDNA High Sensitivity Assay (Thermo Fisher Scientific). For the qPCR measurement, DNA concentrations were adjusted to 5 ng per reaction, and PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen). Bacterial copy numbers were determined by a standard curve. For this purpose, log10 of standard copy numbers were plotted against ct-values.

Cytokine Analysis

For determination of IL-6, IL-10, IL-23, IL-1β concentrations in cell culture supernatants, ELISA kits purchased from BD Biosciences or eBiosciences were used according to manufacturers' instructions. For detection of mouse serum cytokines, the Bio-Plex Pro assays Mouse Cytokine 23-Plex and sets for Mouse IL-17F, Mouse IL-21, Mouse IL-22, Mouse IL-23 and TGFβ1 (Bio-Rad) were performed according to manufacturer's instruction and analyzed on a Bio-Plex 200.

Flow Cytometry Analysis

After harvesting or isolation, mICcl2cells, BMDCs, cLP cells and mLN cells were washed and Fc-receptors were blocked for 15 min at 4°C. Staining with fixable viability dyes (Thermo Fisher Scientific) for 15 min at 4°C was applied for live-dead discrimination. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instructions, washed and resuspended in PBS/1% FCS/0.1% saponin. For intracellular staining and cell surface staining, cells were labeled for 30 min at 4°C with fluorophore-conjugated antibodies (all BD, if not stated otherwise) and washed twice. Flow cytometric analyses were performed on a FACS LSRII (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star Inc., USA). Antibodies: CD11c (HL3)-APC, CD11c (HL3)-PE-Cy7, CD4 (RM4-5)-BV605, CD45 (30-F11)-APC-Cy7, CD45R (RA3-6B2)-PE, CD64 (X54-5/7.1)-PE, Foxp3 (MF23)-AF647, Foxp3 (MF23)-BV421, GATA3 (L50-823)-PE-Cy7, IFNγ (XMG1.2)-PE-Cy7, IFNγ (XMG1.2)-APC, IFNγ (XMG1.2)-FITC, IκBζ (LK2NAP)-PerCP-EF710 (Thermo Fisher Scientific), IL-10 (JES5-16E3)-BV510, IL-10 (JES5-16E3)-FITC (BioLegend), IL-17A (TC11-18H10)-PE, IL-17A (TC11-18H10)-APC-Cy7, IL-4 (11B11)-PE, LY6G/C (RB6-8C5)-PE, I-A/I-E (MHC II) (AF6-120.1)-APC, I-A/I-E (MHC II) (AF6-120.1)-BV421, RORγt (Q31-378)-BV421, and T-bet (4B10)-BV421 (BioLegend).

Statistics

Statistical analysis of the data was performed with the GraphPad Prism 8 Software. Data were tested for normality using the Shapiro-Wilk normality test. Statistical analyses were then performed *via* unpaired student's t test or ANOVA for normally distributed data and Mann-Whitney or Kruskal-Wallis multiple comparison test for nonparametric statistics. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Error bars represent + standard deviation (SD).

RESULTS

Expression of IκBζ Promotes Intestinal Homeostasis in a Mouse Model of Acute Colitis

To confirm the role of IκBζ in the modulation of mucosal immune responses, we analyzed the impact of IκBζ-expression on the course of intestinal inflammation in a mouse model of dextran sodium sulfate (DSS) –induced acute colitis. Wild type (WT) and *Nfkbiz*$^{-/-}$ specific-pathogen-free mice were administered 2.5% DSS for seven days in order to induce acute colitis. The severity of disease was estimated by monitoring the weight of the mice and determining the disease activity index (DAI). *Nfkbiz*$^{-/-}$ mice were found to be significantly more susceptible to DSS colitis, as shown by a significantly increased weight loss and DAI, as well as clear signs of severe colitis as shown by histopathological examination of colon sections (**Figure 1**). Based on these results, we conclude that IκBζ plays an important role in maintaining intestinal homeostasis.

IκBζ Expression in BMDCs and Intestinal Epithelial Cells Is Differentially Modulated by Distinct Commensals

Next, we assessed the contribution of two model gut commensals to IκBζ-dependent activation and maturation of DCs. The mouse gut commensal *B. vulgatus* exhibits low immunogenicity and induces smDCs in the colonic lamina propria (cLP), thus contributing to the promotion of homeostasis and prevention of intestinal inflammation in mouse models for colitis (8, 23, 33). *E. coli*, however, is strongly immunogenic and provokes a pro-inflammatory immune response by inducing mDCs, resulting in intestinal inflammation in *Il-2* deficient mice (8, 23, 34). Since bone marrow-derived dendritic cells (BMDCs) are phenotypically similar tointestinal lamina propria DCs (26) and can be generated in high numbers and comparable maturation status, we used BMDCs to evaluate IκBζ induction by *B. vulgatus* or *E. coli*. Wild type (WT) BMDCs were stimulated with either of the two commensals at a MOI of 1 for 16 h, and *Nfkbiz* gene expression as well as IκBζ protein levels were determined at different time points (**Figures 2A, B**, and **Supplementary Figure 1**). *E. coli* stimulation strongly induced *Nfkbiz* gene expression with a maximal

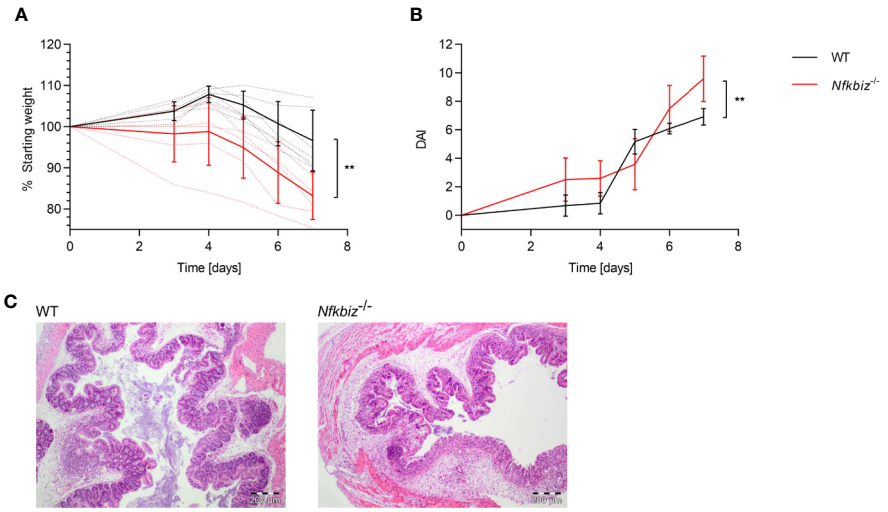

FIGURE 1 | IκBζ expression promotes intestinal homeostasis in the mouse model of DSS colitis. Wild type (WT) (n = 6) and *Nfkbiz*$^{-/-}$ (n = 6) specific-pathogen free (SPF) mice were administered 2.5% DSS (w/v) in drinking water for 7 days to induce colitis. **(A)** Changes in body weight were monitored throughout the experiment: dotted lines indicate each individual, and continuous lines indicate group means ± SD. **(B)** Disease activity index (DAI) was determined according to the criteria mentioned in the material and methods part, with indicated group means ± SD. **p < 0.005 **(C)** Representative H&E stained colon sections.

expression at 2 h post stimulation, followed by a decrease over time to levels close to the starting ones (**Figure 2A**). In contrast, stimulation of WT BMDCs with *B. vulgatus* did not significantly alter the basal levels of *Nfkbiz* gene expression. In agreement with the enhanced mRNA levels, 2 h stimulation of WT BMDCs with *E. coli* strongly increased the IκBζ protein levels in comparison to those in *B. vulgatus*-stimulated BMDCs, which did not differ much from the basal protein levels (**Figure 2B**). Yet, the IκBζ protein levels in *E. coli*-stimulated BMDCs did not decrease as strongly and rapidly as the mRNA levels, indicative of a stable protein. These results suggest that IκBζ expression in BMDCs is differently regulated by commensals, with *E. coli* provoking a strong cell response and *B. vulgatus* a weak one. Hence, the question arises whether IκBζ-mediated cytokine secretion required for T cell polarization is also influenced by commensals.

To address this, we measured the secreted levels of Th17-inducing cytokines IL-6, IL1β and IL-23 as well as anti-inflammatory IL-10 in cell culture supernatants of BMDCs derived from WT and IκBζ-deficient (*Nfkbiz*$^{-/-}$) mice after stimulation (**Figure 2C**). IL-6 and IL-1β are crucial for the induction of RORγt, whereas IL-23 is required for Th17 effector functions, since the receptor for IL-23 (IL-23R) is absent on naïve T cells (35). Upon 24 h-stimulation with *E. coli*, but not with *B. vulgatus*, WT BMDCs secreted significantly higher amounts of IL-6, IL-1β and a clearly higher amount of IL-23 than unstimulated WT BMDCs. In agreement with our previous findings (34), *E. coli* stimulation also significantly enhanced IL-10 secretion by WT BMDCs when compared to stimulation with *B. vulgatus*. Cytokine secretion in *B. vulgatus*-stimulated WT BMDCs was generally very low and did not significantly differ from that in unstimulated WT BMDCs. However, IL-6 and IL-10 secretion by *Nfkbiz*$^{-/-}$ BMDCs stimulated with *E. coli* was significantly lower than that in WT BMDCs, indicating that IL-6 and IL-10 production by BMDCs is dependent on IκBζ. In contrast, deficiency of IκBζ did not significantly reduce cytokine secretion in *B. vulgatus*-stimulated or unstimulated BMDCs. Hence, IκBζ-mediated cytokine secretion by BMDCs seems to be dependent on a strong stimulus, as provided by *E. coli*.

Since DCs are not the only cell type in the gut expressing IκBζ and in direct contact to the microbiota, we also analyzed commensal-mediated effects on IκBζ-expression in mouse intestinal epithelial cells. We stimulated immortalized mouse small intestinal epithelial cells (mICcl2) cells with PBS (mock), *B. vulgatus* or *E. coli* for 2 and 4 h, and measured IκBζ protein and mRNA levels, respectively. Flow cytometry analysis revealed that, similar to what was observed in BMDCs, IκBζ protein levels were significantly higher in *E. coli*-stimulated cells compared to unstimulated or *B. vulgatus*-stimulated cells after 2 h (**Figure 2D** and **Supplementary Figure 2**). After 4 h, a strong induction of *Nfkbiz* gene expression could still be observed in *E. coli*-stimulated, but not *B. vulgatus*-stimulated cells (**Figure 2E**).

These results indicate that commensals display similar immunogenic effects on different cell types of the gut barrier, facilitating a uniform and coordinated immune response by different cell types.

Commensals Trigger Secretion of Th17-Inducing Cytokines in BMDCs *via* TLR4 Signaling

As previously described, immunogenicity of the model commensal bacteria *B. vulgatus* and *E. coli* is mainly mediated by their lipopolysaccharide (LPS) and affects both the maturation status and cytokine secretion of BMDCs (26, 34). To identify the bacterial MAMP and the host TLR responsible for the observed IκBζ induction, *Nfkbiz* gene expression, IκBζ protein and

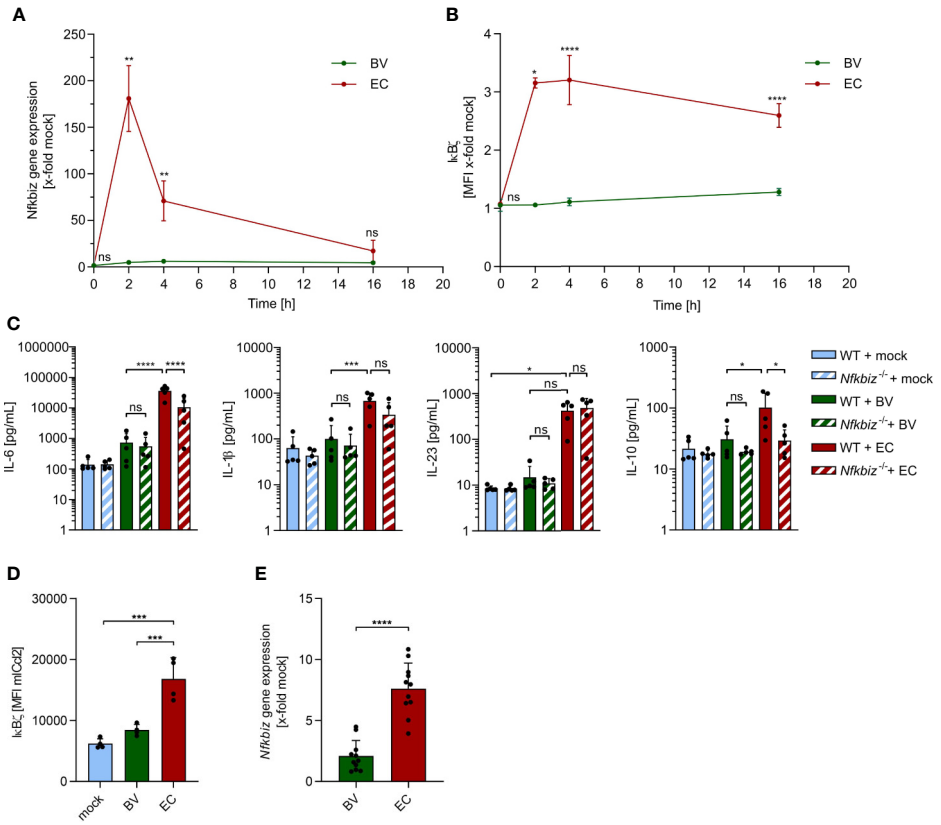

FIGURE 2 | The influence of *B. vulgatus* and *E. coli* stimulation on IκBζ expression in bone marrow-derived dendritic cells (BMDCs) and mICcl2 cells. **(A)** *Nfkbiz* gene expression after stimulation of wild type (WT) BMDCs with *B. vulgatus* (BV) or *E. coli* (EC) at the indicated time points for a total of 16h as determined by RT-PCR (n = 4). **(B)** The respective IκBζ protein levels were determined by flow cytometry analysis. **(C)** Cytokine secretion into cell culture supernatants of WT BMDCs and *Nfkbiz*$^{-/-}$ BMDCs after 24 h stimulation with mock, BV and EC was determined by ELISA (n = 5). **(D)** IκBζ protein levels after 2 h stimulation of mICcl2 cells with mock, BV or EC was determined by flow cytometry and **(E)** *Nfkbiz* gene expression after 4 h stimulation of mICcl2 cells with mock, BV or EC was determined by RT-PCR. Data are represented as geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

secreted cytokine levels were determined in stimulated BMDCs isolated from WT as well as TLR2 (*Tlr2*$^{-/-}$), TLR4 (*Tlr4*$^{-/-}$) and TLR2/TLR4 (*Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$) deficient mice. The significant reduction in *Nfkbiz* gene expression and IκBζ protein levels in *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$ BMDCs, but not *Tlr2*$^{-/-}$ BMDCs, suggested that the TLR4 ligand LPS was mainly responsible for the high IκBζ induction in *E. coli*-stimulated WT BMDCs (**Figures 3A, B**). In *Tlr2*$^{-/-}$ × *Tlr4*$^{-/-}$ BMDCs, the induced levels were even slightly but not significantly lower than those of single knockouts *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ BMDCs, suggesting a synergistic effect of TLR2 and TLR4 signaling upon strong immunogenic stimulation. Deficiency of TLR2 and/or TLR4 did not significantly influence IκBζ induction in *B. vulgatus*-stimulated BMDCs, emphasizing the low immunogenicity of this commensal. TLR4 signaling was also responsible for the secretion of Th17-inducing cytokines, since the amount of secreted IL-6, IL-1β, IL-23 and IL-10 was significantly reduced in *Tlr4*$^{-/-}$ BMDCs despite a strong stimulus, and slightly but not significantly lower in TLR2 and/or TLR4-deficient BMDCs stimulated with weakly immunogenic *B. vulgatus* (**Figure 3C**).

To evaluate the role of IκBζ in TLR-dependent DC maturation, we correlated the percentage of highly mature BMDCs, as indicated by MHC II^{hi} expression, 16 h post stimulation with the IκBζ protein levels measured 2 h after stimulation in these cells (**Figure 3D**). A positive correlation could be observed which decreased upon deficiency for TLR2 and/or TLR4, suggesting a possible role for IκBζ in TLR-ligand induced maturation processes of BMDCs.

To confirm LPS as the main trigger for IκBζ expression, WT BMDCs were stimulated for 2 h with *B. vulgatus*, *E. coli* and the respective LPS (LPS_{BV} and LPS_{EC}). As expected, IκBζ protein levels normalized to levels in unstimulated BMDCs did not significantly differ between *B. vulgatus* and LPS_{BV} as well as between *E. coli* and LPS_{EC} (**Figure 3E**). Furthermore, LPS_{EC}-induced IκBζ levels were significantly higher than the LPS_{BV}-induced protein levels, mirroring the results obtained with *B. vulgatus* and *E. coli* stimulation. This data suggests that the immunogenicity-dependent effects of *B. vulgatus* and *E. coli* on IκBζ expression, cell maturation and cytokine secretion are mediated by their LPS.

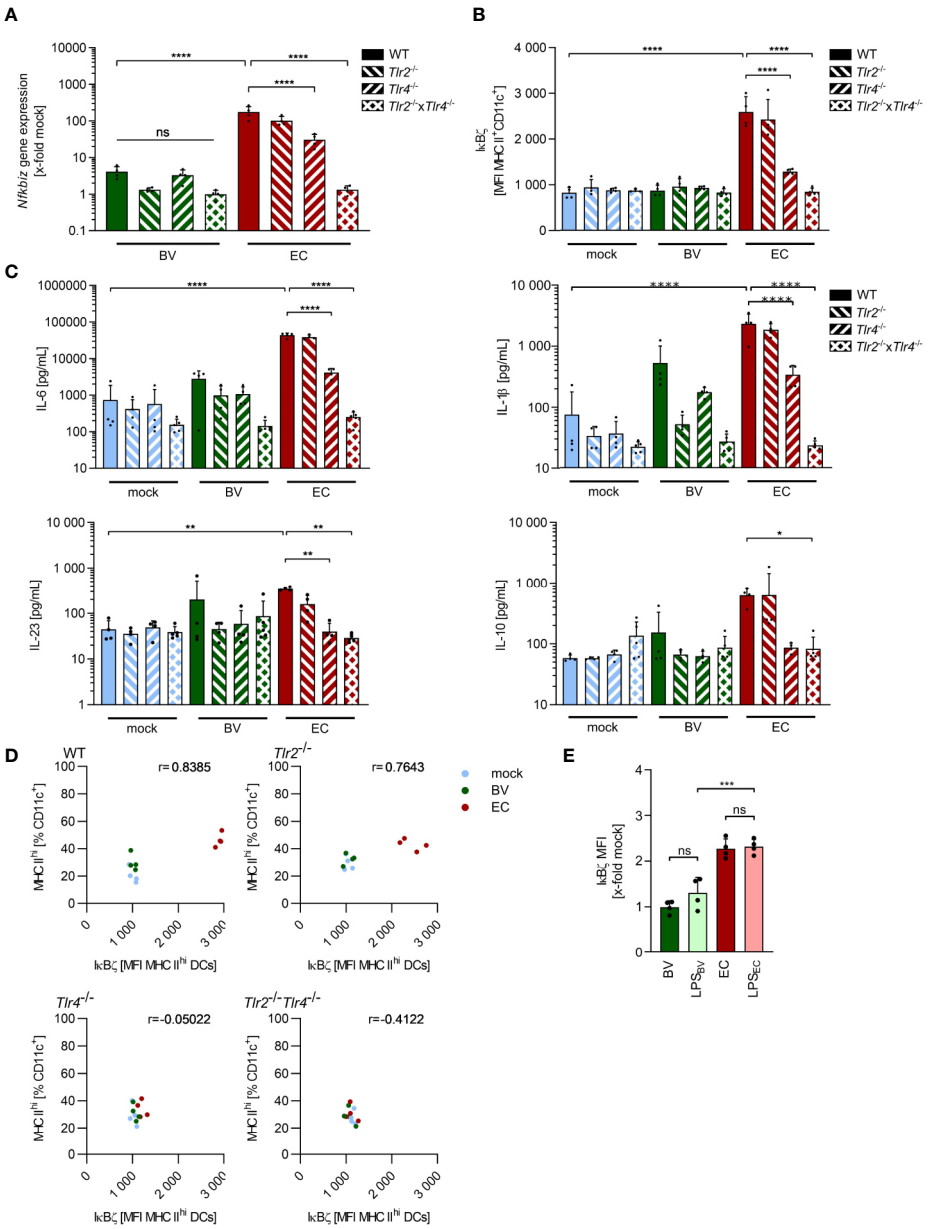

FIGURE 3 | IκBζ induction in bone marrow-derived dendritic cells by commensals is mainly TLR4-dependent. **(A)** *Nfkbiz* gene expression in wild type (WT), *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$x *Tlr4*$^{-/-}$ BMDCs after stimulation with *B. vulgatus* (BV) or *E. coli* (EC) as determined by RT-PCR. **(B)** The respective IκBζ protein levels were determined by flow cytometry after 2 h of stimulation. **(C)** Cytokine secretion into cell culture supernatants after 16 h of stimulation was determined by ELISA. **(D)** Correlation between percentages of MHC II^{hi}-expressing BMDCs after 16 h of stimulation and the IκBζ levels after 2 h with indicated Pearson r **(E)** IκBζ levels in WT BMDCs after stimulation with BV, LPS_{BV} (100 ng/ml), EC, LPS_{EC} (100 ng/ml) for 2 h, normalized to IκBζ levels in unstimulated WT BMDCs. Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

The Unique Composition of the Cytokine Milieu in Response to Various Commensals Differentially Polarizes T Cells

Antigen-inexperienced, i.e., naïve, CD4$^+$ T cells can differentiate into multiple lineages upon activation, depending on the local environment mainly defined by the composition and concentration of the available cytokines (36, 37). As we observed a distinct cytokine secretion pattern in response to *B. vulgatus* and *E. coli*, we analyzed the influence of the different cytokine milieu on CD4$^+$ T cell differentiation. To this aim, we antigen-independently activated naïve CD4$^+$ T cell with plate-bound anti-CD3 and soluble anti-CD28, and defined their polarization fate in response to sterile-filtrated cell culture supernatant (SN) of BMDCs previously stimulated for 16 h

with PBS (mock SN), *B. vulgatus* (BV SN), or *E. coli* (EC SN) (**Figure 4A** and **Supplementary Figure 3**). As control, differentiation in presence of the sole BMDC medium was performed.

