Host immune system modulation mechanisms mediated by commensal bacteria during health and disease

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Table of Contents

Abbreviations **5**

Summary **6**

Zusammenfassung **7**

Topic-specific Publications **9**

Contributions **10**

INTRODUCTION **11**

1. The intestinal microbiota **12**

1.1 Gut microbiota composition 12

1.2 Commensals: symbionts and pathobionts 13

2. Microbiota-dependent autoimmune diseases **14**

3. Microbiota-host interactions **15**

3.1 Outer membrane vesicles 19

4. The mucosal immune system **22**

4.1 Tolerant dendritic cells (DCs) 24

4.2 Regulatory B cells (Bregs) 25

Aim of this work **27**

RESULTS AND DISCUSSION **28**

1. Outer membrane vesicle secretion contributes to *B. vulgatus* –mediated immune response silencing 30

2. Bacterial immunogenicity is critical for the induction of tolerant and regulatory immune cell phenotypes resulting in the suppression of inflammatory immune responses 36

CONCLUSION **42**

References **46**

List of publications **58**

List of posters and presentations **60**

Acknowledgements **62**

Appendix: publications **63**

Abbreviations

AID	Autoimmune disease
AMP	antimicrobial peptides
APC	antigen presenting cell
BMDC	Bone-marrow derived dendritic cell
Breg	regulatory B cell
CD	Cluster of differentiation
ELISA	Enzyme-linked immunosorbent assay
GALT	gut-associated lymphoid tissue
HEK	Human embryonic kidney
IBD	Inflammatory bowel disease
IEC	intestinal epithelia cell
IFNγ	Interferon γ
IgA	immunoglobulin A
IL-	Interleukin-
LPS	Lipopolysaccharide
LBP	LPS binding protein
MAMP	Microbe-associated molecular pattern
MD-2	Myeloid differentiation protein 2
mLN	mesenteric lymph nodes
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OMV	Outer membrane vesicle
PGN	peptidoglycan
PP	Peyer’s patches
PSA	Polysaccharide A
PRR	Pattern recognition receptor
Rag1$^{-/-}$	Recombination-activating protein 1-deficient
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Summary

The composition of the intestinal microbiota affects the host organism in manifold ways by the digestion of nutrient, colonization resistance against pathogens and especially by influencing and priming the immune system. Consequently, a balanced microbiota composition, mainly consisting of symbiotic and pathobiotic commensal bacteria of the *Firmicutes* and *Bacteroidetes* phyla, is beneficial for the host by maintaining a healthy and homeostatic immune state. Symbionts generally possess important immunomodulatory functions to regulate inflammatory immune processes whereas pathobionts cause non-beneficial or detrimental effects on the host under certain genetic or environmental pre-conditions by promoting inflammatory immune processes. Alteration or dysbiosis of a complex intestinal microbiota has already been shown to be associated with the progression of various autoimmune diseases such as inflammatory bowel diseases, multiple sclerosis, type-1 diabetes and even neurological disorders such as autism.

In particular, the interaction of microbes with the host immune system and the subsequent activation and differentiation of certain immune cells are crucial for the development of inflammatory or regulatory processes.

For this reason, we focused on and investigated the immunogenicity-dependent potential of the commensals *Bacteroides vulgatus* (weak immunogenic) and *Escherichia coli* (strong immunogenic) to modulate and regulate the immune system of the host via the direct or indirect interaction with antigen-presenting dendritic cells and B cells. We could (1) identify the production of bacterial outer membrane vesicles as an important inter-kingdom signaling mechanism exhibiting immunomodulatory properties to prime host target cells; (2) demonstrate the activation and differentiation of a tolerant dendritic cell phenotype initiated by symbiotic *B. vulgatus* to maintain and restore immune homeostasis; (3) elucidate the induction of regulatory B cells via pathobiotic *E. coli* as an decisive counter-regulation mechanism suppressing pro-inflammatory processes which are self-triggered and dependent on the bacterial immunogenicity.