To mimic an imbalance between pro- and anti-inflammatory cytokines as reported for the pathogenesis of autoimmune diseases (38), we added neutralizing anti-IL-10 antibody to the cell culture supernatants (medium + anti-IL-10, mock SN+ anti-IL-10, BV SN + anti-IL-10, EC SN + anti-IL-10). Neutralization of extracellular IL-10 appeared to slightly reduce T cell survival after 72 h of incubation (**Figure 4B**). Yet, it also induced a more pronounced differentiation of naïve T cells into Th1 (IFNγ+CD4$^+$) and Th2 (IL-4$^+$CD4$^+$) effector helper subsets. Furthermore, it significantly increased differentiation into Th17 cells (IL-17$^+$ CD4$^+$) when present alone (BMDC medium only) and in combination with BV SN or EC SN. Yet, BV SN and EC SN induced similar levels of Th17 cells, under both balanced and imbalanced cytokine conditions.

Th17 cells are known to have certain plasticity. On the one hand, they are able to convert to Th1-like Th17 cells, co-expressing IL-17 and IFNγ, and contributing to increased inflammatory activity (39). On the other hand, anti-Th17 Treg cells co-expressing IL-17 and Foxp3 were shown to suppress CD4$^+$ T cell proliferation, and found in the inflamed intestinal mucosa of patients with Crohn´s Disease (40). Th17 cells co-expressing IL-17 and IL-10 are instead protective and prevent the accumulation and activity of inflammatory Th17 at sites of inflammation (41). Therefore, we further characterized differentiated Th17 cells with respect to the co-expression of IL-17 with IFNγ, Foxp3 or IL-10 to define their inflammatory or non-inflammatory potential. No significant influence of the differentiation environments on the subsets of Th17 cells was observed (**Figure 4B**, bottom panels). However, the percentage of Foxp3$^+$ IL-17$^+$ T cells and IFNγ$^+$ IL17$^+$ T cells significantly increased upon neutralization of IL-10 in BV SN, suggesting that, even in absence of anti-inflammatory IL-10, a balanced Th17 immune response is guaranteed by an increased number of anti-Th17 Tregs.

E. coli Promotes a Pro-Inflammatory CD4$^+$ T Cell Response in the Mouse Model of T Cell Transfer Colitis

The initial lack of Tregs and induction of inflammatory Th1 and Th17 cells are known to play a role in disease onset in the T cell transfer model of colitis in *Rag1*$^{-/-}$ mice (42, 43). Transfer of naïve T cells into these immune-deficient mice lacking functional T cells and B cells induces a chronic colonic inflammation that is largely dependent on the microbiota composition (44).

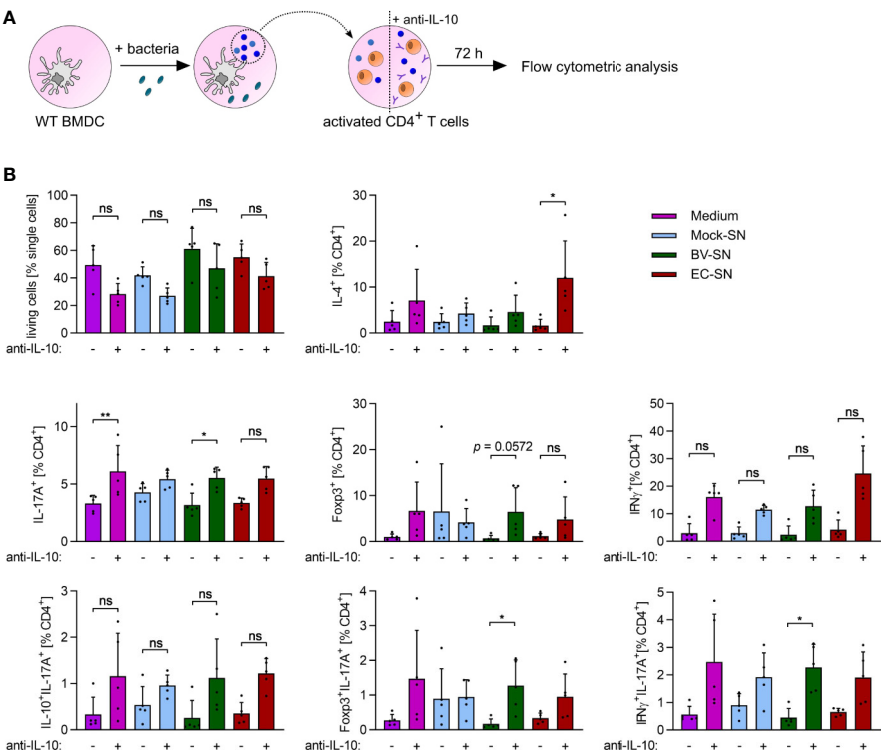

FIGURE 4 | *In vitro* CD4$^+$ T cell polarization in response to the supernatant of BMDCs. **(A)** Overview of the experimental setup: WT BMDCs were stimulated with PBS (mock), *B. vulgatus* (BV) or *E. coli* (EC) for 16 h. The resulting supernatants were sterile-filtrated and used for differentiation of anti-CD3/anti-CD28-activated WT CD4$^+$ T cells with or without addition of neutralizing anti-IL-10 antibodies (10 µg/ml). After 72 h, T cells were analyzed by flow cytometry. **(B)** Flow cytometry analysis of differentiated CD4$^+$ T cell subsets after incubation with T cell medium or supernatants of mock, BV or EC-stimulated WT BMDCs with or without addition of neutralizing anti-IL antibodies (10µg/ml) (n = 5). Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005.

We therefore analyzed the impact of administration of a symbiont or a pathobiont, respectively, on DC responses and T helper cell polarization in the colonic lamina propria (cLP) and mesenteric lymph nodes (mLN). SPF *Rag1*$^{-/-}$ mice were administered either *B. vulgatus* or *E. coli* by continuous administration of 2×10^8 bacteria per mL drinking water, starting one week prior to transplantation of 5×10^5 naïve T cells (**Figure 5A**). Mice were weighed and drinking water renewed twice a week. Mice were sacrificed five weeks after T cell transplantation.

Administration of *B. vulgatus* or *E. coli* did not lead to significant differences in weight loss over time (**Figure 5B** and **Supplementary Figure 4**). However, a slightly accelerated weight loss was observed in *E. coli*-administered mice starting three weeks after T cell transplantation compared to mice administered with either *B. vulgatus* or no bacteria. Furthermore, a high variation within experimental groups was observed, as indicated by large standard deviations in **Figure 5B**. To evaluate the influence of *B. vulgatus*- and *E. coli*-administration on systemic inflammation, the concentration of serum cytokines was determined (**Figure 5C**). No significant differences were observed between the different experimental groups with the exception of increased IL-21 levels in some *E. coli*-administered mice. Serum concentrations of Th17-inducing IL-6 and IL-1β were very low with IL-23 concentrations even under the detection limit in all mice. Concentrations of the anti-inflammatory IL-10 did positively correlate with concentrations of many pro-

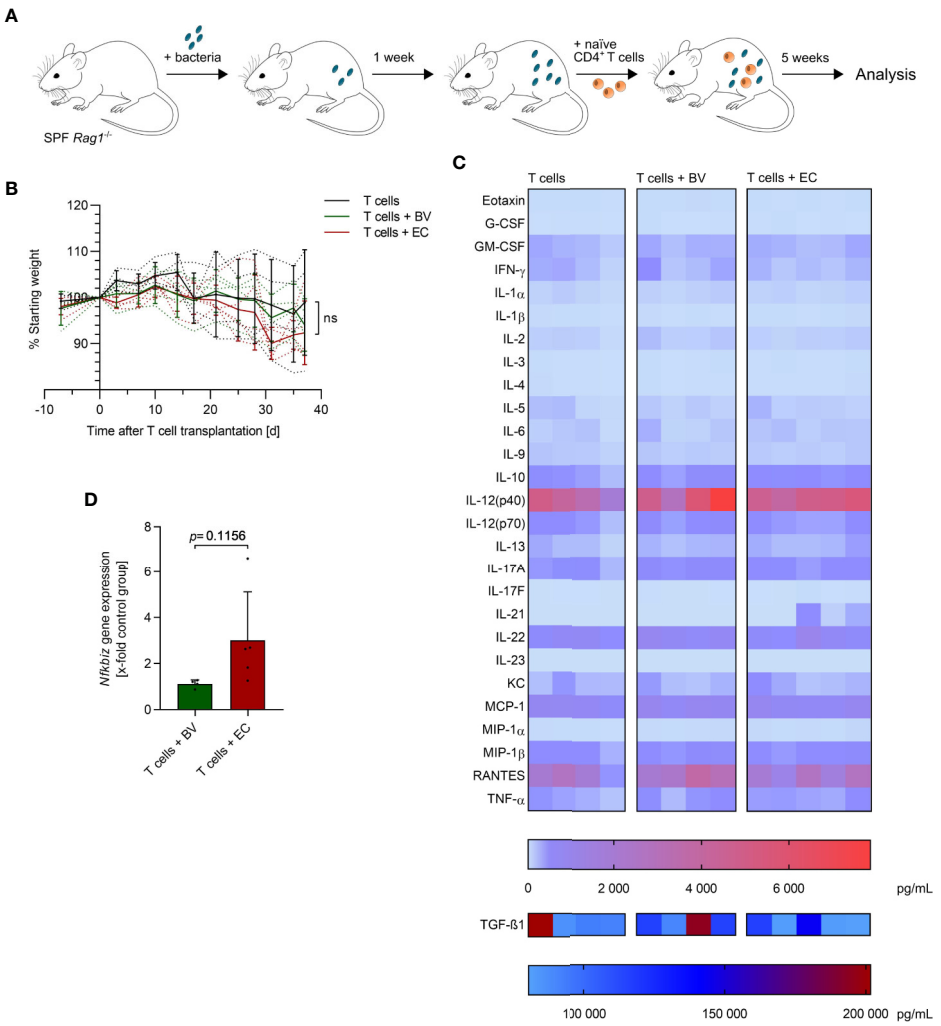

FIGURE 5 | *E. coli*-administration accelerates colitis induction in a T cell transfer model of colitis. **(A)** Overview of the experimental setup: specific-pathogen free (SPF) *Rag1*$^{-/-}$ mice were continuously administered with *B. vulgatus* (n = 4) or *E. coli* (n = 5) *via* drinking water. A control group was left untreated (n = 4). After one week of bacterial association, naïve CD4$^+$ T cells were transplanted. Mice were sacrificed 5 weeks after T cell transplantation. **(B)** The change in bodyweight was monitored throughout the experiment: dotted lines indicate each individual and continuous lines indicate group means ± SD, ns, not significant. **(C)** The concentration of the indicated cytokines was determined in mouse serum, using Bio-Plex assays. Each column represents one individual. **(D)** RNA was isolated from colonic tissue and *Nfkbiz* gene expression was determined by RT-PCR. Data represent geometric mean + SD.

inflammatory cytokines (**Supplementary Figure 5**), indicating a systemic repressive function. Yet, *Nfkbiz* gene expression in colonic tissue was found to be higher upon *E. coli*-administration, compared to *B. vulgatus*-administration, giving first hints of a more pronounced Th17 response to a microbiota rich in *E. coli* (**Figure 5D**).

Flow cytometry analyses revealed that total numbers of cLP DCs were significantly higher in the *B. vulgatus*-administered group compared to the control group, whereas *E. coli*-administration resulted in only slightly increased numbers (**Figure 6A** and **Supplementary Figure 6**). A positive correlation between the maturation status of cLP DCs, as indicated by MHC II^{hi} expression, and IκBζ protein levels in these DCs was observed, with only low percentages of highly mature DCs in *B. vulgatus* or *E. coli*-administered mice (**Figure 6B**, left panel). However, highly mature DCs with low IκBζ levels were observed in mLN of *B. vulgatus* or *E. coli*-administered mice (**Figure 6B**, right panel). In the control group, the percentage of $IκBζ^{hi}$ MHC II^{hi} mLN DCs remained low.

Total numbers of recruited cLP $CD4^+$ T cells were not found to be dependent on microbiota composition (**Figure 6C**). Yet, the polarization of these $CD4^+$ T cells seemed to be conditioned by microbiota composition: enhanced abundance of *B. vulgatus* clearly induced more Tregs than *E. coli*-administration or no microbiota-manipulation. Also the total numbers of $Foxp3^+$ $IL\text{-}17^+$ anti-Th17 Tregs were significantly higher, and total numbers of $IL10^+$ $IL\text{-}17^+$ protective Th17 cells were slightly higher in *B. vulgatus*-administered mice compared to the other groups. In contrast, *E. coli*-administration resulted in significantly higher total numbers of Th1 cells and slightly higher total numbers of Th1-like Th17 cells than the control group, but at levels similar to those detected in the *B. vulgatus*-administered group. Total numbers of Th2 cells were significantly higher in *B. vulgatus*- or *E. coli*-administered groups compared to the control group. Taken together, polarization of $CD4^+$ T cells in the cLP is tilted to rather anti-inflammatory and regulatory phenotypes in *B. vulgatus*-administered mice whereas in *E. coli*-administered mice, cLP $CD4^+$ T cells express rather pro- inflammatory markers, promoting colonic inflammation.

In order to evaluate the role of DCs in the induction of the observed phenotypes and overall disease progression, we correlated IκBζ-expression in cLP DCs with $CD4^+$ T cell phenotypes in cLP and mLN (**Figure 6D**). IκBζ-expression in cLP DCs was negatively correlated with total numbers of cLP Th1 cells in all experimental groups (**Figure 6D**, left panel). Furthermore, induction of the anti-Th17 Tregs seemed to be dependent on intermediate IκBζ-levels as observed in the *B. vulgatus*- administered group (**Figure 6D**, right panel). High and low IκBζ-levels in cLP DCs in control or *E. coli*-administered mice did not correlate with high numbers of anti-Th17 Tregs.

With respect to the classical Th1/Th2 balance, a shift towards autoimmune-disease promoting Th1 cells was observed in *E. coli*-administered mice whereas in *B. vulgatus*- administered mice the number of Th2 cells exceeded the number of Th1 cells (**Figures 6C, E**) (45). Consistently, the grade of colonic inflammation negatively correlated with the induction of protective Th17 cells in mLN, emphasizing the anti-inflammatory role of these cells (**Figure 6F**).

Increased Abundance of *B. vulgatus* in Microbiota Dampens the Secretion of Pro-Inflammatory Cytokines by BMDCs

The above presented results indicate that the enhanced intestinal abundance of *B. vulgatus* leads to an increase in regulatory/anti-inflammatory $CD4^+$ T cell subsets whereas higher numbers of *E. coli* promote differentiation of pro-inflammatory $CD4^+$ T cells in an immune-compromised host with a presumably dysbiotic microbiota. However, a differential activation of DCs by the two commensals could not be clearly observed in these mice. To directly link our *in vitro* results with those obtained in the model of T cell transfer colitis, we collected fecal samples from representative *Rag1*$^{-/-}$ mice with a presumably dysbiotic microbiota (DYS) prior to bacterial administration and from T cell transplanted mice with or without commensal enrichment after development of colitis (DYS + TC, DYS + TC + BV, DYS + TC + EC). Heat-inactivated fecal samples were then used to stimulate WT BMDCs (**Figure 7A**). Increased abundance of *E. coli* induced significantly higher *Nfkbiz* gene expression (**Figure 7B**) and IκBζ–protein levels (**Figure 7C**) than all the other microbiota. However, we did not observe a significant increase in the secretion of pro-inflammatory cytokines in response to DYS + TC + EC- stimulation as compared to DYS+TC (**Figure 7D**). Rather, 4 h stimulation of BMDC with DYS + TC + BV decreased the secretion of pro-inflammatory cytokines, with a slight but not significant decrease of secreted IL-6, and a significant lower secretion of IL-23 compared to DYS + TC or DYS + TC + EC. The levels of IL-10 decreased as a result of T cell transfer.

DISCUSSION

The impact of the intestinal microbiota on health and disease is indisputably large. Due to the close link between microbiota and host immunity, it is not surprising that dysbiosis is associated with many diseases linked to a malfunctioning immune system, e.g., autoimmune diseases. A local impact of a disturbed microbiota is well described for inflammatory bowel diseases (IBD) such as Crohn's disease or ulcerative colitis). Moreover, many extra-intestinal diseases such as type 1 diabetes, rheumatoid arthritis, asthma or multiple sclerosis have been reported to be influenced by the microbiota (3, 46).

The first model commensal used in this study, *B. vulgatus*, belongs to the phylum of Bacteroidetes, one of the most abundant phyla in the mammalian gut, and was found to reduce inflammation in mouse models of colitis (23, 47). A decrease in *Bacteroides* species was reported in patients with IBD, together with the simultaneous increase of facultative anaerobes such as *E. coli*, the second model commensal used in this study (48). *E. coli* is a colitogenic pathobiont,that can promote intestinal inflammation in genetically predisposed hosts (23). Differences in bacterial immunogenicity as well as in the interaction with several cell types of the innate and adaptive

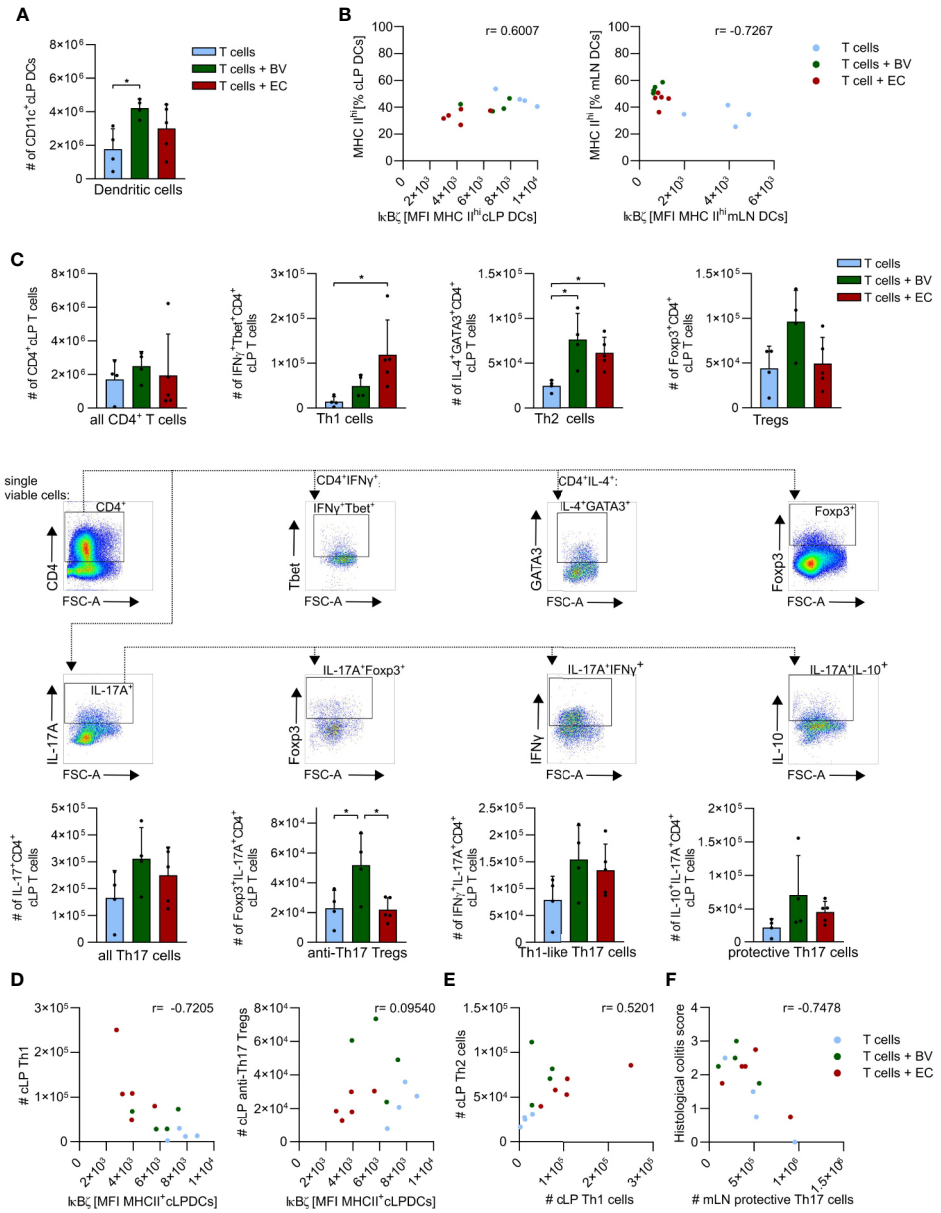

FIGURE 6 | *B. vulgatus* promotes induction of immune regulative mechanisms in a T cell transfer model of colitis. Dendritic cells (DCs) and CD4$^+$ T cells were isolated from the colonic lamina propria (cLP) and mesenteric lymph nodes of T cell transplanted *Rag1*$^{-/-}$ mice and analyzed by flow cytometry (see Fig.5) **(A)** Total number of cLP CD11c$^+$ DCs as determined by flow cytometry. **(B)** Correlation between percentages of MHC II^{hi} expressing cLP (left panel) and mLN DCs (right panel) and their IκBζ levels, indicated Pearson r. **(C)** CD4$^+$ T cell phenotypes in cLP with corresponding gating. **(D)** Correlations between IκBζ expression in cLP DCs and cLP Th1 cells or CLP anti-Th17 Tregs are indicated Pearson r. **(E)** Th1/Th2 balance in cLP. **(F)** Correlation between histological colitis score and number of cLP protective Th17 cells, as indicated Pearson r. Data represent geometric mean + SD, *p < 0.05.

immune system are accountable for these contrary outcomes. In this study, we focused on the interaction of commensals with DCs and the resulting CD4$^+$ T cell response *in vitro* and *in vivo* in an autoimmune-driven mouse model of colitis (49).