Zusammenfassung

Die Zusammensetzung der intestinalen Mikrobiota beeinflusst den Wirtsorganismus auf vielfältige Weise durch die Unterstützung beim Verdau von Nährstoffen, die Bildung einer Besiedlungsresistenz gegen Krankheitserreger und insbesondere durch die Aktivierung und Modulation des Immunsystems. Deshalb ist eine ausgewogene Mikrobiota-Zusammensetzung, die hauptsächlich aus symbiotischen und pathobiotischen kommensalen Bakterien der Phyla *Firmicutes* und *Bacteroidetes* besteht, für den Wirt von Vorteil, da sie einen gesunden und homöostatischen Immunzustand aufrechterhält. Symbionten besitzen im Allgemeinen wichtige immunmodulatorische Funktionen, um entzündliche Immunprozesse zu regulieren, wohingegen Pathobionten unter bestimmten genetischen oder umweltbedingten Voraussetzungen keine vorteilhaften oder sogar schädliche Wirkungen auf den Wirt ausüben, indem sie inflammatorische Prozesse fördern. Es wurde bereits gezeigt, dass eine Veränderung oder Dysbiose einer komplexen Darmmikrobiota mit der Entstehung und Fortschreiten verschiedener Autoimmunerkrankungen wie chronisch entzündlichen Darmerkrankungen, Multipler Sklerose, Typ-1-Diabetes und sogar neurologische Störungen assoziiert ist.

Insbesondere die Interaktion von Mikroben mit dem Wirts-Immunsystem und die anschließende Aktivierung und Differenzierung bestimmter Immunzellen sind entscheidend für die Entwicklung entzündlicher oder regulatorischer Prozesse.

Aus diesem Grund konzentrierten und untersuchten wir das immunogenitätsabhängige Potenzial der kommensalen Bakterien *Bacteroides vulgatus* (schwach immunogen) und *Escherichia coli* (stark immunogen), um das Immunsystem des Wirts über die direkte oder indirekte Wechselwirkung mit Antigen-präsentierenden dendritischen Zellen und B Zellen zu modulieren und zu regulieren. Wir konnten (1) die Produktion von bakteriellen Vesikeln der Außenmembran als einen wichtigen speziesübergreifenden Signalmechanismus identifizieren, der immunmodulatorische Eigenschaften aufweist, um Wirtszielzellen zu primen; (2) die Aktivierung und Differenzierung eines toleranten dendritischen Zellphänotyps nachweisen, der durch den Symbiont *B. vulgatus* ausgelöst wird, um die Immunhomöostase aufrechtzuerhalten und wiederherzustellen; (3) aufzeigen, dass die Induktion von regulatorischen B-Zellen durch den Pathobiont *E. coli* ein entscheidender Gegenregulationsmechanismus zur Unterdrückung von entzündungsfördernden

Prozessen darstellt und dass diese Aktivierung von der Immunogenität des interagierenden Bakteriums abhängig ist.

Topic-specific Publications

a **Maerz J.K.**, Steimle A., Lange A., Bender A., Fehrenbacher B., Frick J.S.: *Outer membrane vesicles blebbing contributes to* B. vulgatus *mpk-mediated immune response silencing*. Gut Microbes 9 (1): 1-12 (2018)

b 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., Öz H.H., Pichler B.J., Autenrieth I.B., Molinaro A., Frick J.S.: *Weak Agonistic LPS Restores Intestinal Immune Homeostasis.* Molecular Therapy. pii: S1525-0016(19)30319-3. (2019)

c **Maerz J.K.**, Trostel C., Lange A., Parusel R., Michaelis L., Schäfer A., Yao H., Löw H. and Frick J.S.: *Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses*. Front. Immunol. 10:3093.doi: 10.3389/fimmu.2019.03093 (2020)

d 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(6):e3000334 (2019)

e Parusel R., Steimle A., Lange A., Schäfer A., **Maerz J.K.**, Bender A., Frick J.S.: *An important question: Which LPS do you use?* Virulence 8(8): 1890-1893 (2017)

f 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)

Contributions

Publication a

I designed the project together with Anna Lange and Alex Steimle. I established all protocols, performed all experiments in the laboratory and analyzed the resulting data. The manuscript was written by me and Julia-Stefanie Frick.

Publication b

I took part in designing the experiments and contributed in editing the manuscript. I participated in the experimentation.