The role of IκBζ has already been extensively studied in various autoimmune diseases and cell types. For instance, keratinocyte-derived IκBζ was found to drive psoriasis (50, 51); and mice deficient in IκBζ are resistant to EAE due to a defect in Th17 development, explainable by the fact that IκBζ enhances IL-17 expression by directly binding to the regulatory region of the *Il-17* gene (16). However, IκBζ-deficient epithelial cells provoke a Sjögren's syndrome-like inflammation in mice, and IκBζ-deficient hepatocytes showed defective proliferation due to impaired TLR4-signaling (21, 52). Furthermore, IκBζ exerts both inhibitory and transcription-promoting effects on NFκB activity. The transcription factor NFκB plays an

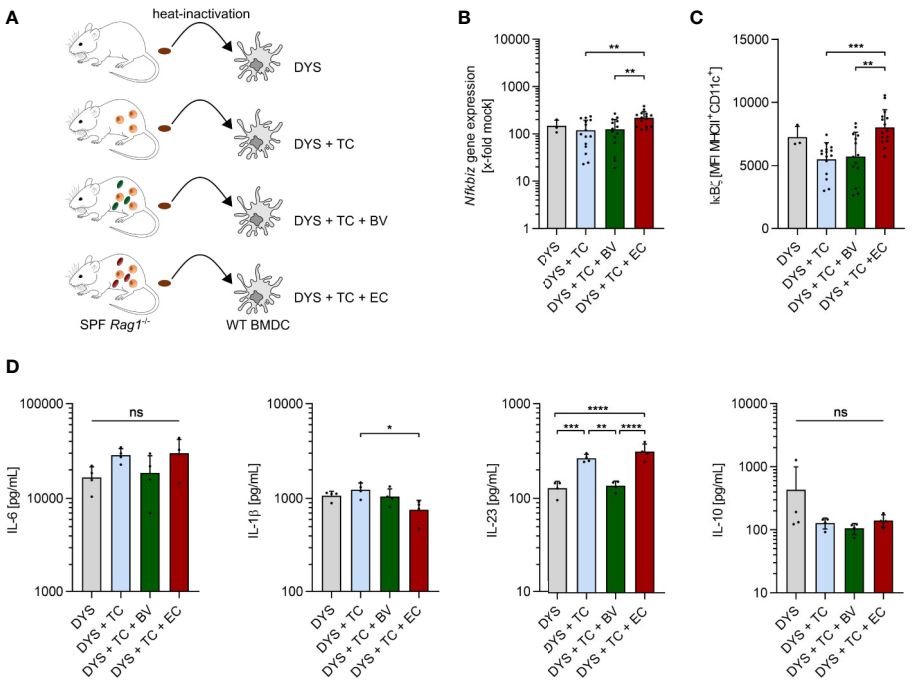

FIGURE 7 | Enhanced abundance of *E. coli* increases IκBζ levels in dendritic cells. **(A)** Overview of the experimental setup: fecal samples were collected from SPF *Rag1*$^{-/-}$ mice prior to bacterial administration and T cell transplantation (DYS) as well as from T cell transplanted mice with established colitis, and left untreated (DYS + TC) or administered with *B. vulgatus* (DYS + TC + BV) or *E. coli* (DYS + TC + EC) *via* drinking water. Samples were dissolved in PBS, heated for 15 min at 80°C, filtered and used in a concentration of 100 µg/ml for a 2 h and 4 h stimulation of wild type (WT) bone marrow-derived dendritic cells (BMDCs). **(B)** *Nfkbiz* gene expression, as determined by RT-PCR and **(C)** IκBζ protein levels, as determined by flow cytometry, after 2 h stimulation of WT BMDCs. **(D)** Secreted cytokines after 4 h stimulation of WT BMDCs, as determined by ELISA. Data represent geometric mean + SD, ns, not significant, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005.

importantrole in cellular responses to stress, injury and inflammation (53). Its subunits p50, p52, p65 (RelA), RelB and c-Rel can form various homo- and heterodimers which bind to specific DNA elements to induce target gene expression of e.g., IL-6, IL-1β, IL-23, or IL-10 (54–56). More recently, upregulation of *Nfkbiz* has been detected in inflamed intestinal tissue of UC patients, suggesting that an altered function of IκBζ may contribute to the development of the disease (57). We could demonstrate a deleterious effect of IκBζ deficiency in the mouse model of acute DSS-induced colitis (**Figure 1**). *Nfkbiz*$^{-/-}$ mice progressed towards a significantly more severe disease than WT mice, indicating an important role of IκBζ for intestinal inflammatory responses to DSS administration. Taken together, these data suggest that the function of IκBζ needs to be tightly regulated in many cell types: too much or too little of its activity can lead to disease.

We could also show that bacterial immunogenicity regulates IκBζ expression in DCs thus driving either an inflammation-promoting or tolerogenic DC phenotype. In our previous studies, we demonstrated that *B. vulgatus* induces smDCs, characterized by a lower expression of maturation markers, such as MHC II, CD40, CD80 and CD86, as well as lower secretion of pro-inflammatory cytokines compared to mDCs induced by *E. coli* (8, 58). These smDCs are tolerant towards maturation-inducing stimuli and are unable to induce pro-inflammatory Th1 and Th17 responses (8). Here, we could relate the expression levels of IκBζ to the degree of DC maturation and induction of T cell differentiation: *E. coli*, but not *B. vulgatus* increased the mRNA and protein levels of IκBζ (**Figures 2A, B**). Based on this, we propose that the transition from smDCs to mDCs requires a relief of the tight regulation on IκBζ expression and activity.

IκBζ is generally induced by stimulation with MAMPs or the cytokines IL-1, IL-17 and IL-18 (16, 20, 21, 57). Here, we could demonstrate that *E. coli*-induced IκBζ expression as well as BMDC maturation is mainly mediated by LPS *via* TLR4 signaling (**Figure 3**). Despite being one of the most conserved structures in Gram-negative bacteria, differences in immune-activating activities of LPS have been observed before: isolated LPS_{BV} displayed only weak agonistic interactions with the host MD2/TLR4 receptor complex, thus inducing smBMDCs, whereas isolated LPS_{EC} potently activated the MD2/TLR4 receptor complex, causing rather pro-inflammatory signaling by mBMDCs (26). We additionally demonstrate that the extent of LPS-induced TLR4 signaling impacts the ability of BMDCs to induce a Th17 response: a stronger activation significantly enhances secretion of Th17-promoting cytokines by BMDCs (**Figures 2C** and **3C**). In addition, we observed a synergistic effect of TLR2 and TLR4 signaling upon a strong stimulus, indicated by a decreased response in BMDCs deficient for both receptors compared to BMDCs deficient for only one of these TLRs. This observation supports earlier findings, describing a marked increase in pro-

inflammatory cytokine secretion by mouse peritoneal macrophages upon co-stimulation with TLR2 and TLR4 ligands compared to the stimulation of either receptor alone (59). As previously reported, only secretion of IL-6 and IL-10 was found to be IκBζ-dependent (21, 60). IL-6 is mainly induced by p65/p50 NFκB heterodimers and IL-10 by p50/p50 NFκB homodimers (56, 61). IκBζ preferentially associates with p50 present in p65/p50 heterodimers or p50/p50 homodimers, stabilizes promoter binding and thus assists expression of IL-10 and IL-6 (17, 60, 61). IL-1β is mainly induced by subunits p65 and cRel and, thus, presumably not preferentially bound by IκBζ (62). Nevertheless, an indirect influence of IκBζ activity on IL-1β secretion has been elucidated recently: IκBζ upregulates the transcription of the *Nlrp3* gene, which encodes the inflammasome component NLRP3 (63). Activation of the NLRP3 inflammasome leads to the cleavage of inactive pro-IL-1β into active IL-1β, which can then be secreted by the cell. Kim et al. reported that *Nfkbiz* deficiency results in impaired IL-1β secretion, which we could confirm (**Figure 2C**). An IκBζ-dependent regulation of IL-23 secretion by BMDCs was however not observed.

We tested whether the cytokine milieu of stimulated BMDCs is sufficient for determining the differentiation fate of already activated T cells. We could not observe significant induction of effector T cells (Th1, Th2, Tregs, Th17 subsets) (**Figure 4**). A slightly but insignificantly increased survival of T cells could be observed upon differentiation with the cytokine mix originating from *B. vulgatus*- and *E. coli*- stimulated BMDCs. Cytokines such as IL-6 serve as T cell survival factors and are secreted in higher amounts by BMDCs upon contact with bacterial antigens (**Figures 2C** and **3C**) (64). IL-10 is known to exert a critical role in limiting immune-mediated inflammation and to prevent autoimmune pathologies. IL-10 is broadly expressed by many cell types of the innate and adaptive immune system, serving as feedback negative regulator of the innate effector functions of macrophages, DCs and, indirectly, T cells. Furthermore, IL-10 stimulates its own production by enhancing differentiation of IL-10-secreting Tregs (65). Interestingly, creating an "imbalanced" pro-inflammatory cytokine environment by neutralizing IL-10 in the cytokine mixes led to a slightly decreased survival in all conditions tested. Nevertheless, it also increased differentiation of effector T cells. The cytokine mix secreted by *E. coli*–stimulated BMDCs induced significantly higher amounts of Th2 cells upon neutralization of IL-10, suggesting a Th2-inhibiting action of IL-10 upon exposure to strong stimuli. Since overshooting Th2 responses provoke allergic reactions, a strategy for inducing IL-10-secreting DCs with strong stimuli such as bacteria or bacterial components is of large therapeutic interest (66). Furthermore, DC-secreted IL-10 also appears to inhibit Th1 differentiation upon a strong stimulation, here represented by *E. coli*-stimulation. This effect could be abolished by neutralization of IL-10 and was less evident with the other cytokine mixes used. Neutralization of IL-10 in *B. vulgatus*-induced cytokine mixes resulted in significantly increased differentiation of Th17 cells, especially of those expressing Foxp3 and IFNγ. However, the percentage of induced $CD4^+$ T cells was relatively low, questioning the biological relevance of the observed differences. Comparatively high amounts of induced $Foxp3^+$ Tregs could be observed by cytokine mixes produced by unstimulated immature BMDCs, which was not significantly influenced by IL-10-neutralization. Immature DCs are known to promote T cell anergy and generate Tregs (5). Here, we suggest a Treg-promoting effect by immature DCs independent of antigen presentation and IL-10, which needs further investigation.

When we evaluated the immuno-modulating effects of *B. vulgatus* and *E. coli* under inflammatory conditions in a genetically predisposed host with a presumably dysbiotic microbiota, *E. coli* administration induced colitis slightly but not significantly quicker than an unchanged microbiota or *B. vulgatus*administration as indicated by accelerated weight loss beginning 3 weeks after T cell transfer (**Figure 5**). Nonetheless, flow cytometry analysis of the cLP immune cells revealed significant differences in $CD4^+$ T cell subsets even though the absolute numbers of $CD4^+$ T cells remained equal: In *B. vulgatus*-administered T cell-transplanted *Rag1*$^{-/-}$ mice, numbers of regulatory and anti-inflammatory T helper subsets were higher than upon *E. coli* administration or in T cell-transplanted control mice, indicating a potent regulation of inflammation (**Figure 6**). In contrast, *E. coli* administration resulted in high numbers of pro-inflammatory Th1 and Th1-like Th17 cells in the cLP, indicating an uncontrolled inflammation, which was not dampened by low numbers of regulatory $CD4^+$ T cell phenotypes. Administration of commensals thus seems to manipulate the Th1/Th2/Th17/Treg balance as well as the pathogenicity of induced Th17 cells. In previous studies, we had already observed that *B. vulgatus* impaired inflammation in T cell-transplanted *Rag1*$^{-/-}$ mice, whereas transplantation of *Enterobacteriaceae*-rich microbiota strongly exacerbated the course of colitis (27, 33). So far, the cellular mechanisms underlying the protective effect of *B. vulgatus* remained unknown. Here, we could shed light on the influence of the two commensal bacteria on T cell polarization and disease progression.

In addition to the *in vitro* experiments, we observed increased *Nfkbiz* gene expression in inflamed colonic tissue isolated from T cell-transplanted *Rag1*$^{-/-}$ mice administered with *E. coli*-compared to control or *B. vulgatus*-administered mice. This finding could not be completely traced back to intestinal DCs as sole source of IκBζ-expressing cells. Since mouse small intestinal epithelial cells also increased IκBζ expression upon stimulation with *E. coli in vitro* (**Figures 2D, E**), we assume that the measured *Nfkbiz* expression originated from intestinal epithelial cells.We are aware of the limitation that small intestinal cells do not fully recapitulate the response of colonic tissue and might not be a colonization site for these commensals.

In IBD, the colonic barrier is weakened, resulting in a close contactof the commensals with the epithelial layer (67). This increased contact to epithelial cells represents an antigen-overload, and can lead to an inappropriate and dysregulated response of $CD4^+$ T cells, resulting in pro-inflammatory phenotypes and, eventually, in chronic inflammation.

We cannot rule out a contribution of DC-derived IκBζ to the consolidation of inflammation. Intestinal DCs migrate to the mLN upon antigenic challengewhere they present microbiota-derived antigens to naïve T cells thus initiating an adaptive immune response. Recognition of the cognate antigen along with DC-secreted lineage specifying cytokines leads to the differentiation and proliferation of effector T cells, which migrate to the effector

site, e.g., the cLP (68, 69). On a first sight, low IκBζ levels observed *in vivo* in cLP and mLN DCs in bacteria-administered T cell-transplanted *Rag1*$^{-/-}$ mice would contradict the *in vitro* findings. Ir is however conceivable that cLP and mLN DCs represent DCs in different stages of maturation and differentiation. An earlier accumulation of IκBζ results in suppression of NFκB-induced gene transcription due to the inhibitory activity of IκBζ, creating a self-limiting negative feedback loop. We speculate that the *in vivo*-induced IκBζ expression levels are already diminished in the analyzed cLP and mLN DCs as a result of its self-limitation at a later time point of the maturation stage. WT BMDCs stimulated with heat-inactivated microbiota samples of T cell-transplanted *Rag1*$^{-/-}$ mice from the experiment discussed above confirmed the commensal-dependent IκBζ expression in DCs: Supporting the inflammation-dampening influence of *B. vulgatus* is the significantly decreased secretion of IL-23 by BMDCs stimulated with the microbiota of *B. vulgatus*-administered mice, a cytokine responsible not only for the maintenance of Th17 cells but also for the innate immune-based pathology (70).

In conclusion, our study suggests that modulating the host's immune response by commensal bacteria can define the outcome of a Th17-mediated disease, at least in part, *via* regulation of IκBζ in DCs. These findings can be applied for the optimization of microbiota-based therapeutic strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration. Protocols and experiments involving mice were reviewed and approved by the responsible Institutional Review Committee and the local authorities within H5/10, H1/15, §4 09.01.2015, §4 14.06.2017 and §4 28.09.2017 approval.

AUTHOR CONTRIBUTIONS

LM, AlS, KS-O, and J-SF conceived and designed the experiments. LM, MT, H-CL, JK, CK, AL, AG, AnS, and SM performed the experiments. LM, MT, HC-L, and J-SF analyzed the data. LM, AL, and J-SF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612336/full#supplementary-material

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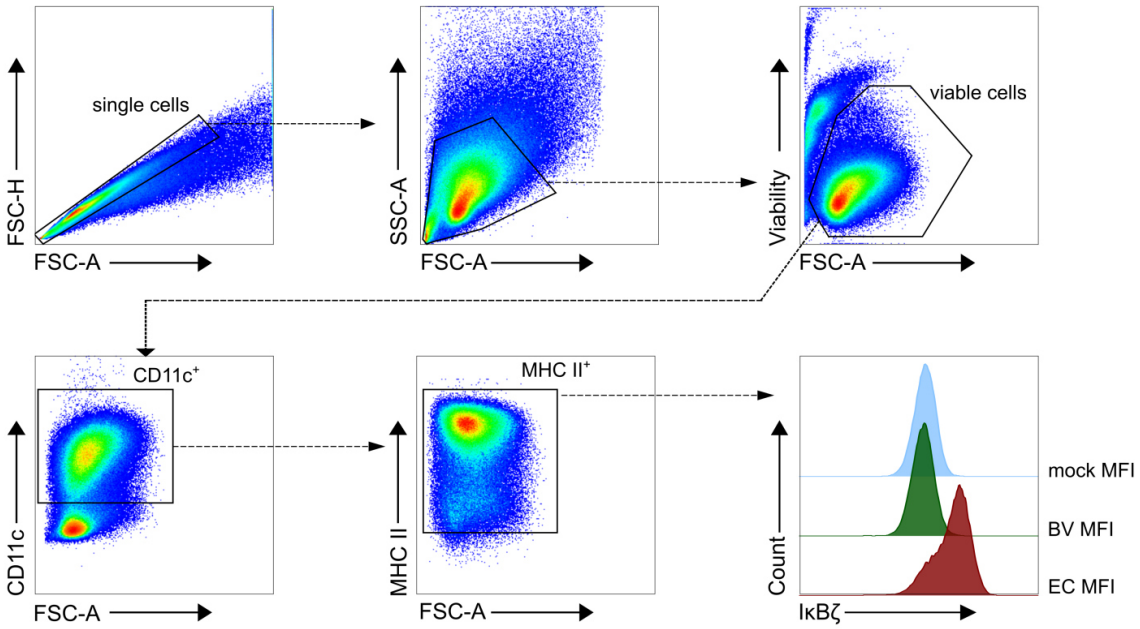
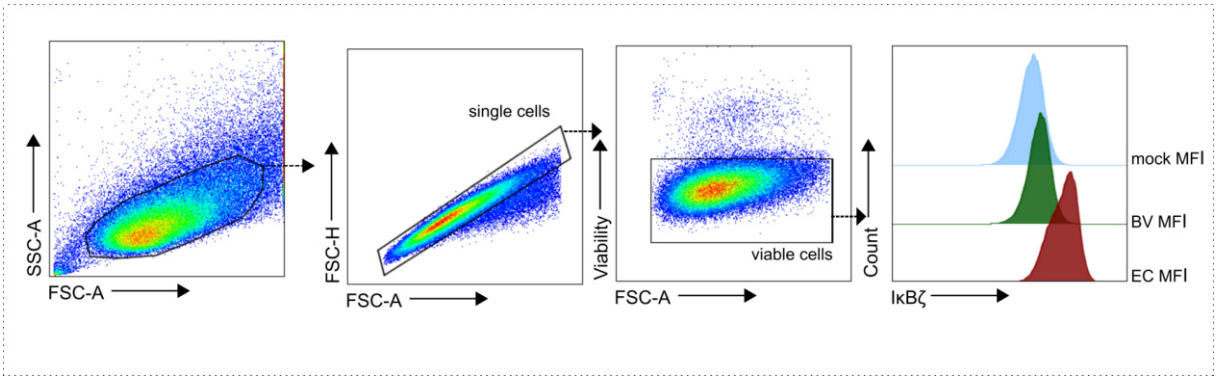
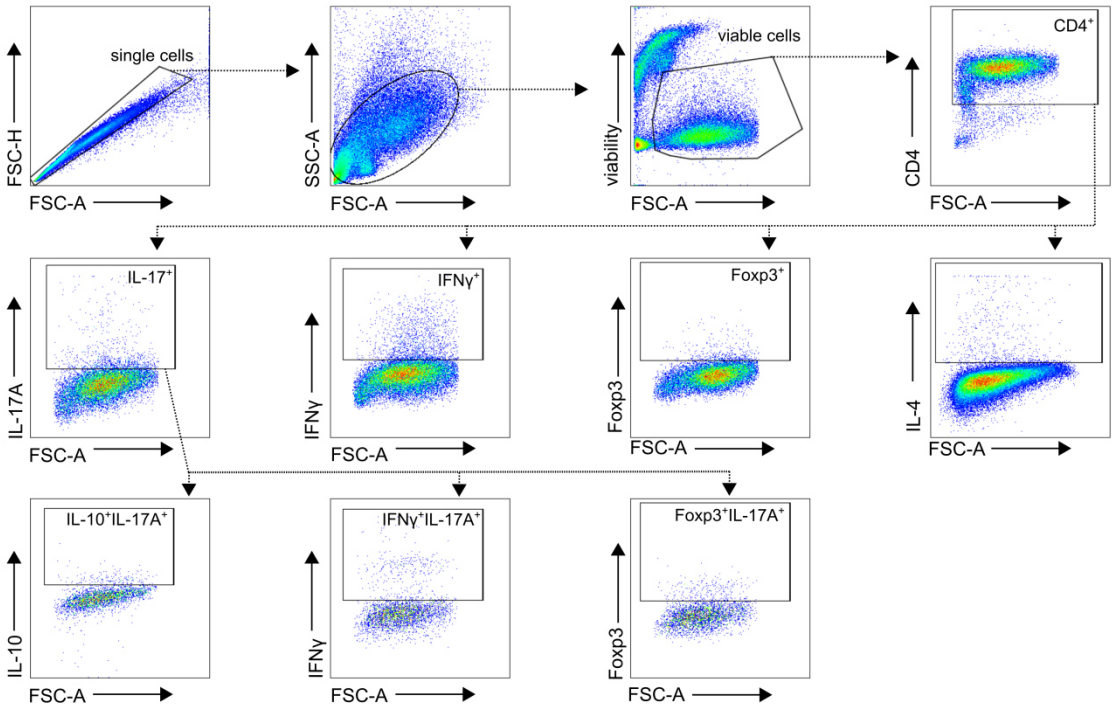


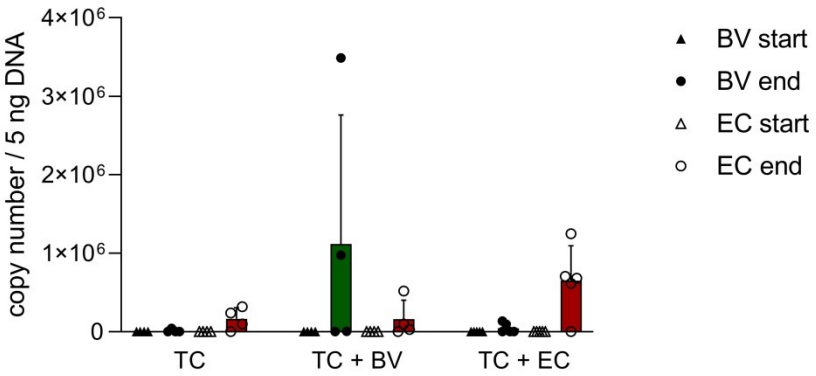
Supplementary Figure 1: Gating strategy applied for the flow cytometry analysis of bone marrow derived dendritic cells (BMDCs). In order to quantify IκBζ protein levels in BMDCs, cell doublets (FSC-A/FSC-H), cell debris (FSC-A/SSC-A) and dead cells (fixable viability dye $^{+}$) were excluded from further analysis. Mean fluorescence of IκBζ was determined in CD11c^{+} MHCII^{+} BMDCs. Here, representative histograms for IκBζ MFI in BMDCs stimulated with PBS (mock), *B. vulgatus* (BV) and *E. coli* (EC) are depicted.