Publication c

I devised the hypothesis and designed the project. I developed the protocols and performed the experiments in the laboratory and analyzed data and results with restricted assistance of Constanze Trostel. The manuscript was written by me, Anna Lange and Julia-Stefanie Frick.

Publication d

I contributed in editing the manuscript. I participated in the experimentation.

Publication e

I took part in designing the project and contributed in editing the manuscript.

Publication f

I contributed in editing the manuscript. I participated in the experimentation.

INTRODUCTION

1. The intestinal microbiota

During their development, complex eukaryotic organisms are associated with large numbers of microbes including bacteria, fungi, viruses, and parasites [1, 2]. Colonization with commensal microbes starts in newborns right after birth and establishes its highest complexity in adults resulting in the formation of a versatile microbial ecosystem – the microbiota [3]. Microbes that live inside and on mammals and other complex organisms outnumber the eukaryotic host by an estimated 10-fold and thus function as a major selective force shaping eukaryotic evolution. This symbiotic relationship leads to the genesis of a metaorganism, sharing a common metabolome despite having a separate metagenome, in which both partners benefit from each other [4, 5].

Besides the skin, the respiratory tract and the genitals, the highest numbers and variety of commensal bacteria reside in the intestine [6]. The intestinal microbiota harbors approximately 10^{13} microorganisms per gram luminal content predominantly occurring in the colon and distal ileum [6]. The mutualistic interaction of the host and intestinal microbiota influences and modulates various processes in the host such as (I) the extraction and processing of nutrients, (II) the integrity of the intestinal mucosal barrier, (III) angiogenesis, (IV) metabolism, (V) the colonization of pathogens, (VI) the development of innate and adaptive immune system and even (VII) the activity of the enteric nervous system [7-14]. Despite the microbiota has crucial effects, the mechanisms by which the gut microbial community influences the biology of the host organism remain insufficiently understood.

1.1 Gut microbiota composition

The composition of the microbiota is subject to a permanent and dynamic transformation. Due to many extrinsic and intrinsic properties, the gut microbiota composition is highly variable among distinct individuals [15]. Various environmental factors, diet, the presence of toxins and medication such as antibiotic treatments, influence the assembly of the microbiota. In addition, inherent properties of the host such as age, genetic background and psychology individually affect the constitution of the intestinal microbiota [16, 17].

The intestinal microbiota of a healthy adult is mainly composed of seven major bacterial phyla: Gram-positive *Firmicutes* and *Actinobacteria* as well as Gram

negative *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* [18]. Among these, *Firmicutes* and *Bacteroidetes* bacteria represent 90% of all bacterial strains in intestinal microbiota [3, 15]. In detail, almost about 2000 different bacterial species that encode more than 5 million genes (microbiome) were identified, with numbers continuously increasing [19].

Despite of a permanent dynamic change of this tremendous microbial society, there is a constant balanced state in healthy organisms with the aim of maintaining host-microbiota homeostasis. However in a predisposed host influenced by intrinsic or extrinsic factors, a certain microbiota composition can be potentially harmful for the organism. This condition is termed as “dysbiosis” or “dysbacteriosis” and may predispose the host to a wide range of chronic diseases and infections [20].

1.2 Commensals: symbionts and pathobionts

The bacterial composition of the intestinal microbiota of a healthy host is diverse and consists of a heterogeneous pool of microorganisms. Thereby, microbiota members can not only be classified according to their phylogenetic origin but also divided into different functional groups, dependent on their effect on the host immune system [1]. In general, the gut microbiota is composed of commensals which are nonpathogenic or even beneficial for the host [2]. Furthermore, commensals can be subgrouped into probiotic, autobiotic/symbiotic and pathobiotic bacteria. Probiotics are usually obtained from diet and food supplements and defined by the Food and Drug Administration (FDA) and WHO as “live microorganisms which when administered in adequate amounts confer a health benefit to the host” [21]. Probiotic strains such as *Lactobacillus casei* and *Bifidobacterium bifidum* are especially prescribed to patients with an altered or disturbed microbiota after gastroenteritis or treatments with antibiotics [2, 21]. Symbionts are part of the basic microbiota and are permanently associated with the host organism. Symbiotic bacteria such as *Faecalibacterium prausnitzii*, *Bacteroides fragilis* and *Bacteroides vulgatus* possess important immunomodulatory functions to regulate inflammatory immune processes and to maintain host immune homeostasis [2, 22-24]. Pathobionts are defined as resident bacteria of the microbiota which can cause non-beneficial or detrimental effects on the host under certain genetic or environmental pre-conditions when the composition