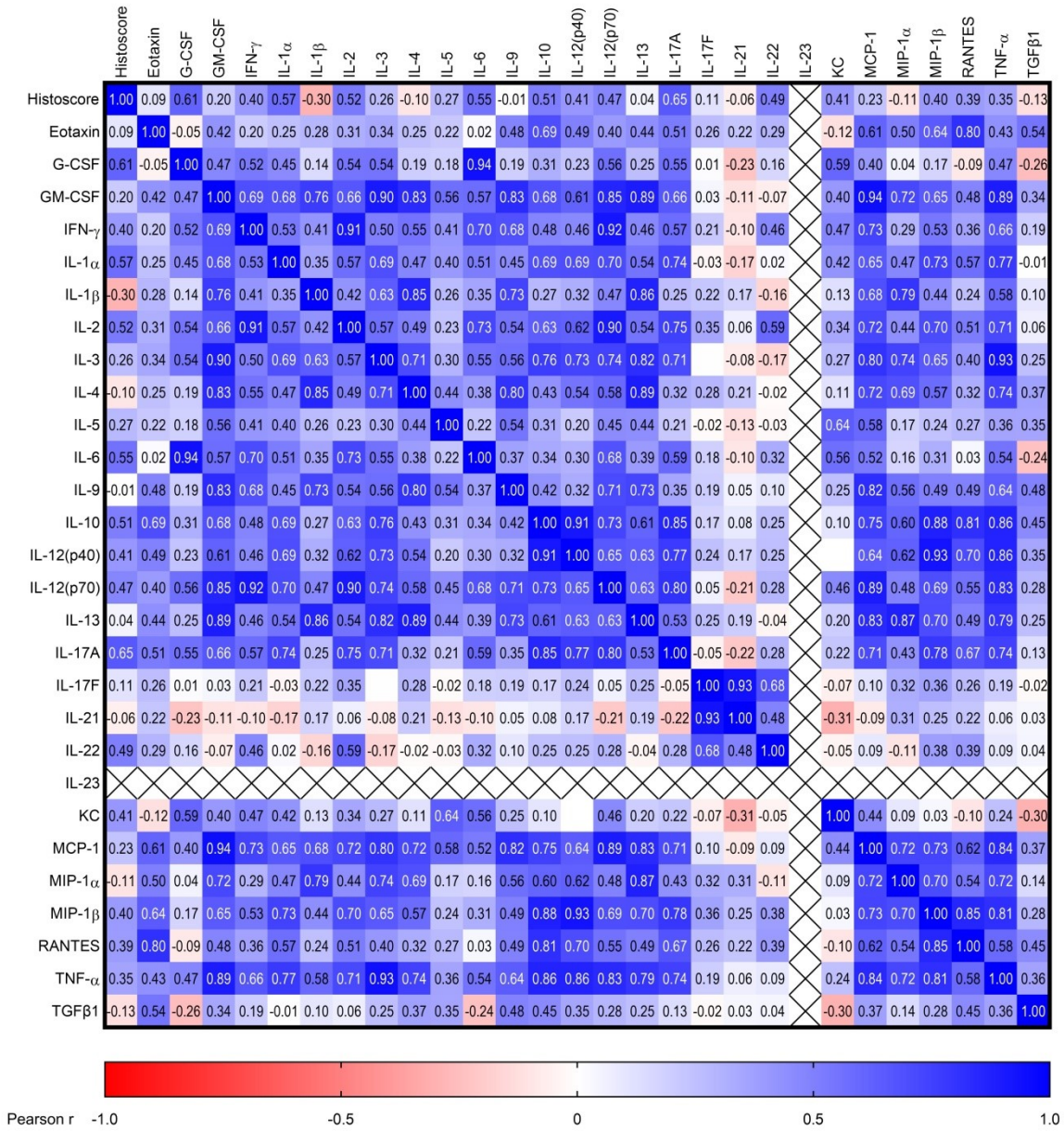
Supplementary Figure 2: Gating strategy applied for the flow cytometry analysis of mICcl2 cells. In order to quantify IκBζ protein levels in mICcl2 cells, cell debris (FSC-A/SSC-A), cell doublets (FSC-A/FSC-H) and dead cells (fixable viability dye $^{+}$) were excluded from further analysis. Mean fluorescence of IκBζ was determined in single, viable cells. Depicted here are representative histograms for IκBζ MFI in mICcl2 cells stimulated with PBS (mock), *B. vulgatus* (BV) and *E. coli* (EC).



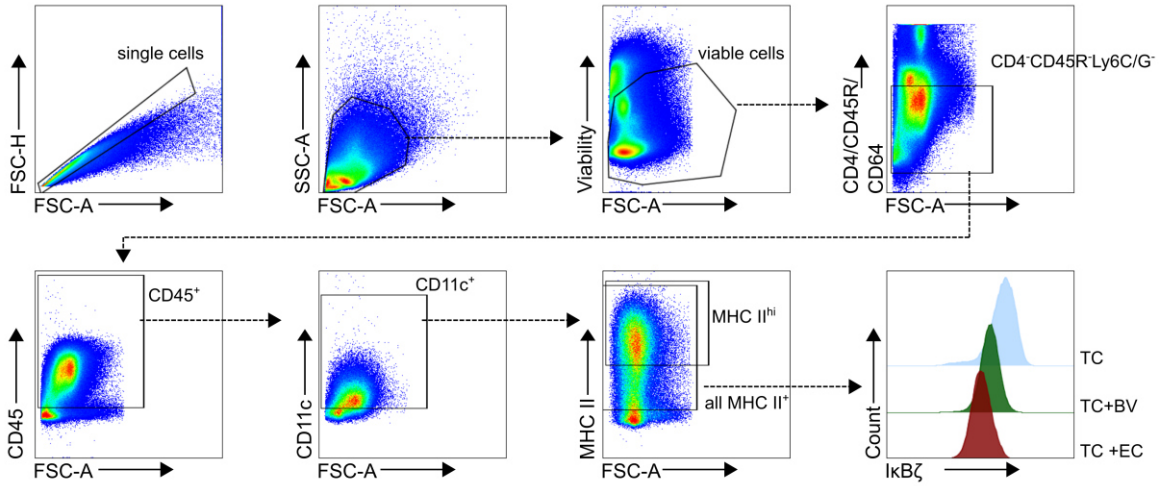
Supplementary Figure 3: Gating strategy applied for the flow cytometry analysis of CD4$^+$ T cell subsets. In order to analyze different CD4$^+$ T cell subsets, cell doublets (FSC-A/FSC-H), cell debris (FSC-A/SSC-A), and dead cells (fixable viability dye $^+$) and CD4$^-$ cells were excluded from further analysis. Subsets were determined with specific gates set according to FMO controls.



Supplementary Figure 4: Concentration of *B. vulgatus* and *E. coli* in fecal samples of *Rag1*$^{-/-}$ mice. Fecal samples were collected prior bacterial administration (BV start, EC start) and at the end of the experiment (BV end, EC end). Copy numbers of *B. vulgatus*- and *E. coli*-specific 16S rDNA genes were determined from 5 ng of gDNA



Supplementary Figure 5: Correlation matrix of histological colitis scores and respective serum cytokines of T cell-transplanted *Rag1*$^{-/-}$ mice for the experiment described in Fig. 5. Positive Pearson r value= positive correlation (blue), negative Pearson r value =negative correlation (red). IL-23: no Pearson r calculable since measured concentrations were under the detection limit.



Supplementary Figure 6: Gating strategy for flow cytometry analysis of cLP and mLN DCs. Cells were isolated from cLP and mLN of gnotobiotic WT mice or T cell transplanted *Rag1*$^{-/-}$mice from experiments described in Fig. 4 and Fig. 5. In order to quantify IκBζ protein levels in DCs, cell doublettes (FSC-A/FSC-H), cell debris (FSC-A/SSC-A), dead cells (fixable viability dye $^{+}$) and CD4^{+}CD45R^{+}Ly6C/G^{+} cells were excluded from further analysis. CD45^{+} CD11c^{+} cells were regarded as DCs and mean fluorescence of IκBζ was determined in MHC II^{+} DC and MHC II^{hi} DC populations. Here, representative histograms for IκBζ MFI in MHC II^{+} DCs isolated from T cell transplanted (TC) mice without or with *B. vulgatus* (TC + BV) or *E. coli* (TC + EC) administration are depicted.

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Bacterial Immunogenicity Is Critical for the Induction of Regulatory B Cells in Suppressing Inflammatory Immune Responses

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B cells fulfill multifaceted functions that influence immune responses during health and disease. In autoimmune diseases, such as inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis, depletion of functional B cells results in an aggravation of disease in humans and respective mouse models. This could be due to a lack of a pivotal B cell subpopulation: regulatory B cells (Bregs). Although Bregs represent only a small proportion of all immune cells, they exhibit critical properties in regulating immune responses, thus contributing to the maintenance of immune homeostasis in healthy individuals. In this study, we report that the induction of Bregs is differentially triggered by the immunogenicity of the host microbiota. In comparative experiments with low immunogenic *Bacteroides vulgatus* and strong immunogenic *Escherichia coli*, we found that the induction and longevity of Bregs depend on strong Toll-like receptor activation mediated by antigens of strong immunogenic commensals. The potent B cell stimulation via *E. coli* led to a pronounced expression of suppressive molecules on the B cell surface and an increased production of anti-inflammatory cytokines like interleukin-10. These bacteria-primed Bregs were capable of efficiently inhibiting the maturation and function of dendritic cells (DCs), preventing the proliferation and polarization of T helper (Th)1 and Th17 cells while simultaneously promoting Th2 cell differentiation *in vitro*. In addition, Bregs facilitated the development of regulatory T cells (Tregs) resulting in a possible feedback cooperation to establish immune homeostasis. Moreover, the colonization of germfree wild type mice with *E. coli* but not *B. vulgatus* significantly reduced intestinal inflammatory processes in dextran sulfate sodium (DSS)-induced colitis associated with an increase induction of immune suppressive Bregs. The quantity of Bregs directly correlated with the severity of inflammation. These findings may provide new insights and therapeutic approaches for B cell-controlled treatments of microbiota-driven autoimmune disease.

Keywords: immune regulation, homeostasis, microbiota, regulatory B cells (Bregs), interleukin 10 (IL-10), immunogenicity, inflammatory bowel disease (IBD), Toll-like receptors

INTRODUCTION

The fact that B cells play a critical role during the onset and course of inflammatory processes is indisputable and has been demonstrated in many studies in both mice and humans. However, these studies focused on the production of autoantibodies with B cells being understood as the cause for the development of inflammation, e.g., multiple sclerosis (MS) (1–3). Nevertheless, it is important to discriminate between the versatile functions of B cells. More and more studies have revealed that the depletion of B cells leads to an aggravation of disease in many autoimmune disorders, such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (4). B cells were shown to mediate an anti-inflammatory effect in mice that spontaneously develop chronic colitis, exhibiting more severe disease in the absence of B cells (5–9). Even in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, CD20 antibody-mediated B-cell depletion substantially exacerbated the disease when the treatment was initiated before EAE induction (10–12). This may link the activation of functional B cells with a suppressive effect in inflammation by promoting immune tolerance.

The beneficial influence of B cells during inflammatory processes is primarily attributable to a specific B cell population—regulatory B cells (Bregs) (13). Hitherto, no definitive phenotype with specific markers has been identified for Bregs. Certain phenotypes are characterized and described, but they differ in their expression of surface proteins. However, these phenotypes possess similar functionalities which explain the reason why the definition of Bregs is based on their immune-regulatory and anti-inflammatory capabilities (14). The main features of Bregs include the potent suppression of type 1 T helper (Th) cell differentiation, the inhibition of autoimmune pathogenesis and the maintenance of immune homoeostasis (15). The three most intensely studied Breg subsets are splenic transitional 2 marginal-zone precursor (T2-MZP) cells ($CD19^+CD21^{hi}CD23^{hi}CD24^{hi}$), B10 cells ($CD19^+CD5^+CD1d^+IL10^+$), and Tim-$1^+$ B cells ($CD19^+$Tim-1^+) (16–21). A common and most important feature of these B cell subsets is the production and secretion of the anti-inflammatory cytokine interleukin 10 (IL-10). IL-10 fulfills regulatory functions by effectively suppressing cell-mediated inflammatory responses, thus restoring Th1/Th2 balance (22–25). IL-10-producing B cells have also been identified in humans (26, 27). The powerful immune downregulation quality of IL-10-producing Bregs has already been shown in various autoimmune diseases, such as EAE, collagen-induced arthritis, lupus and inflammatory bowel disease (9, 28–31). Depending on the type of inflammatory response, there are two major mechanisms by which Bregs suppress inflammation via IL-10: (I) in autoimmune diseases, such as IBD where both innate and adaptive immune responses are involved, Bregs directly dampen the production of proinflammatory cytokines by macrophages (32–34); (II) during inflammatory processes in, for example, EAE and RA, in which an overshooting T cell-mediated response is the driving force for inflammation, Bregs balance Th1/Th2 immune homeostasis (9, 28). Moreover, Bregs shift T cell differentiation to a regulatory phenotype (Tregs) in both mice and humans (19, 35). The influence of regulatory B cells on the induction of Tregs polarization has been verified in B cell-deficient μMT mice and mice harboring a B cell-specific deletion of IL-10. These genotypes revealed a reduction of Treg numbers with a simultaneous increase of proinflammatory Th1 and Th17 cells (19, 36–38).

In addition to the production of IL-10, Bregs express and secrete suppressive molecules and thus possess further mechanisms to regulate immune responses in an IL-10-independent manner: CD73 is a cell-surface enzyme that converts adenosine monophosphate to adenosine with potential immunosuppressive effects (39); PD-L1 (programmed death ligand 1) is an inhibitory costimulatory molecule that restricts T cell differentiation (40–43); FasL (Fas ligand) bound with its receptor induces apoptotic cell death (44–46); GITRL (glucocorticoid-induced tumor necrosis factor receptor-related protein ligand) induces proliferation of Tregs (47); EBI3/IL-35 (Epstein-Barr virus induced gene 3) regulates inflammatory immune responses through induction of Tregs (48–51). In combination, these molecules perfect regulatory B cells to a strong immune-suppressive cell subset.

However, only a few studies have been published that investigate the activation B cells by a direct interaction with viable bacteria *in vitro* and *in vivo*. It has thus far been shown that several bacterial and viral pathogens, as well as parasites, manipulate B cell function directly to modulate host immune responses as part of an immune evasion strategy facilitating their survival and prolonging infection (51–55). Recently published studies highlighted the influence of the resident microbiota on the activation of B cells that modulate intestinal inflammation and promote immune homeostasis (16, 56). The intenseness of B cell activation and differentiation depends on the composition of the host microbiota and the involved resident bacteria which interact and stimulate various immune cells (immunogenicity) (56–58). The immunogenicity of bacteria is pivotal for the strength of provoking an immune response. As demonstrated in recently published studies, the immunogenicity is dependent on the structure of different MAMPs (e.g., LPS) and consequently to the binding affinity to PRRs (59). We thus investigate the immunogenicity-dependent potential of the two model commensals *Bacteroides vulgatus* (weak immunogenic) and *Escherichia coli* (strong immunogenic) to modulate and regulate the immune system of the host via B cells. In this context, we could already show that a weak immunogenic signal provided by *B. vulgatus* is beneficial in genetically predisposed host (deficient for Rag1 or IL-2) in the course of inflammation. In contrast, the administration of strong immunogenic *E. coli* aggravates the disease progression due to the lack of a functional B cell immunity which can restore immune tolerance in a healthy host by counter-regulating the induced pro-inflammatory immune response (59–61).

In this study we demonstrated the following: (I) B cells can be activated directly by commensal members of the host microbiota and, depending on the immunogenic potential of the encountered bacterial species, B cells can mint strong regulatory cell phenotypes to promote immune tolerance; (II)

the intensified induction of Bregs by *E. coli* can counter-regulate pro-inflammatory immune responses in a healthy host inherently caused by the same bacteria; (III) this regulation mechanism may serve as a feedback loop to maintain immune homeostasis and even attenuate inflammatory processes in autoimmune disease.

MATERIALS AND METHODS

Bacteria Cultivation

E. coli mpk was grown in Luria-Bertani (LB) medium under aerobic conditions at 37°C. *B. vulgatus* mpk was grown in Brain-Heart-Infusion (BHI) medium and anaerobic conditions at 37°C.

Mice

C57BL/6NCrl mice and C57BL/6-Tg(TcraTcrb)425Cbn/Crl (OT-II) mice were purchased from Charles River Laboratories. Toll-like receptor 2 and 4 double KO mice (*Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$) were provided by Jackson Laboratory. All animals were kept and bred under SPF conditions. For isolation of B cells, T cells and bone marrow, only female mice aged 8–10 weeks were used. Germfree C57BL/6J mice were bred and housed in our own gnotobiotic facility. Animal experiments were reviewed and approved by the responsible institutional review committee and the local authorities.

Purification and Cultivation of Naïve B Cells and Naïve T Cells

B cells were purified from spleens of WT or TLR2$^{-/-}$ × TLR4$^{-/-}$ mice by magnetic isolation using negative selection with microbeads (Miltenyi Biotec) according to the manufacturer's instruction. The purity of CD19^{+}CD43^{-}CD4^{-}Ter119^{-} B cell population was >95%. B cells were cultured in complete medium (RPMI1640 supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 25 mM Hepes, 1% non-essential amino-acids, 1% sodium pyruvate and 1% penicillin/streptomycin) at a density of 1 × 10^{6} cells/mL in flat-bottom plates for subsequent stimulation experiments. The purity of naïve CD4^{+}CD44^{-}CD8a^{-}CD11b^{-}CD11c^{-}CD19^{-} CD25^{-}CD45R^{-}CD49b^{-}CD105^{-}MHCII^{-}Ter-119^{-}TCRγ/δ^{-} T cell population was >94%. After purification, T cells were directly used for co-culture experiments. For proliferation assays, isolated splenic B cells or T cells were adjusted to a concentration of 10^{6} cells/mL in PBS/1% FCS, resuspended in 10 μM CFSE and incubated at 37°C for 20 min.

Cultivation of Bone Marrow-Derived Dendritic Cells (BMDCs)

Bone marrow cells were isolated from femurs and tibias of WT mice and cultivated for differentiation in GM-CSF supplemented media as described previously (62). Cells were supplemented with fresh DC media on days 3 and 5. Seven days after isolation, the resulting CD11c^{+} bone marrow-derived dendritic cells (BMDCs) were harvested and used for co-culture stimulation experiments.

Stimulation of Naïve B Cells and Bone Marrow-Derived Dendritic Cells

1 × 10^{6}/mL splenic B cells or BMDCs were stimulated with PBS, *B. vulgatus* mpk or *E. coli* mpk at a Multiplicity of infection (MOI) of 1 at 37°C ("-stimulated B cells/BMDCs"). One μL/mL gentamicin was added to prevent bacterial overgrowth. According to the experimental setting, cells were harvested following stimulation at different time points.

Co-culture Experiments

For B-T cell co-culture experiments B cells served as APCs and were stimulated for 24 h with commensals as described previously ("-primed B cells"). Prior to co-cultivation with naïve CFSE-labeled OT-II CD4^{+} T cells, B cells were incubated with 10 μg/mL Ova-Peptide (ISQAVHAAHAEINEAGR, EMC) for 2 h at 37°C. B cells were washed and supernatant was exchanged with fresh media ("-pulsed B cells"). Primed and pulsed B cells and naïve T cells were co-cultured at different ratios for 72 h at 37°C and 100 ng/mL purified anti-mouse IL-10 antibody (Clone: JES5-2A5, BioLegend) was added to certain samples.

For CD11c^{+} dendritic cells maturation assay, naïve B cells and differentiated immature BMDCs were simultaneously stimulated with bacteria at MOI 1 and co-cultured at a ratio of 5:1 (B cells/BMDCs). To show the effect of indirect cell-cell interaction, naïve B cells and BMDCs were additionally co-cultured in Transwells (pore-size 0.4 μm) and stimulated with bacteria at MOI 1. To mimic the influence of soluble IL-10 produced by B cells on the maturation of BMDCs, 10 μg/mL recombinant mouse IL-10 (BioLegend) were added to BMDC mono-cultures.