of the microbiota or the host's immunity is disturbed [2, 25]. This subgroup includes *Escherichia coli*, *Helicobacter hepaticus* and *Clostridium difficile* [1, 26].
These different properties of symbiotic and pathobiotic bacteria, modulating and triggering inflammatory immune reactions in the host, have been demonstrated in various studies of our working group focusing on the induction of chronic and acute colitis in mice [24, 27-29].

2. Microbiota-dependent autoimmune diseases

Autoimmune diseases (AIDs) arise from a malfunctioning immune system or an abnormal immune response to self-tissue causing inflammation and destruction of tissues and/or organs [30]. The pathogenesis is not understood completely, but it is proposed that AID is a multifactorial disease in which environmental factors (life-style, diet, drugs, infections) and certain genetic backgrounds contribute to the pathology [31, 32]. Based on their immunogenicity, intestinal symbionts and pathobionts have the ability to influence different physiological aspects of the host such as the immune system or metabolism and consequently might be a major player in autoimmunity and thus contributing to the pathogenesis of several diseases, including AIDs [13]. Thereby, the development of pathogenesis can result from an inappropriate and overshooting immune response directed against normally harmless commensal microbes or an ineffective clearance of immune-stimulatory microorganisms by an adequate proinflammatory immune response leading to a continuous activation of the immune system [13, 33].

The manifestation of inflammatory bowel disease (IBD), which is characterized by chronic relapsing intestinal inflammation located either transmural in the whole gastrointestinal (GI) tract (Crohn's Disease, (CD)) or only in the mucosa of the colon (Ulcerative Colitis, (UC)), is associated with changes in the microbiota composition in which a decrease in general complexity but also an increase in certain bacterial species is observable [34-36]. A shifted microbiota composition in the colon of IBD patients was demonstrated in various studies, for example, the proportion of *Bacteroides* species is markedly decreased in UC patients [32, 37-42]. However, no single bacterial strain or combinations of strains have been shown to directly cause or prevent IBD in humans.

In addition, there are correlative evidences that host gut microbiota can directly initiate autoimmunity and influence autoimmune disease that target tissues other than the intestine, such as Type 1 diabetes, multiple sclerosis, arthritis and psoriasis [43-46]. For instance, mice treated with antibiotics or reared in germ-free conditions show attenuated progression of experimental autoimmune encephalitis (EAE) a mouse model for multiple sclerosis [47-49]. Moreover, according to latest reports, the cause for neurodevelopmental disorders including schizophrenia and autism are connected to inflammatory processes suggesting that these diseases could also be associated to changes and dysbiosis in the intestinal microbiota [50-54]. These findings highlight the crucial influence of a disturbed or altered host microbiota composition on the progression of autoimmune disease located in intra- and extra-intestinal tissue.

3. Microbiota-host interactions

The gastrointestinal tract is the main site of interaction between microorganisms and antigens with the host immune system. To control this intense cross-talk caused by a microbial load of >10^{12} bacteria/cm^3 intestinal content, the immune system continuously monitors the resident microbiota and evolved certain antimicrobial mechanisms to prevent overgrowth of the colonizing microbes which could result in serious health consequences including inflammation and sepsis. Simultaneously, the Immune system has the challenge of remaining tolerant to food antigens and the commensal microbiota. The first strategy of the host to maintain immune homeostasis is the minimization of direct contact between intestinal bacteria and the epithelial cell surface (stratification), an area of approximate 200 m^2 in humans. Stratification of microbes by the host is achieved *via* a microbial and chemical barrier, mainly composed of a ~150 µm thick viscous mucus layer [55]. This bisected mucus layer is assembled by intestinal goblets cells secreting mucin glycoproteins. The inner mucus layer additionally contains secretory immunoglobulin A (sIgA) produced by laminal propria plasma cells and antimicrobial peptides (AMPs) secreted by Paneth cells preventing microbial translocation across the epithelial barrier [56, 57]. The second line of defense consists of a single cell layer (10 µm) of intestinal epithelia cells (IECs), including distinct subpopulations connected with tight junctions

and associated with cytoplasmic actin and myosin network regulating intestinal permeability, which form a continuous physical barrier [58]. The third line of defense is composed of a multitude of different lamina propria immune cells forming a more specialized immunological barrier directed against penetrant bacteria to confine their exposure to the systemic immune system (compartmentalization) [59-62].