DSS-Induced Colitis

Germfree (GF) C57BL/6J mice were colonized for 7 days with 1 × 10^{8} *B. vulgatus* or *E. coli* per mL sterile drinking water. After day 8, mice received sterile drinking water without bacteria. Colonization was checked weekly by collecting fresh feces and determining CFU on selective growth agars (**Supplementary Figure 1**). After 4 weeks of colonization, mice were administered 2% DSS (molecular weight 36–50 kDa; MP Biomedicals, Santa Ana, CA) in their drinking water for 7 days, followed by regular drinking water for 2 days. The disease activity index (DAI) was determined daily by assessment of body weight, stool consistency, and detection of rectal bleeding. On day 9, mice were sacrificed. Colon, spleen and mesenteric lymph nodes (mLN) were removed and cleaned for histological analysis and cell isolation. For the latter, colons were finely minced and incubated in HBSS containing 5 mM EDTA at 37°C for 15 min in motion for 2 cycles. Then, tissue was digested in RPMI 1640 containing 0.4 mg/ml collagenase D (Roche) and 0.01 mg/ml DNase I (Roche) for 20 min at 37°C on a shaking platform. After collagenase digestion, mononuclear cells were collected by centrifugation at 400 g for 5 min. In addition, cells of mesenteric lymph nodes (mLN) and spleen were extracted by gently disrupting the tissue with a sterile syringe plunger and passing through a nylon cell strainer (40-μm mesh) with PBS containing 1% FBS.

Viable cells were counted and cultured for 4 h with 2 μl/mL Leukocyte Activation Cocktail with Brefeldin A (BD

Bioscience). After incubation, cells were prepared for flow cytometrical analysis.

Flow Cytometrical Analysis (FCM)

Harvested cells were washed and FC-receptors were blocked to avoid non-specific binding. Cell death was measured by LIVE/DEAD Cell Viability kit (ThermoFisher) staining for 20 min at 4°C. Intracellular staining was performed using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's description. For intracellular and cell-surface staining, cells were labeled for 25 min at 4°C with specific fluorophore-conjugated antibodies, washed, and resuspended in staining buffer (PBS/1% FCS). Multi-color FCM analyses were performed on a FACS LSRII (BD Biosciences). For compensation, fluorescence minus one (FMO) samples served as controls. Doublets were excluded using FSC-A/FSC-H gating. Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Antibodies: CD11c-APC, CD19-BV421, CD1d-AF647, CD21/CD35-BV605, CD23-BB515, CD24 AF700, CD365(TIM-1)-PE, CD4-APC, CD4-Bv605, CD40-APC, CD40-Bv421, CD45R(B220)-FITC, CD5-Bv605, CD80-PerCP-Cy5.5, CD86-PE-Cy7, CD178(Fas-L)-PE, CD274(PD1-L)-PE, CD73-PE, CD210(IL10R)-PE, FoxP3-AF647, I-A/I-E(MHC-II)-FITC, IFNγ-PE-Cy7, IgM-APC, IL10-PE, IL17A-APC-Cy7, IL4-PE, Vβ5.1,5.2-Bv605, GITR-L-PE, Ebi3-PE. All fluorophore-coupled antibodies were purchased from BD Biosciences.

Cytokine Analysis by ELISA

For determination of TNF and IL-10 concentrations in cell culture supernatants, ELISA Kits purchased from BD Bioscience were used according to the manufacturer's instructions.

Statistics

Data have been tested for normality using the Shapiro-Wilk normality test. Statistical analyses were then performed via unpaired student's *t*-test or ANOVA for data with Gaussian distribution and via Mann-Whitney or Kruskal-Wallis test for non-parametric statistics. Correlation analyses were performed via Spearman's rank correlation coefficient (*r*). Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Error bars represent ± standard deviation (SD).

RESULTS

B Cell Activation and Maturation via Strong Immunogenic *E. coli* Are TLR-Dependent

B cells are mainly associated with the production of antibodies against invaders during adaptive immune responses (4). Activation and proliferation of B cells are achieved by either T cell- dependent or -independent interaction, whereas the latter is coordinated by antigen-recognition via membrane-bound immunoglobulin on the B-cell surface, known as B-cell receptor (BCR) (63). However, B cells express additional receptors for the specific and initial recognition of microbial antigens: Toll-like receptors (TLRs) (64). These surface-bound molecules play a key role in the identification of microbe-associated molecular patterns (MAMPs), such as LPS (via TLR4) or PGN (TLR2) by professional antigen-presenting cells (65).

In order to show that B cells are activated and differentiated upon the MAMP recognition via TLRs and that the activation level is dependent on the immunogenicity of encountered microbes, B cells isolated from wild type (WT) and $TLR2^{-/-} \times TLR4^{-/-}$ (KO) mice were stimulated with two different immunogenic bacteria: *B. vulgatus* mpk and *E. coli* mpk (66–69).

Survival of $CD19^{+}$ B cells was investigated *in vitro* after 0, 24, 48, and 72 h of stimulation with *B. vulgatus* or *E. coli*. The viability of B cells decreased significantly 24 h after stimulation with PBS (Mock) or *B. vulgatus* in comparison to *E. coli*-stimulated B cells, indicating that the survival of B cells is strongly dependent on a potent stimulus with strong immunogenic *E. coli* (**Figure 1A**). In addition, no significant differences between *E. coli*, *B. vulgatus* or PBS stimulated $TLR2^{-/-} \times TLR4^{-/-}$ B cells were observable, indicating that the activation of naïve B cells depends on interaction of bacterial MAMPs with the corresponding TLRs. This is in line with the analyzed B cell proliferation in response to bacterial encounter after 72 h: a robust bacterial interaction with TLR2 or TLR4 on the surface of B cells is critical for the induction of B cell proliferation (**Figure 1B**). Stimulation with *E. coli* results in an increased proliferation of naïve B cells expressing TLR2 and TLR4. *E. coli* and *B. vulgatus* failed to activate naïve $TLR2^{-/-} \times TLR4^{-/-}$ B cells, resulting in a lack of proliferation. Additionally, maturation and activation markers characteristic for APCs on the surface of splenic B cells were measured after stimulation with bacteria. As shown in **Figure 1C**, the production and expression of MHC-class-II, CD80, CD86, and IgM were strongly upregulated in response to *E. coli* and increased distinctly over time. In contrast, *B. vulgatus*-challenged B cells expressed only moderate levels of cell activation markers with a constant surface protein expression during 72 h of cultivation. In line with B cell survival and proliferation, the activation of B cells was shown to be TLR2/4 dependent, as $TLR2^{-/-} \times TLR4^{-/-}$ splenic B cells did not express significant levels of activation markers, regardless of the bacterial stimulus.

Strong Immunogenic *E. coli* Induces Differentiation of Regulatory B Cell Phenotypes

We first demonstrated that B cells can interact directly with MAMPs via TLRs resulting in differently pronounced survival, activation and proliferation of B cells depending on the recognized microbial antigen. We next determined the cell phenotype of B cells stimulated with *E. coli* or *B. vulgatus*. In order to demonstrate that, in a healthy host, immunogenic bacteria are able to counter-regulate a pro-inflammatory immune response, hence contributing to the maintenance of immune homoeostasis, we focused on the differentiation of naïve B cells to regulatory B cells (Bregs). We determined the percentage of the best characterized Breg subsets: B10 cells, T2-MZP cells, and Tim-1^{+} B cells, at several time points after stimulation with strong immunogenic *E. coli* or low immunogenic *B. vulgatus* in comparison to naïve, unstimulated B cells (**Figure 2A**) (70). As shown in **Figure 2B**, the induction of the regulatory B cell phenotypes $CD19^{+}CD5^{+}CD1d^{+}IL10^{+}$ (B10 cells) and $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}$ (T2-MZP cells)

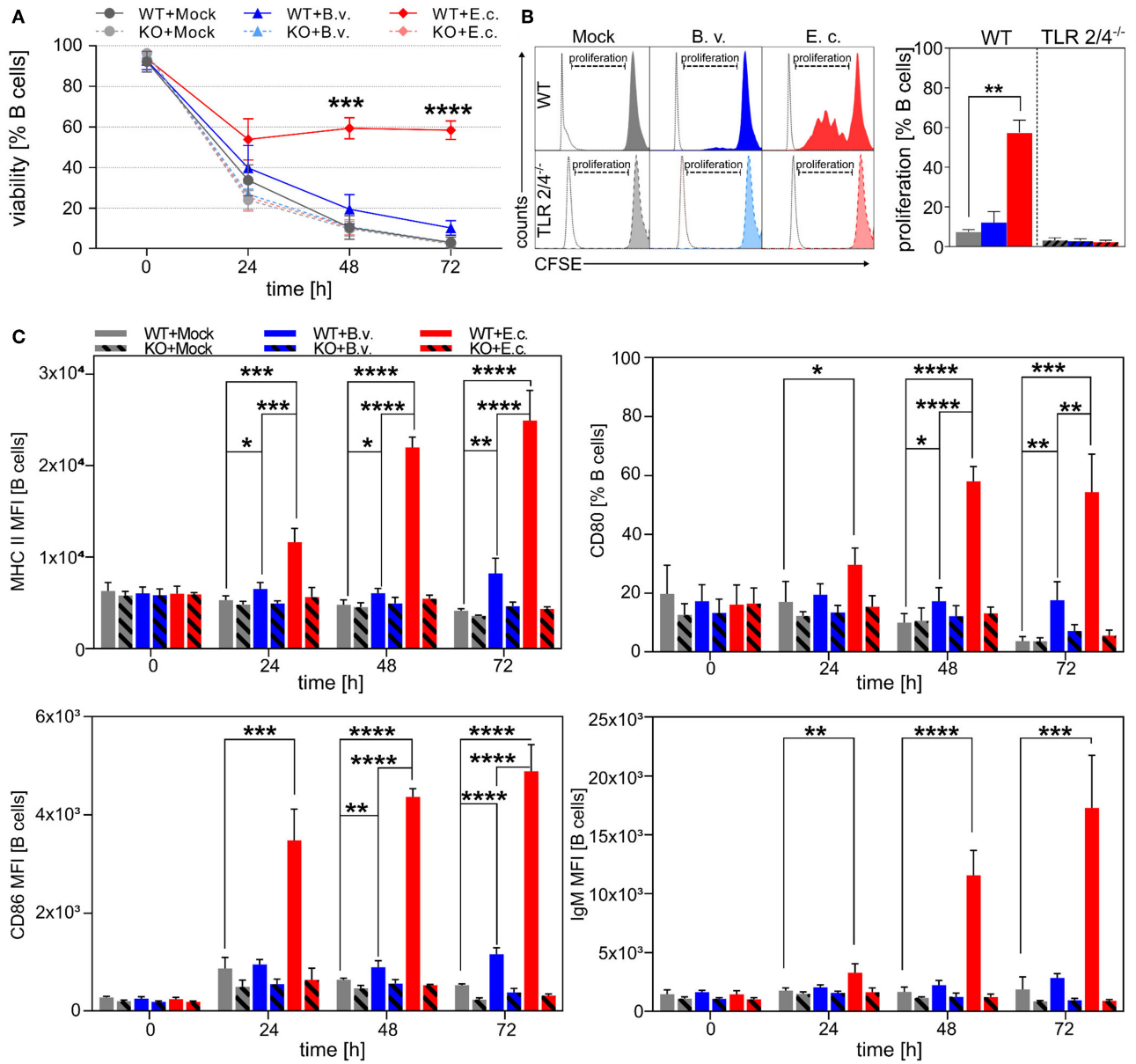

FIGURE 1 | Toll-like receptor-dependent activation of naïve B cells by *E. coli*. Naïve B cells were isolated from spleens of WT or TLR2$^{-/-}$ × TLR4$^{-/-}$ (KO) mice via magnetic cell sorting. B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) for various time points (0, 24, 48, and 72 h). After incubation, cells were blocked for non-specific binding of antibodies, permeabilized for intracellular antibody staining and fixed prior flow cytometric analysis. **(A)** Survival of CD19^{+} B cells was assessed by staining with life-dead dye. **(B)** B cell proliferation of CD19^{+} B cells was analyzed via CFSE cell labeling. Left panel shows exemplary histograms for each stimulus (colored peaks) including unstained control (white peak). **(C)** Surface expression of IgM, MHC-II, CD80, and CD86 was measured to determine B cell activation after bacterial stimulation ($n = 3$–4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

were significantly enhanced after being challenged with *E. coli* in comparison to *B. vulgatus* or PBS stimulation, reaching the maximum proportion after 48–72 h of incubation (**Figure 2B**; **Supplementary Figures 2**, **3**). Furthermore, *E. coli*-stimulated B cells differentiated to a higher proportion into CD19^{+} Tim-1^{+} cells (Tim-1^{+} B cells), an additional regulatory B cell phenotype compared to PBS and *B. vulgatus* stimulation (**Figure 2B**, right; **Supplementary Figure 4**). Tim-1^{+} B cells are found in the spleen and are reported to produce IL-10 and suppress effector CD4^{+} T cells (70). To further characterize the regulatory properties of PBS, *B. vulgatus* or *E. coli*-stimulated B cells, we determined the concentrations of pro- and anti-inflammatory cytokines secreted in cell culture supernatant. The concentrations of secreted IL-10 were significantly increased upon stimulation of B cells with *E. coli* compared to stimulation with PBS

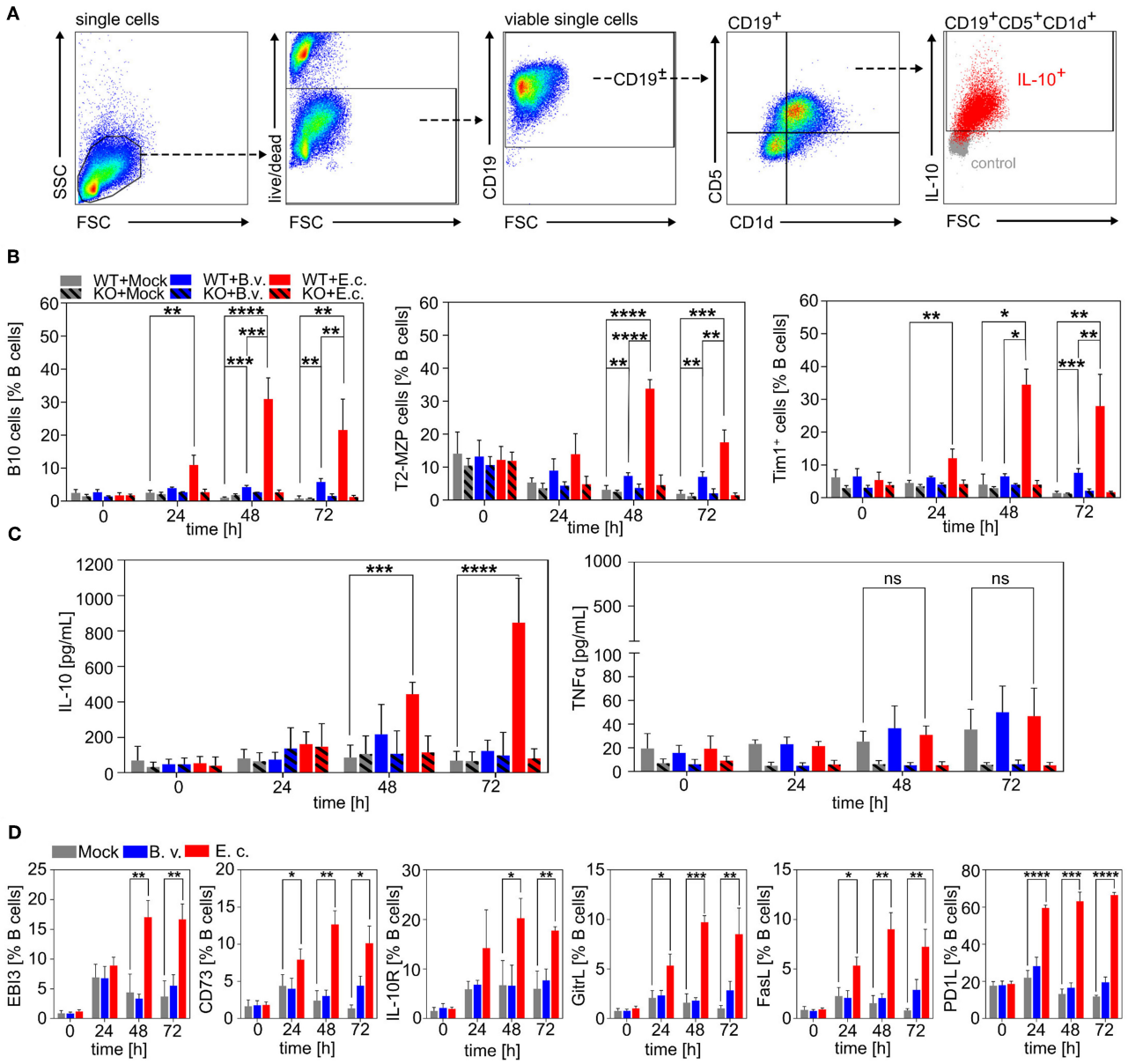

FIGURE 2 | Induction of regulatory B cell phenotypes via *E. coli*. Naïve B cells were isolated from spleens of WT or TLR2$^{-/-}$ × TLR4$^{-/-}$ (KO) mice via magnetic cell sorting. B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) for various time points (0, 24, 48, and 72 h). **(A)** Exemplary flow cytometry gating strategy for CD19^{+}CD5^{+}CD1d^{+}IL10^{+} (B10 cells) of purified and stimulated splenic B cells **(B)** Induction of regulatory B cell phenotypes CD19^{+}CD5^{+}CD1d^{+}IL10^{+} (B10 cells), CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi} (T2-MZP cells) and CD19^{+}Tim-1^{+} (Tim-1^{+} B cells) as determined by flow cytometry. **(C)** Concentration of secreted cytokines IL-10 and TNFα by stimulated B cells measured via ELISA. **(D)** Expression of suppressor molecules on B cell surface after bacterial stimulation as determined by flow cytometry ($n = 3$–4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

or *B. vulgatus* (**Figure 2C**). In contrast, only low levels of the pro-inflammatory cytokine TNFα were detectable (<80 pg/mL) and not different in the various treatments (**Figure 2C**). Furthermore, we analyzed different suppressor molecules produced by immune cells that are not directly linked to a specific Breg phenotype: CD73, PD-L1, FasL, GITRL, and EBI3. *E. coli*, but not *B. vulgatus* stimulation of naïve splenic wild type B cells led to significantly elevated productions of all analyzed immune suppressive molecules starting after 24 h of stimulation (**Figure 2D**).

E. coli-Stimulated B Cells Inhibit DC Activation and Maturation

In the next step we wanted to elucidate whether *E. coli*-activated B cells featuring regulatory properties via the intensified

production and upregulated expression of suppressive molecules directly influence the function of other immune cells, e.g., dendritic cells (DCs). We primarily focused on the maturation of differentiated $CD11c^+$ bone marrow-derived dendritic cells co-cultured with bacteria-stimulated B cells (**Figure 3A**). Interaction of immature DCs (iDCs) with MAMPs (e.g., Lipopolysaccharide, LPS) results in DC maturation characterized by an enhanced expression of MHC-II, CD40, CD80, and CD86 and increased secretion of TNFα (61, 68, 69, 71). Activated DCs, as professional APCs, are responsible for a robust immune response via antigen-recognition, -processing, and -presentation resulting in a proper activation of T cells (72, 73).

In line with our group's previous work, stimulation of iDCs for 24 h with strong immunogenic *E. coli* led to a fully mature DC phenotype with a strong upregulation of surface-bound MHC-II, CD40, CD80, and CD86 (**Figure 3B**, DC dataset, red columns) and high levels of secreted TNFα (**Figure 3C**, upper panel, DC dataset, red columns). In contrast, the stimulation with low immunogenic *B. vulgatus* resulted in a semi-mature DC phenotype with an intermediate expression of T cell activation markers and alleviated production of TNFα (**Figures 3B,C**, DC dataset, blue columns) (66, 68, 69). To demonstrate the effect of stimulated B cells on DC maturation, we co-cultured iDCs with B cells either in direct contact (DC + B cells) or separated via Transwell membranes (DC | B cells) and stimulated the cells with PBS, *B. vulgatus* or *E. coli*. *E. coli* challenged B cells inhibit DC maturation during stimulation, as indicated by a notably reduced expression of MHC-II, CD40, CD80, and CD86 (**Figure 3B**, DC + B cells and DC|B cells dataset, red columns) and significantly diminished secretion of the pro-inflammatory cytokine TNFα (**Figure 3C**, upper panel, DC + B cells and DC|B cells dataset, red columns). This effect was observed in both direct and indirect DC-B cell co-cultures stimulated with *E. coli,* leading us to conclude that soluble factors able to translocate through the Transwell membrane possess the strongest potential for inhibiting DC maturation. However, the maturation was slightly more suppressed in direct interaction with B cells stimulated with *E. coli*.

As IL-10 has been described as an important regulatory factor of Bregs, mainly driving the modulation of immunological functions of other cell types, we mimicked the effect of IL-10 on DC maturation by adding recombinant IL-10 to DC culture (**Figures 3B,C**, DC + IL10 dataset) (70). We propose that the stimulation of iDCs with *B. vulgatus* or *E. coli* in the presence of recombinant IL-10 led to a reduced maturation of DCs, indicated by the tendency of lower expression of MHC-II, CD40, CD80, and CD86 in comparison to the stimulation without recombinant IL-10 (**Figure 3B**, DC + IL10 and DC dataset). We support this hypothesis by the detection of significantly lower levels of secreted TNFα (**Figure 3C**, upper panel, DC + IL10 and DC dataset) (**Figure 3C**, upper panel, DC + IL10 and DC dataset).

To investigate the effect of IL-10 secretion by *E. coli*-stimulated B cells on DC maturation, we neutralized IL-10 in the DC-B cell co-culture by adding anti-IL-10 (**Figures 3B,C**, orange columns). The use of anti-IL-10 mAb partially blocks the inhibitory effect of Bregs on DC maturation, resulting in a restored production of pro-inflammatory cytokine TNFα compared to the stimulation of DCs with *E. coli*-stimulated B cells (**Figure 3C**, upper panel, orange and red columns). Further, we measured the concentration of IL-10 in the cell culture supernatant of all samples and proved the equal distribution of secreted cytokines through the Transwell membranes in the upper and bottom chamber (**Figure 3C**, lower panel). As expected, and already shown in **Figure 2B** in single cell cultures, the concentration of IL-10 in co-culture supernatant was highest in *E. coli*-stimulated samples (**Figure 3C**, lower panel, red columns).

E. coli-Primed B Cells Inhibit T Cell Activation and Induce Treg Differentiation

As presented above, we were able to show that commensal primed B cells significantly shape DC maturation and consequently influence antigen presentation and the potential to activate T cells. Next, we investigated the direct effect of commensal activated B cells on the proliferation and polarization of T cells without the involvement of professional APCs. We primed WT B cells with PBS, *B. vulgatus* or *E. coli* for 24 h, pulsed the cells with Ova-peptide and co-cultured these B cells at different cell ratios (1:1, 1:5) for 72 h with naïve CFSE-labeled $CD4^+CD44^-$ T cells isolated from OT-II mice, which express an Ova peptide-specific TCR (**Figure 4A**). The proliferation of naïve $CD4^+$ T cells was significantly lower in co-cultures with *E. coli*-primed B cells, with a T cell-B cell ratio of 1:1 in comparison to PBS or *B. vulgatus*-primed B cells (**Figure 4B**). Increasing the number of antigen-pulsed B cells (ratio T/B 1:5) resulted in a potent reduction of T cell proliferation in both *E. coli*- and *B. vulgatus*-primed cells (**Figure 4B**). B cells as *bona fide* APCs, usually direct the induction of Th2 responses by suppressing the differentiation of Th1 and Th17 cells and promoting regulatory T-cell expansion (74–76). Th1 ($CD4^+IFN\gamma^+$), Th2 ($CD4^+IL4^+$), Th17 ($CD4^+IL17A^+IL4^-IFN\gamma^-$) cell, and Treg ($CD4^+FoxP3^+$) differentiation in B-T cell co-culture was analyzed by flow cytometry. PBS-primed naïve B cells appeared to favor the differentiation of naïve T cells to Th1 and Th17 cells, whereas *E. coli*-primed B cells led to a significantly enhanced T cell polarization toward Tregs. In addition, co-culture of *E. coli*-primed B cells with naïve T cells resulted in a polarization of Th2 cells at a T cell-B cell ratio of 1:5 (**Figure 4C**) and the frequency of Th1 cells significantly decreased with a simultaneous increase of Th2 cells compared to PBS control.

Induction of Begs by Strong Immunogenic *E. coli* Contributes to the Counter-Regulation of DSS-Induced Inflammation

In the *in vitro* experiments, we could show that B cells, especially regulatory B cells, play a crucial role during the initiation of immune responses and that B cells can directly or indirectly affect the function of other immune cells by their regulatory properties.

This led us to the hypothesis that in a host having functional B cell immunity, *E. coli* with its immunogenic potential counter-regulates inflammatory processes via the strong induction of regulatory cell populations and can consequently help to

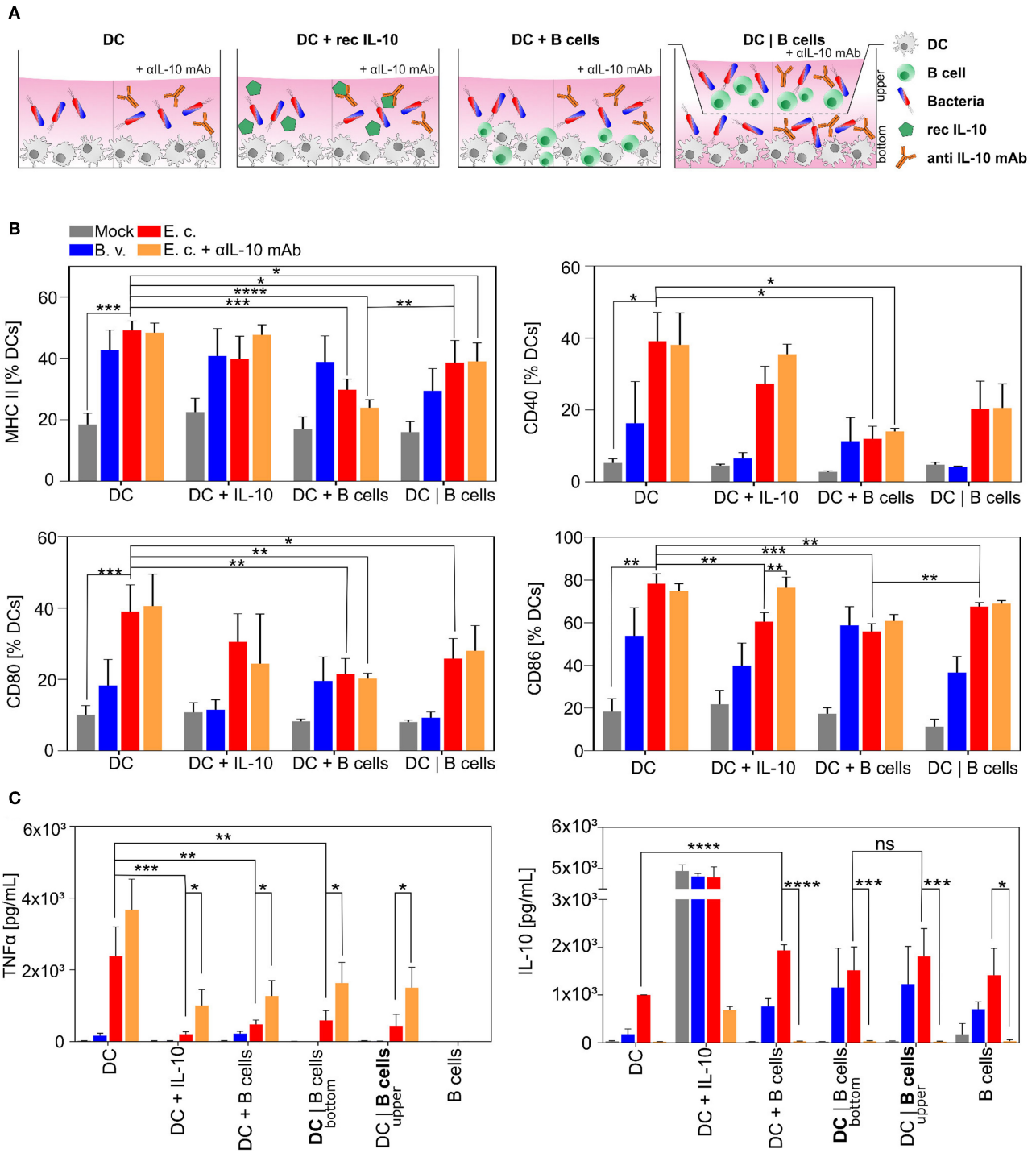

FIGURE 3 | *E. coli*-induced regulatory B cells inhibit maturation of professional APCs *in vitro*. Naïve B cells were isolated from spleens of WT mice and stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 in co-culture with bone marrow-derived dendritic cells (DC) for 24 h. **(A)** Differentiated bone marrow-derived DCs were cultured alone (DC), with recombinant IL-10 (DC + IL10) or co-cultured with primed B cells without (DC + B cells) or with Transwells (DC|B cells) and challenged with bacteria. **(B)** Maturation of CD11c$^+$ APCs was determined through the expression of MHC-II and co-stimulatory markers CD40, CD80, and CD86. **(C)** Concentrations of secreted pro- and anti-inflammatory cytokines TNFα and IL-10 of mono- and co-culture stimulation were determined in the cell culture supernatants. In Transwell stimulations, supernatant of bottom and upper chambers was analyzed ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

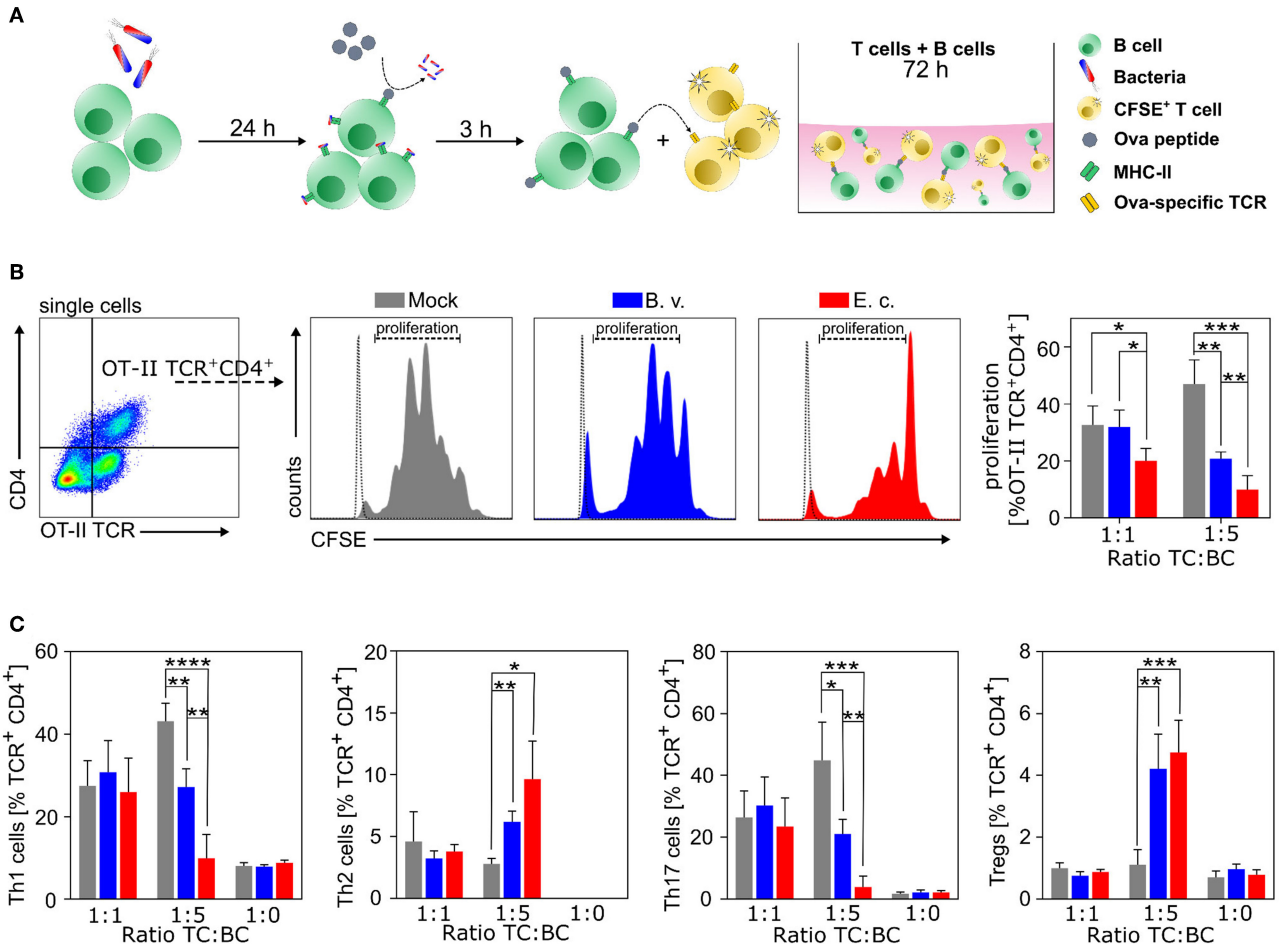

FIGURE 4 | *E. coli*-induced Bregs inhibit T cell proliferation and direct T cell polarization *in vitro*. **(A)** Naïve B cells were isolated from spleens of WT mice and primed with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 for 24 h. T cell proliferation and polarization assays were performed with Ova peptide-specific TCR-expressing naïve T cells in combination with primed and with Ova peptide-pulsed B cells at various ratios. **(B)** Left panel shows exemplary flow cytometry gating strategy. Middle panel shows exemplary histograms for each stimulus (colored peaks) including unstained control (white peak). Percentage of proliferated TCR^+CD4^+ T cells was assessed by CFSE staining (right panel). **(C)** Polarization of naïve T cells incubated for 72 h with different stimulated B cell ratios: Th1 ($TCR^+CD4^+INF\gamma^+$), Th2 ($TCR^+CD4^+IL\text{-}4^+$), Th17 ($TCR^+CD4^+IL\text{-}17a^+$) cells, and Treg ($TCR^+CD4^+FoxP3^+$) cells ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

maintain immune homeostasis. Therefore, we used the Dextran Sulfate Sodium (DSS)-induced colitis model in gnotobiotic C57BL/6 mice which have a physiological B and T cell response. We colonized germfree WT mice with *B. vulgatus* or *E. coli* for 4 weeks via drinking water (**Supplementary Figure 1**) and administered 2% DSS for 7 days in order to correlate the bacteria-dependent induction of regulatory B cells in spleen and mesenteric lymph nodes (mLN) with the disease pathology (**Figure 5A**).

Colonization of germfree mice with *E. coli*, but not with *B. vulgatus*, resulted in significantly reduced weight loss and a lower disease activity index in comparison to germfree mice in response to DSS-administration (**Figures 5B,C**). Additionally, the histological score of tissue damage in the colon was significantly attenuated in *E. coli*-colonized mice but not in *B. vulgatus*-associated mice as compared to germfree mice (**Figure 5D**). Further, the increased absolute numbers of $CD11b^+Gr1^+$ neutrophils in the spleen and the infiltration of these neutrophils in the colonic tissue implied a severe inflammatory state in germfree mice after DSS treatment in comparison to *E. coli*-colonized mice (**Figure 5E**). Interestingly, the influx of neutrophils was also reduced in *B. vulgatus*-colonized mice as compared to germfree mice. The percentage of $CD4^-CD19^+IL10^+$ regulatory B cells in the spleen and mLN was significantly increased in *E. coli*-colonized mice in comparison to *B. vulgatus*-colonized and germfree animals before and after DSS-administration (**Supplementary Figure 5**, **Figure 6A**). This enhanced Breg induction correlated with a lower histological score, whereas highly inflamed mice showed a minimal activation of regulatory B cells in the spleen and mLN (**Figure 6B**). In addition, we analyzed the polarization of $CD4^+$ T cells in all three DSS-administered

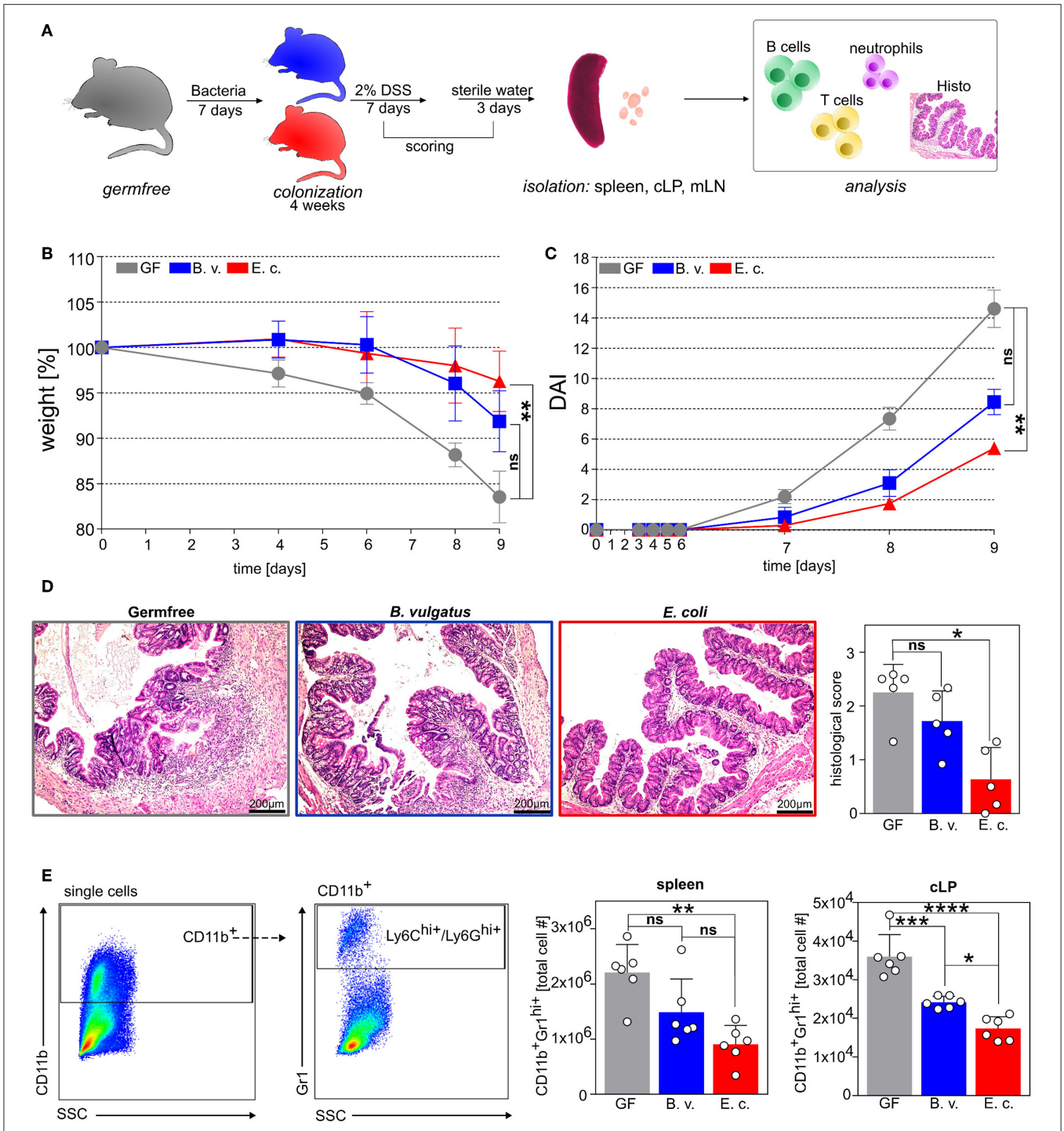

FIGURE 5 | *E. coli* colonization of germfree mice ameliorates DSS-induced colitis. **(A)** Germfree wild type mice and *B. vulgatus*- or *E. coli*-colonized wild type mice were administered with 2% DSS in drinking water for 7 days, followed by regular drinking water for an additional 2 days. **(B)** Bodyweight changes and **(C)** disease activity index (DAI). **(D)** Representative colon histological sections stained with hematoxylin and eosin (H&E) and histological score. **(E)** Exemplary flow cytometry gating strategy and analysis of absolute infiltrated $CD11b^{+}GR1^{+}$ neutrophils in spleen and colonic lamina propria (cLP) (GF: $n = 5$; B.v/E.c: $n = 5$–6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

groups. No significant differences were observed in the Th2 cell differentiation between the groups (**Figure 6C**). However, *E. coli*-colonized mice showed a significant increase of regulatory T cells ($CD19^{-}CD4^{+}CD25^{+}FoxP3^{+}$) in the spleen and mLN, whereas the proportion of Th1 ($CD19^{-}CD3^{+}CD4^{+}IFN\gamma^{+}$) and Th17 ($CD19^{-}CD3^{+}CD4^{+}IL\text{-}17^{+}$) cells was significantly

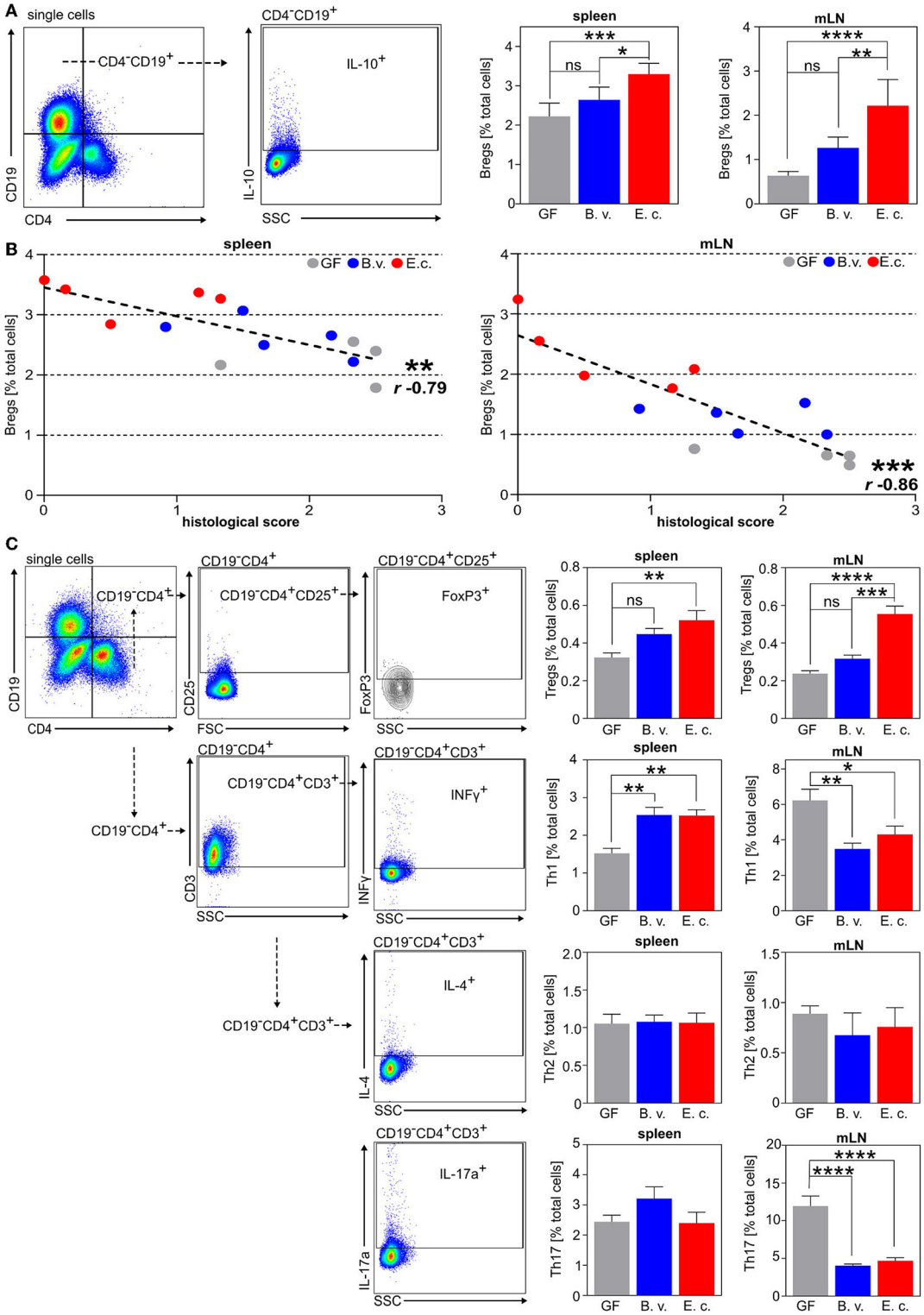

FIGURE 6 | *E. coli* colonization of germfree mice triggers the induction of regulatory B cells accompanied by an increase of Treg cells during DSS-induced colitis. **(A)** Exemplary flow cytometry gating strategy and analysis of $CD4^{-}CD19^{+}IL\text{-}10^{+}$ regulatory B cells (Bregs) in the spleen and mesenteric lymph nodes (mLN). **(B)** Correlation between histology colitis score and percentage of $CD4^{-}CD19^{+}IL\text{-}10^{+}$ Bregs in the spleen (left panel) and mLN (right panel) of colonized and DSS-administered mice (r = Spearman's rank correlation coefficient). **(C)** Exemplary flow cytometry gating strategy and analysis of Th1 ($CD19^{-}CD4^{+}INF\gamma^{+}$), Th2 ($CD19^{-}CD4^{+}IL\text{-}4^{+}$), Th17 ($CD19^{-}CD4^{+}IL\text{-}17a^{+}$) cells, and Treg cells ($CD19^{-}CD4^{+}CD25^{+}FoxP3^{+}$) in the spleen and mesenteric lymph nodes (mLN) (GF: $n = 5$; B.v/E.c: $n = 5$–6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

reduced in the mLN in comparison to germfree DSS-treated mice (**Figure 6C**).