A crucial step for the interplay between microbes and the host immune system and activation of the immunological barrier at intestinal epithelial interface is the adequate recognition of microbes and microbial compounds by immune cells. Thereby, the mucosal immune system can implement a preliminary discrimination between commensal bacteria, which provide basal signals to maintain a tolerant immune state, or pathogenic bacteria, which provoke maturation of the immune system and activation of specialized immune responses [63-65]. Intestinal epithelial, innate and adaptive immune cells are able to detect and recognize conserved bacterial molecular structures named microbe-associated molecular patterns (MAMPs) *via* the expression of pattern recognition receptors (PRRs) resulting in initiation of downstream signaling pathways [66]. Known signaling receptor families are RIG-like receptors (RLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and Toll-like receptors (TLRs). RLRs initiate antiviral responses after recognition of viral RNAs [67]. CLRs are a large family of PRRs divided into 17 groups which bind carbohydrate ligands to induce immunity to fungi, bacteria, viruses, helminths and protozoa [68-70]. NLRs are cytoplasmic receptors whose activation caused by intracellular pathogenic microbes elicit the expression of cytokines, chemokines and defensins [71]. Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 are specialized NLRs that participate in detecting invading pathogenic microbes which multiply intracellularly [72]. The best-characterized PRRs in the intestinal mucosa are the type I transmembrane proteins of the TLR family. TLRs are composed of an extracellular domain made of Leucine-rich repeats (LRR) that recognizes specific MAMPs, a single transmembrane domain (TMD) and an intracellular Toll-interleukin 1 receptor (TIR) domain which is responsible for downstream signal transduction [73]. Ten human and twelve murine TLRs have been characterized, TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice [74]. Nearly all TLRs are present in the human colon and are expressed on the cell surface or in internal cell compartments of IECs, macrophages,

dendritic cells or B cells [24, 75-78]. The signal transduction after interaction of TLRs with the respective MAMPs is mediated *via* interaction with one of the four identified accessory molecules MyD88, MyD88-adaptor-like (Mal/TIRAP), TIR domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) and triggers the activation of transcription factors such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), interferon regulatory factor (IRF) and activator protein 1 (AP-1) [79-82]. The activation of the transcription factors results in the synthesis of certain inflammatory cytokines and type I and type III interferons (INFs) dependent on the recognized antigen [83-87].

The most intensively researched MAMPs that trigger the intestinal immunity of the host include lipoproteins/lipopeptides (Lpp), muramic acid which is part of the peptidoglycan (PGN) layer of bacterial cell wall, flagellin, unmethylated bacterial DNA CpG motifs and lipopolysaccharides (LPS) (Table 1, Figure 1). Extracellular binding of Lpps or PGN components leads to a heterodimerization of TLR2 with TLR1 or TLR6 respectively whereas the recognition of bacterial flagellin promotes the formation of TLR5-homodimers on the cell surface [88, 89]. The presence of CpG DNA in endosomes boost the activation of TLR9 [90]. The MAMP with a decisive immune response stimulatory property for the host immune system (immunogenicity) is LPS which is anchored in the bacterial outer membrane to protect the cell from surrounding and potentially harmful environment influences [91, 92] (Figure 1). In general, LPS consists of a lipid A part which is considered to mediate the immunogenicity of LPS and a core oligosaccharide (OS) (“rough” LPS). LPS which contains an additional O-antigen built from repeating units of usually not more than five different sugar moieties adjacent to the core OS are named “smooth” [93, 94]. The recognition of this general conserved structure by the MD-2/TLR4 receptor complex with the help of LPS binding protein (LBP) and soluble or membrane bound CD14 results in a conformational change of the MD-2/TLR4 heterodimer to initiate a $(MD\text{-}2/TLR4)_2$ heterotetramerization [76, 95-100].