In summary, we showed *in vitro* that the activation of naïve B cells and the differentiation of Bregs are highly dependent on the strength of immunogenicity of the interacting commensal bacteria. Moreover, the enhanced induction of Bregs by strong immunogenic *E. coli* in combination with the differentiation of Tregs may contribute to the regulation and even suppression of inflammatory processes *in vivo* as compared to *B. vulgatus*.

DISCUSSION

In healthy individuals, a complex interaction between the host immune system and commensal microbiota is required to maintain intestinal homeostasis.

Alterations of the gut microbial communities can cause an aberrant and dysregulated immune system resulting in the onset or aggravation of inflammatory bowel diseases, such as Crohn's disease (CD) and ulcerative colitis (UC) (77–79). Furthermore, a disturbed immune system-microbiota balance has also been described in disease pathologies affecting tissues other than those of the intestine (e.g., Grave's disease, multiple sclerosis, type-1 diabetes (T1D), systemic lupus erythematosus, psoriasis, schizophrenia, and autism spectrum disorders) and can be the cause for inflammatory processes during disease development (80–89).

Beside pathogens which are typically acquired from the environment, often provoking acute infections and are the target of immunity, also members of the resident microbiota, found in most healthy hosts, may be the cause for several inflammatory disorders in a genetic or environmental predisposed organism (90–94). The exact mechanisms mediating pathology remain largely unclear, but commensal bacteria are capable of triggering inflammatory disease in immunosufficient rodents by altering barrier function, invading the gut epithelium and stimulating local inflammatory responses (pathobionts) (93, 94).

However, it is conceivable that pathobionts do not actively use specific mechanisms to harm the host under certain circumstances; rather, mechanisms, signaling pathways or cell functions are malfunctioning or are lacking in an immune-compromised host, which pathobionts usually activate in a healthy host to counter-regulate inflammatory processes.

But which immune response mechanisms are consulted by commensals in a healthy host to maintain immune homeostasis? One possible mechanism could be the induction of regulatory B cells.

Most studies investigate the interaction of pathogenic bacteria with B cells during infection. Alternatively, we wanted to demonstrate that the induction of regulatory B cell populations is a mechanism which commensals use to suppress self-caused pro-inflammatory immune responses in a healthy host (54, 95). We also wanted to confirm that Bregs, although present in a minor cell frequency, are an interface in immune-regulation and thus an important player in counter-regulation of inflammatory immune responses.

Primarily, naïve B cells are activated via the help of Th cells which present microbial antigens to the B cell receptor (BCR) followed by the uptake of the antigen through receptor-mediated endocytosis, degradation and presentation of peptide in complex with MHC-class-II molecules on the B cell membrane (96). The recognition of this complex by the corresponding T cell receptor (TCR) of Th cells and the concurrent binding of CD40L with the CD40 receptor on the surface of B cells, as well as the secretion of co-stimulatory cytokines, such as IL-4 and IL-21 by the T cells, promote B cell proliferation, immunoglobulin class switching and somatic hypermutation (T cell-dependent activation) (96, 97). However, we propose a T cell-independent activation and induction of Bregs by commensal bacteria via the direct interaction of MAMPs with TLRs expressed on B cells. Especially the exposure of naïve B cells to LPS and peptidoglycan (PGN), the two most prominent immune-stimulatory bacterial cell components, were responsible and necessary for a potent B cell proliferation and differentiation of Bregs since activation markers like MHC-II, CD80 and CD86 were at lower levels expressed on B cells lacking TLR2 and TLR4 after bacteria encounter (65, 98–101). We could provide evidence for a T cell-independent activation of B cells even without other co-stimulatory factors [e.g., CD40 engagement, CD80/CD86, B cell-activating factor (BAFF), or BCR-TLR crosstalk] that, according to many studies, are pre-requisited to promote an optimal B cell activation (102–105).

Further, we suggest that not only the presence of pattern recognition receptors (PRRs), such as TLRs on the B cell surface is crucial for B cell activation. Additionally, the antigen origin and its immune-stimulatory level might be decisive for the strength of activation and thus the differentiation and survival of Breg subsets.

We could demonstrate that commensal bacteria possess different potentials for directly activating B cells, prolonging their survival and inducing Breg phenotypes as well. In this comparative study of *B. vulgatus* (low immunogenic) and *E. coli* (strong immunogenic), we were able to show that the potent stimulation of naïve B cells with *E. coli* resulted in a strong activation and differentiation of Breg phenotypes characterized by a significantly elevated secretion of anti-inflammatory IL-10 and higher production of suppressive molecules like EBI3, CD73, GITRL, FasL, and PD-L1. In addition, the secretion of the pleiotropic modulatory cytokine IL-6, that exerts either pro-inflammatory or anti-inflammatory properties, was upregulated past stimulation of B cells with *E. coli* whereas only low levels of pro-inflammatory TNFα were detectable (**Supplementary Figure 6**). IL-6 can directly promote Breg cell differentiation and IL-10 production *in vivo* and therefore could be an important trigger for the proceeding induction of Bregs (16, 106, 107).

Important for all following experiments, we demonstrated in *in vitro* stimulation a functioning direct interaction of commensal bacteria with naïve B cells. Nevertheless, the encounter of naïve B cells with microbial antigens *in vivo* is more complex and subject to many limitations, such as intestinal barrier functions and the translocation of microbial structures to secondary lymphoid organs as with the lymph nodes and

the spleen. Furthermore, B cells are not capable of recognizing and taking up microbial components via cell protrusions as dendritic cells perform (108). However, Hudak et al. detected a large number of $CD19^+$ B cells in colonic lamina propria cells which were positive for the uptake of PGN of D-amino acid hydroxycoumarin amino-D-alanine (HADA)-labeled bacteria 4 h after oral gavage (109). This study indicated that $CD19^+$ B cells act as APC in the lamina propria and hence contribute to the regulation of immune processes by directly recognizing MAMPs.

We determined that the bacteria-B cell interaction with the accompanying induction of regulatory B cells had extensive effects on other immune cells and the overall immune response. We demonstrated this in the interplay between bacteria-induced Bregs and DCs. It is already known that DCs, as professional APCs, have crucial influences on B cell function by capturing unprocessed antigen and transferring this antigen to naïve B cells, resulting in the initiation of antigen-specific antibody response and the provision of B cell isotype class switching (110–112). Here, we represented the effect of activated B cells on DC differentiation, maturation and function. The stimulation of immature DCs (iDCs) with *B. vulgatus* led to a semi-mature, tolerant phenotype characterized by a low expression of MHC-II, CD40, CD80, and CD86 and modest secretion of TNFα and IL-1ß. In contrast, the challenge of iDCs with *E. coli* resulted in a high expression of MHC-II and co-stimulatory markers with increased secretion of pro-inflammatory cytokines (60, 66–69, 113, 114) (**Figure 7**).

However, in our co-culture experiments, the interaction of DCs with *E. coli*-stimulated B cells significantly inhibited DC maturation, marked by a reduced expression of DC activation and maturation markers and an alleviated secretion of pro-inflammatory cytokines. This suppression of DC maturation was mediated by distinct regulatory properties of *E. coli*-induced Bregs. We could show in Transwell co-cultures that the regulatory functions of Bregs on DC maturation were not solely mediated by the anti-inflammatory cytokine IL-10 since the addition of recombinant IL-10 was not as effective as the complete repertoire of proteins secreted by stimulated B cells. Consequently, additionally secreted molecules, such as EBI3/IL-35 can contribute to the DC maturation inhibition properties of *E. coli*-stimulated B cells in Transwell co-culture experiments (51) (**Figure 2D**).

Furthermore, maturation of DCs was even more reduced in co-culture stimulation where DCs and *E. coli*-stimulated B cells had direct cell-cell contact. This is in line with our previously findings that the stimulation of B cells with *E. coli* led to an increased upregulation of suppressive molecules like PD-L1, FasL and GITRL on the B cell surface and that these proteins, in combination with secreted cytokines, characterize the strong anti-inflammatory feature of Bregs (47, 51, 116). Moreover, although the expression of T cell activation markers and the production of pro-inflammatory cytokines by DCs were suppressed via *E. coli*-stimulated B cells, the concentration of anti-inflammatory IL-10 in the DC-B cell co-culture was significantly higher than in single cell cultures of DCs and B cells. This led us to the hypothesis that *E. coli*-challenged DCs and B cells may even form a positive feedback loop, resulting in an accumulated anti-inflammatory milieu to regulate overshooting immune responses and dampen inflammatory processes in a healthy host (117, 118).

We further demonstrated that this B cell-driven regulatory mechanism had relevant impacts on the subsequent processes of the adaptive immune response: the activation, proliferation and polarization of T helper cells. Schmidt et al. revealed that Ag-pulsed splenic B cells possess a stronger T cell stimulatory capacity than $CD11c^+$ DCs and that activated $CD4^+$ T cells favor Th2 polarization *in vitro* (119). Saze et al. investigated T cell activation properties of B cells in more detail. They compared the proliferation of T cells incubated with either naïve B cells or activated B cells and showed that freshly purified B cells co-cultured with separated $CD4^+$ T cells augment proliferation of the T cells as well as their cytokine production whereas activated B cells significantly inhibit T cell proliferation (120). By using two commensals featuring different immunogenic properties for B cell stimulation, we could complement and confirm these findings; furthermore, we could expand our understanding of the mechanism behind microbiota-mediated B cell regulation. The stimulation of CFSE-labeled Ova peptide-specific TCR^+CD4^+ T cells with *E. coli*-primed and Ova peptide-pulsed B cells resulted in a significant inhibition of T cell proliferation at a T cell-B cell ratio of 1:1. In contrast, the co-incubation of T cells with *B. vulgatus*-primed B cells did not result in decreased proliferation. Only after increasing the T cell-B cell ratio to 1:5, the low immunogenic bacterium possessed T cell proliferation-preventing effects. The cultivation of T cells with naïve unstimulated B cells led to an intense T cell proliferation and a polarization toward Th1 and TH17 cells. In contrast, T cells incubated with *E. coli*-primed B cells favored a polarization shifted toward Th2 cells and Tregs. *B. vulgatus*-primed B cells also polarized T cells in a Th2 and Treg direction but simultaneously induced Th1 and Th17 cells leading to a more pro-inflammatory Th1/Th2/Th17/Treg balance (121–123). The cause for the strong T cell activation, proliferation and polarization by naïve unstimulated B cells could be the basic expression levels of MHC-II and co-stimulatory proteins in the absence of anti-inflammatory cytokines and surface molecules, which were upregulated in *E. coli*-primed B cells, as shown in previous experiments (124). In addition, after induction of Tregs via *E. coli*-primed Bregs, these two regulatory cell populations could cooperate to generate an IL-10-driven feedback loop to initiate their reciprocal activation and consequently increase their cell emergence (8, 125, 126).

The inflammation-suppressive role of Breg subsets in autoimmune disease has been demonstrated previously in *in vivo* mice models for IBD, MS, T1D, and RA, primarily achieved by the adoptive transfer before or during inflammation of *ex vivo*-activated B cells in wild type, B cell-depleted (anti-CD20 mAb treatment), or B cell-deficient (μMT) mice (10, 28, 47, 127–132). Consequently, the ability of Bregs to contribute to the control of the immune response during inflammation development and disease progression is evidently. Nevertheless, the influence of the host commensal microbiota on the induction of this regulatory cell phenotype needs to be investigated in more detail.

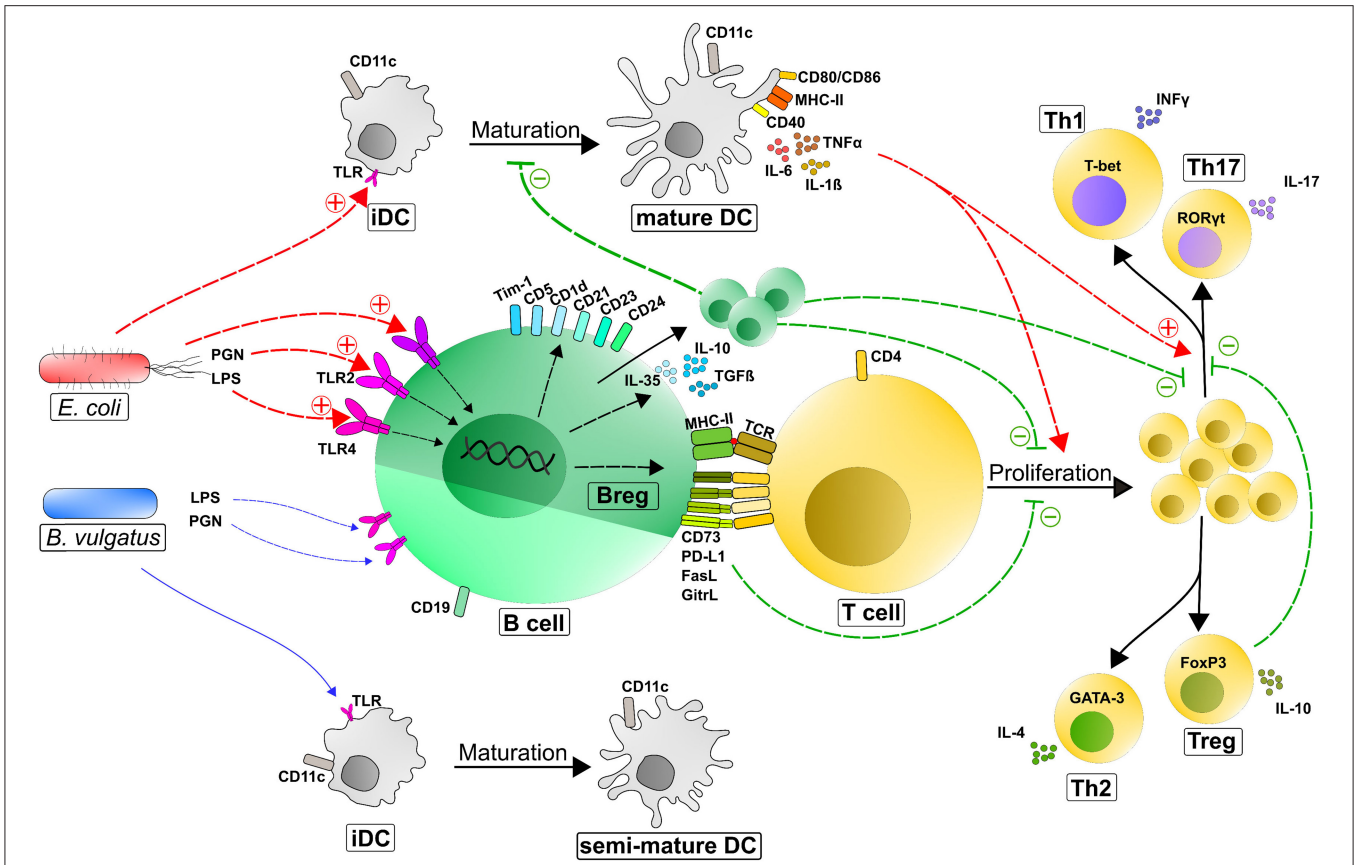

FIGURE 7 | Counter-regulatory mechanisms of Bregs after the induction of immune responses via immunogenic bacteria. Depending on the immunogenicity of a bacterial antigen, APCs (immature DCs and naïve B cells) are activated through the recognition of MAMPs (e.g., LPS or PGN) via Toll-like receptors to different degrees. Potent stimulation of B cells (e.g., by strong immunogenic *E. coli*) induces the differentiation and proliferation of regulatory B cell phenotypes, such as B10 cells ($CD19^+CD5^+CD1d^+IL10^+$), T2-MZP cells ($CD19^+CD21^{hi}CD23^{hi}CD24^{hi}$), and Tim-$1^+$ B cells ($CD19^+$ Tim-1^+) characterized by an upregulated expression and strong secretion of suppressive mediators. Primarily through secreted IL-10, Bregs can inhibit DC and macrophage maturation and function and consequently dampen their antigen presentation capacity to activate and polarize T cells. Moreover, Bregs can regulate T-cell responses by suppressing the proliferation and polarization of effector T cells (in particular TH1 and TH17 cells). These effects are mediated by secreted factors (IL-10, TGFß, and Ebi3/IL-35) and membrane-bound molecules including MHC-II, CD73, PD-L1, FasL, and GitrL at the interface between B cells and T cells (115). Regulatory B cells also crosstalk with Treg cells to promote their expansion and support their function. +, induction/activation; –, inhibition; semi-mature DCs, intermediate expression of DC maturation markers; LPS, lipopolysaccharide; PGN, peptidoglycan; TLR, toll-like receptor; iDC, immature dendritic cell; CD, cluster of differentiation; PD-L1, programmed death ligand 1; FasL, Fas ligand; GitrL, glucocorticoid-induced tumor necrosis factor receptor-related protein ligand; IL–, interleukin–; TCR, T cell receptor; TGFß, transforming growth factor ß; Th, T helper cells; Tim-1, T cell Ig and mucin 1; IFNγ, interferon γ; TNFα, tumor-necrosis factor α; T-bet, T-box transcription factor; RORγt, RAR-related orphan receptor gamma; GATA-3, Trans-acting T-cell-specific transcription factor GATA-3; FoxP3, Forkhead box protein P3.

In earlier studies, we could demonstrate the effect of different immunogenic commensals on the differentiation and maturation of certain immune cells (e.g., $CD11c^+$ DCs), concluding that a potent TLR activation provided by strong immunogenic bacteria leads to enhanced immune responses, aggravating the course of disease (60, 61, 66, 68, 69, 133, 134). Thereby, our and other groups used genetically susceptible colitis mouse models (Rag1-, IL-2-, and IL-10-deficient) exhibiting a dysregulated immune system to mimic disease development in an immune suppressed host (60, 61, 135). In detail, B cell immunity is inoperative, either due to the lack of mature B cells ($Rag1^{-/-}$), the disturbed proliferation and induction of Bregs ($IL\text{-}2^{-/-}$), or the dysfunction of Bregs ($IL\text{-}10^{-/-}$) in all three colitis mouse models. Hence in these colitis models, a potent immune-stimulatory signal mediated by strong immunogenic *E. coli* exacerbated inflammation, since important counter-regulation mechanisms (such as the induction of Bregs) for the compensation of overshooting immune responses malfunctioning. Similar observation could be made with the bacterium *Helicobacter hepaticus*. *H. hepaticus* is a member of the mouse microbiota colonizing the lower intestine and activating innate immunity via Toll-like receptors without inducing immune pathology in a healthy host (136). *H. hepaticus* thus induces an anti-inflammatory immune response through the activation of regulatory macrophages to maintain immune homeostasis (137). However, in immune-deficient $IL\text{-}10^{-/-}$ or $Rag2^{-/-}$ mice, *H. hepaticus* triggered exacerbated intestinal inflammation as a result of aberrant regulatory T cell

function (138–140). In conclusion, strong immunogenic bacteria, which are benignant commensals in a healthy host, provoke an uncontrolled activation of the immune system in hosts with a dysfunctional immune response, leading to inflammatory processes and the exacerbation of disease severity.

However it has already been published in mouse models for IBD and T1D that strong immunogenic bacteria, such as *E. coli* Nissle or *Helicobacter pylori* have inflammation-suppressive properties and can even prevent the onset of disease, on the condition that the host provides a functional immune system (141–143). In our study, we wanted to demonstrate the specific impact of commensal bacteria featuring different immunogenic properties on the activation of the immune system and the development of inflammation while deciphering the crucial role of regulatory B cells in these processes. Therefore, we colonized DSS-administered germfree wild type mice providing a full-featured immune system (no genetic susceptibility) to induce intestinal inflammation.

The comparison of germfree, low immunogenic *B. vulgatus*- or strong immunogenic *E. coli*-associated mice emphasizes the importance of a potent immune stimulus in DSS-induced inflammation since the colonization with *E. coli* significantly prevented intense weight loss, alleviated disease symptoms and reduced inflammation in the affected tissue. In contrast, colonization with *B. vulgatus* alleviated disease symptoms slightly, but not significantly, compared to germfree mice. Reason for that could be the weak immunogenic properties of *B. vulgatus* still provoking a low activation of B cells, minor induction of Bregs and modest inhibition of T cell proliferation and polarization, as observed in *in vitro* experiments. In addition, other bacteria-associated host immune modulating effects which are B cell- and T cell-independent, such as the restoration and education of the immature immune system and mucosal barrier present in germfree mice could be causal for the attenuated inflammation development shown in *B. vulgatus*-colonized mice. However, these potential immunogenic-independent regulatory mechanisms may be insufficient to significantly reduce inflammation processes in DSS-induced colitis. Though, these findings could explain the non-significant differences between *B. vulgatus*- and *E. coli*-colonized mice in some read outs (144, 145).

Moreover, we provide evidence that the induction of Bregs directly correlated with the severity of colonic inflammation and therefore negatively correlated with the immunogenicity of the colonizing bacteria. These findings are in line with other studies reporting a decrease of intestinal regulatory B cells in colonic inflammation in mice and humans (146–148). In line with our *in vitro* experiments, the colonization of germfree mice with *E. coli* prior to DSS-administration, and the accompanying induction of Bregs, resulted in a significantly enhanced development of Tregs and inhibition of a Th1 and Th17 polarization (**Figure 7**). Even though *B. vulgatus*-colonization resulted in a slightly and non-significant increased induction of Bregs, the differentiation of T helper cells is not altered in comparison to germfree mice. In contrast, the significant enhanced induction of Bregs *in vitro* and *in vivo* in *E. coli*-associated mice leading to an anti-inflammatory balance of Th1/Th2/Th17/Treg cells, marked by a pronounced differentiation of Tregs, which is decisive for the development of inflammation and consequently the possible reason for the extenuated inflammation in *E. coli*-colonized and DSS treated mice (121–123).

Thus, the increased accumulation of Bregs and Tregs established an immune-homoeostatic state *in vivo*. In combination, these two crucial regulatory cell populations might develop a powerful anti-inflammatory milieu capable of suppressing inflammation in various autoimmune diseases (149).

In conclusion, Bregs are an important interface in microbiota-driven immune regulation. They contribute to maintenance of immune homeostasis in a healthy host and counter-act emerging inflammatory processes in immune-compromised hosts via the production of suppressive molecules and the interaction with other immune cell populations. The induction and longevity of Bregs specifically depend and correlate with the strength of TLR-ligation and subsequent cell activation, provided by commensal antigens. The presented results directly link different characteristics of commensal bacteria with the immune response of the host and thus provide new insights in the inter-kingdom communication between commensals and their hosts.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration. Protocols and experiments involving mice were reviewed and approved by the responsible Institutional Review Committee and the local authorities within H1/15, H1/17, §4 09.01.2015, §4 14.06.2017.

AUTHOR CONTRIBUTIONS

JM and J-SF conceived and designed the experiments. JM, CT, AL, RP, LM, AS, HY, and H-CL performed the experiments. JM, CT, and J-SF analyzed the data. JM, AL, and J-SF wrote the manuscript. All authors gave final approval to publish the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03093/full#supplementary-material

Supplementary Figure 1 | Determination of colony forming units (CFU) of germfree mice after colonization with *B. vulgatus* or *E. coli*. Feces of every mouse were collected under germ free conditions before and after colonization with bacteria and DSS-administration and were plated timely to determine the CFU.

Supplementary Figure 2 | Representative flow cytometry plots of B10 cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets wee excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. B10 cells were defined as $CD19^+CD5^+CD1d^+IL10^+$.

Supplementary Figure 3 | Representative flow cytometry plots of T2-MZP B cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets were excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. T2-MZP B cells were defined as $CD19^+CD21^+CD23^+CD24^+$.

Supplementary Figure 4 | Representative flow cytometry plots of Tim-1^+ B cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets were excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. Tim-1^+ B cells were defined as $CD19^+$ Tim-1^+.

Supplementary Figure 5 | Analysis of Bregs in germfree mice after colonization with *B. vulgatus* or *E. coli* prior DSS administration. Changes in Bregs amount of germfree, *B. vulgatus* or *E.coli* -colonized mice 2 weeks after colonization and 2 weeks prior DSS-administration measured via flow cytometry. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Supplementary Figure 6 | Concentration of secreted cytokine IL-6 by stimulated B cells measured via ELISA. Naïve B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) for various time points (0, 24, 48, and 72 h) ($n = 4$). $^*p < 0.05$, $^{**}p < 0.01$.

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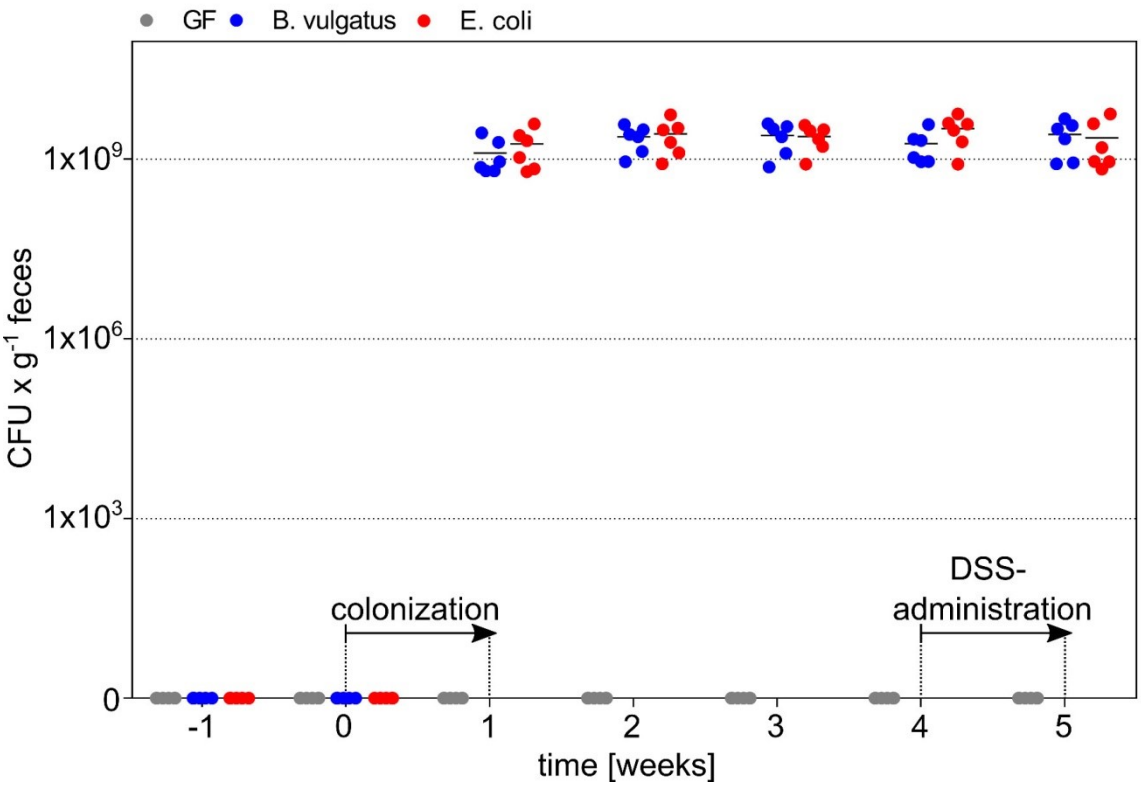
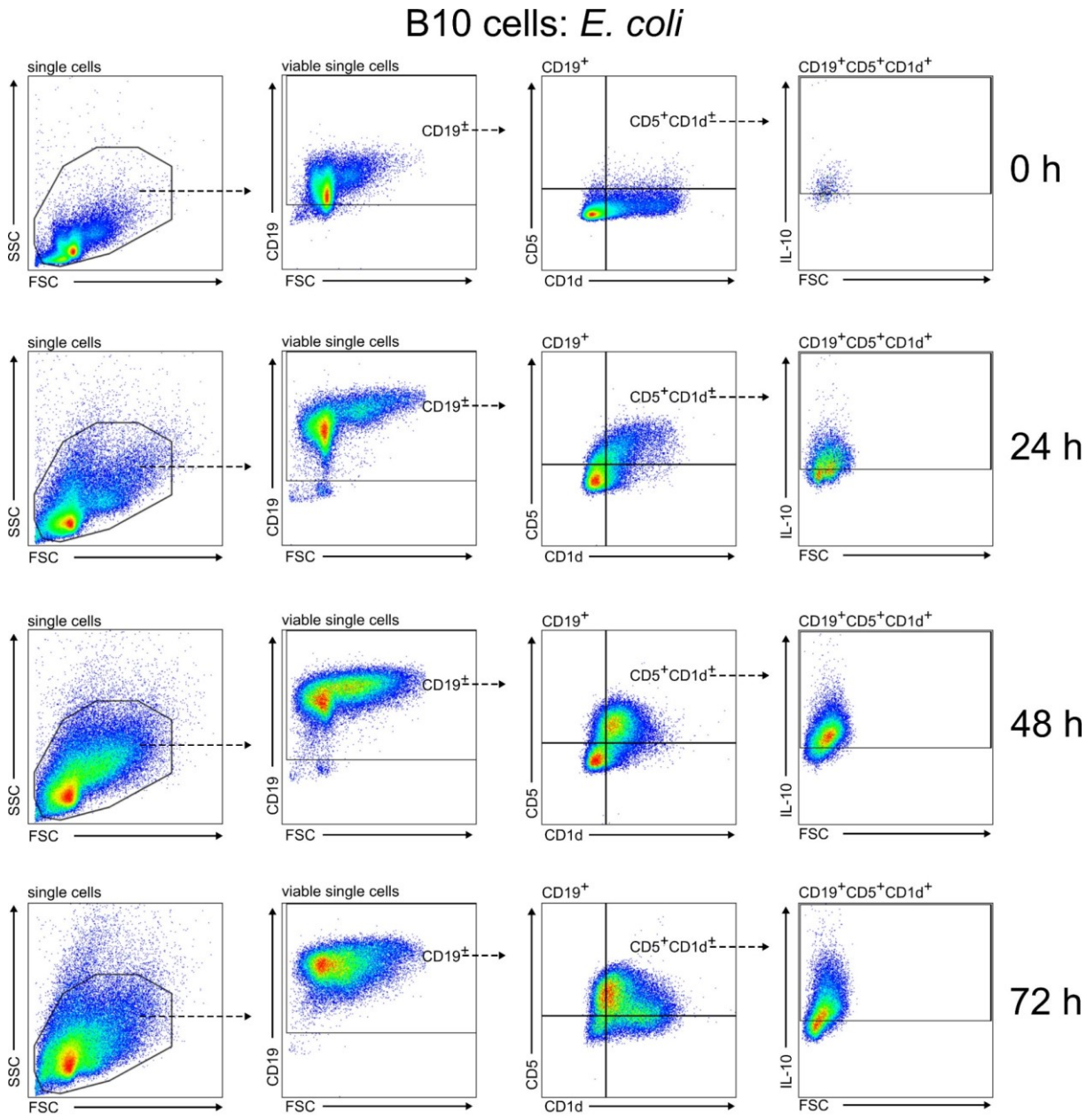
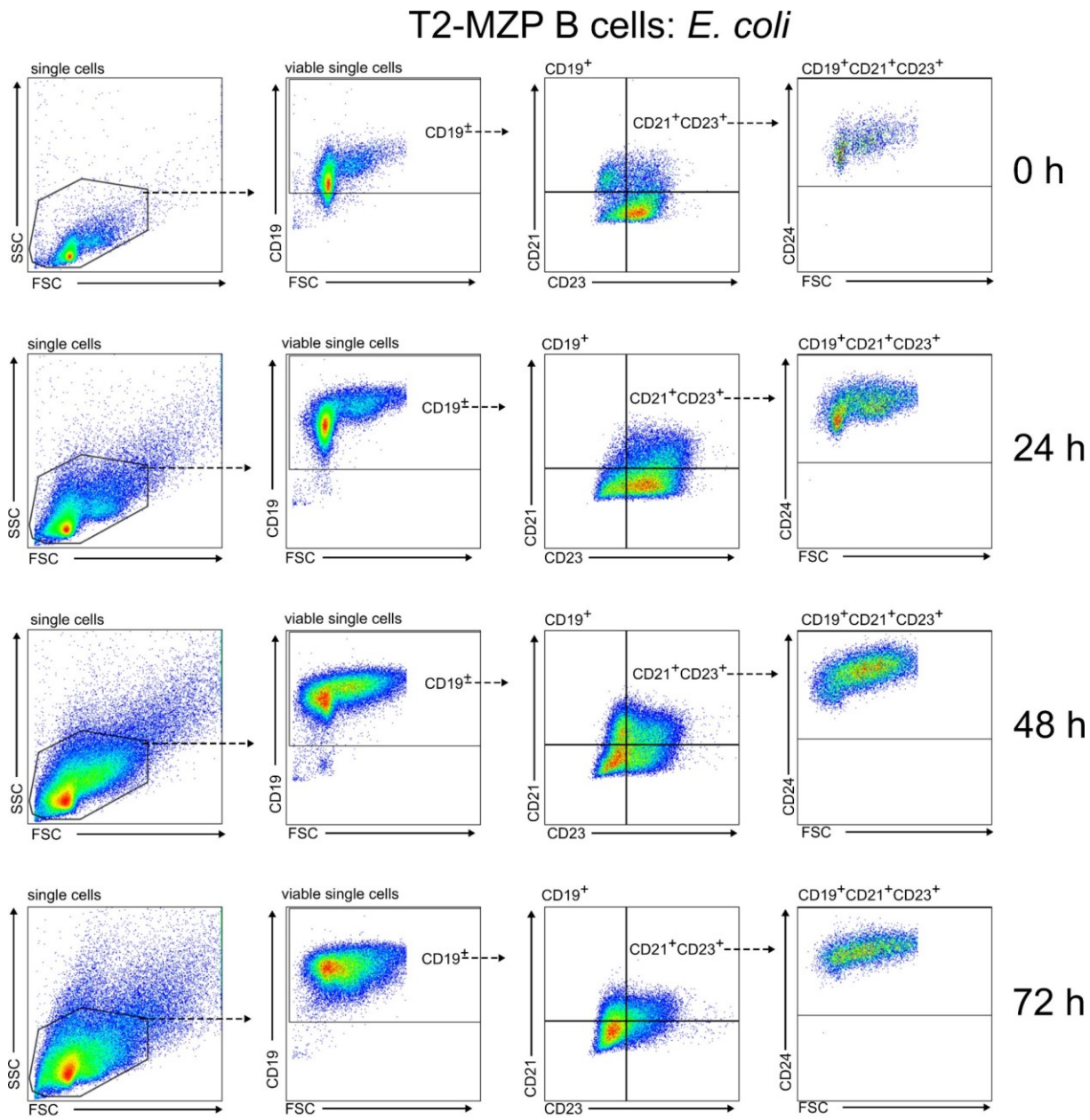
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



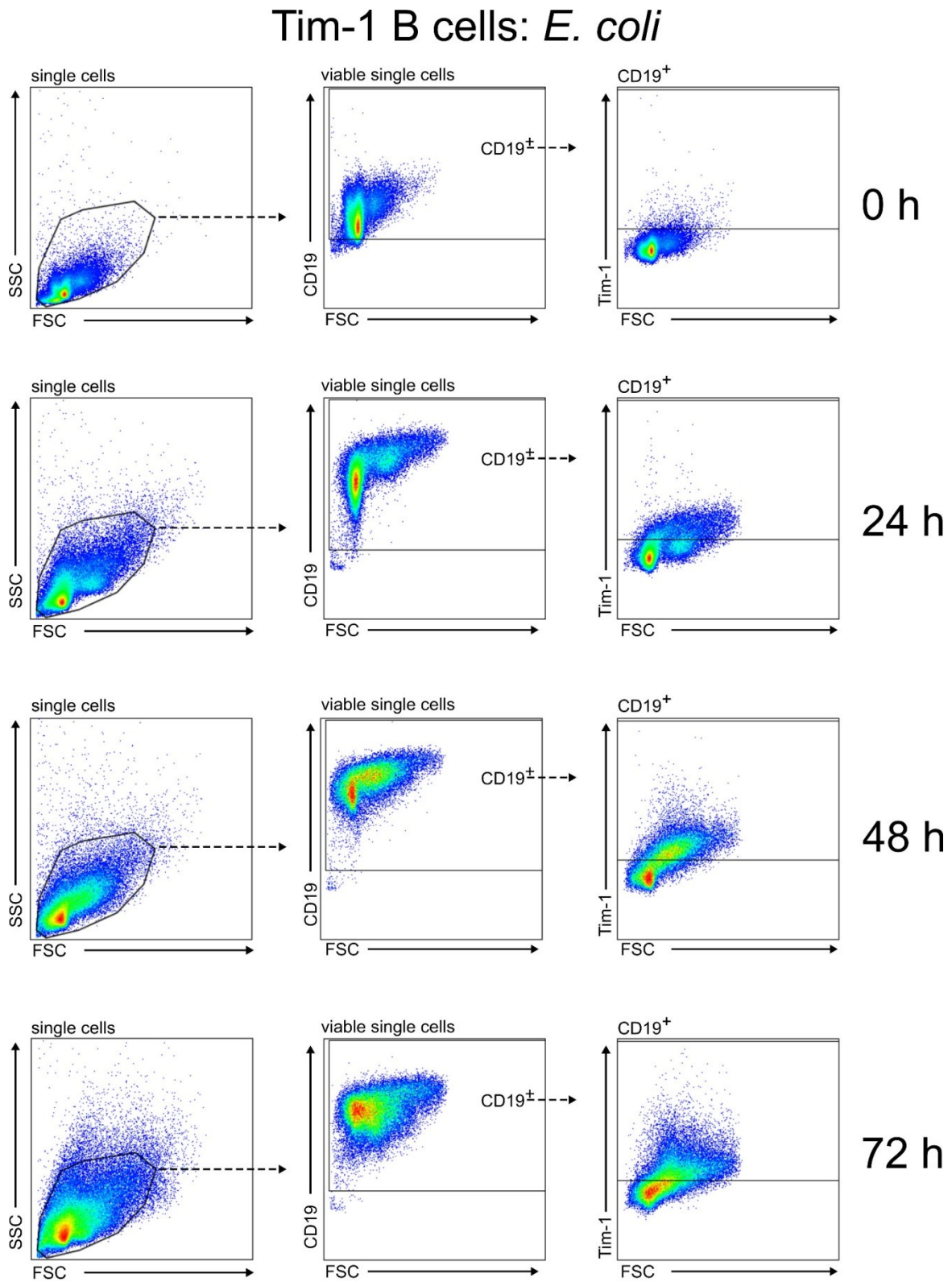
Supplementary Figure 1. Determination of colony forming units (CFU) of germfree mice after colonization with *B. vulgatus* or *E. coli*. Feces of every mouse were collected under germ free conditions before and after colonization with bacteria and DSS-administration and were plated timely to determine the CFU.



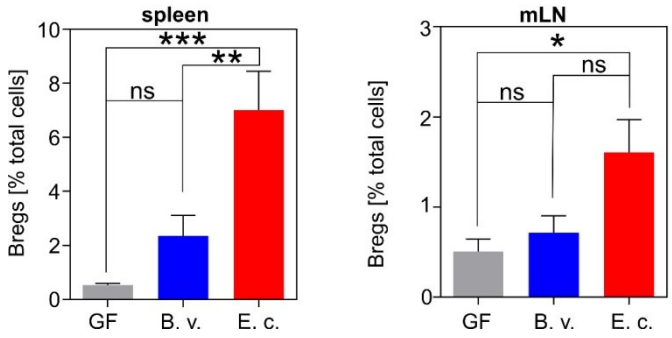
Supplementary Figure 2. Representative flow cytometry plots of B10 cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets wee excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. B10 cells were defined as $CD19^{+}CD5^{+}CD1d^{+}IL10^{+}$.



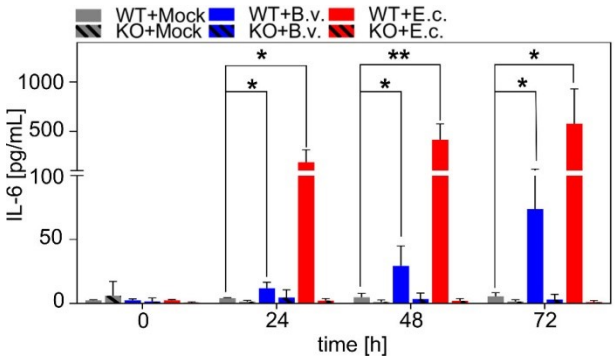
Supplementary Figure 3. Representative flow cytometry plots of T2-MZP B cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets were excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. T2-MZP B cells were defined as $CD19^{+}CD21^{+}CD23^{+}CD24^{+}$.



Supplementary Figure 4. Representative flow cytometry plots of Tim-1^{+} B cells induced by *E. coli* stimulation. Naive B cells were stimulated with *E. coli* at MOI 1 for 0, 24, 48, and 72 h. Doublets were excluded via FSC-A/FSC-H gating and death cells were excluded by fixable viability dye. Tim-1^{+} B cells were defined as CD19^{+} Tim-1^{+}.



Supplementary Figure 5. Analysis of Bregs in germfree mice after colonization with *B. vulgatus* or *E. coli* prior DSS administration. Changes in Bregs amount of germfree, *B. vulgatus* or *E.coli*-colonized mice 2 weeks after colonization and 2 weeks prior DSS-administration measured via flow cytometry. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.



Supplementary Figure 6. Concentration of secreted cytokine IL-6 by stimulated B cells measured via ELISA. Naïve B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) for various time points (0, 24, 48, and 72 h) ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$.