Table 1. Toll-like receptors with corresponding antigens

Toll-like Receptors (TLRs)

TLRs	agonists/ligands	source	literature
TLR1/TLR2	Triacyl lipopeptides	Bacteria	[101, 102]
TLR2	Lipoproteins	multiple Pathogens	[103]
	Peptidoglycan (PGN)	Bacteria	[104]
	Porins	Bacteria	[105]
	Zymosan	Fungi	[106]
	N-Glycan	Fungi	[107]
	GPI-mucin	Protozoa	[108]
	Envelope glycoproteins	Viruses	[109]
TLR2/TLR6	Diacyl lipopeptides	Bacteria	[110]
	Lipoteichoic acid (LTA)	Bacteria	[111]
TLR3	Double-stranded RNA	Viruses	[112, 113]
	Poly (I:C)	Synthetic analog of double-stranded RNA	[114]
TLR4	Lipopolysaccharide (LPS)	Bacteria	[91, 115]
	Glycoinositol-phospholipids	Protozoa	[116]
	Envelope glycoproteins	Viruses	[117]
	Host-derived HMGB1 and HSPs	Endogenous	[118, 119]
TLR5	Flagellin	Bacteria	[29]
TLR7	Single-stranded RNA	Viruses	[120-122]
TLR8	Single-stranded RNA	Viruses	[121, 123]
TLR9	Unmethylated CpG DNA	Bacteria Protozoa Viruses	[124]
	Mitochondrial DNA	Endogenous	[125, 126]
TLR10	unknown	unknown	-
TLR11	unknown	uropathogenic Bacteria	[127]
	Profilin	Parasites	[128]
TLR12	unknown	unknown	-
TLR13	unknown	unknown	-

3.1 Outer membrane vesicles

For the inter-kingdom cross-talk between microbe and host and the interaction with the immune system, involvement of a whole and viable bacteria cell is not fundamental, since, as described previously, specific bacterial structures (PGN, LPS, Flagellin, DNA, LPS) are sufficient to promote the activation of an immune response. With the release and secretion of MAMPs which are small enough to transfer through the mucus and IEC layer, bacteria are able to manipulate and modulate the immune system of the host without a direct physical interaction with the target cells [129] [130]. Moreover, microbes not only locally influence the immune status at the sites of colonization, they can also affect peripheral immune cell populations and contribute to disease pathology at distal sites [86, 131].

In this context, the biogenesis and release of outer membrane vesicles (OMVs) by Gram-negative bacteria represent a secretion pathway combining the long-distance traveling ability of small soluble bacterial products with mimicking the characteristics of the whole bacterial cell [24, 132, 133]. Usually, these spherical structures, ~20-250 nm in diameter, are composed of the outer membrane (OM) of the bacterial cell surrounding a periplasmic space. Consequently by the assembly, OMVs contain a multitude of cellular structures and components which differ from vesicle to vesicle depending on the parental cell. The protein profile of OMVs is comprised of OM structure proteins, porins, ion channels, transporter molecules as well as periplasmic and cytosolic enzymes and proteins [134, 135]. In particular incorporated enzymes such as proteases, peptidase, nucleases and β- lactamases and also toxins and other virulence factors have a decisive influence on the interaction with the host. In addition to proteins, OMVs deliver phospholipids as important agonist for PRRs of host target cells [136, 137]. Interestingly, the phospholipid composition of the OM of a viable bacterial cell differs in comparison to the composition of the OM of secreted vesicles. In *Pseudomonas aeruginosa,* the OM consists predominantly of phosphatidylethanolamine whereas *P. aeruginosa* OMVs include a phospholipid bilayer with higher proportion of phosphatidylglycerol [135]. As important MAMP, LPS are also present as component of OMVs [137]. Furthermore, RNA and DNA are transported *via* OMVs in the form of circular plasmids, linearized plasmids and chromosomal fragments after active transfer across the periplasm during the biogenesis or after the uptake of extravesicular DNA fragments [138, 139].

In the first step of the OMV biogenesis, the OM is detached from the peptidoglycan due to faster growth of the OM membrane compared to the inner membrane (IM) [140, 141]. Additionally, the accumulation of periplasmic materials such as misfolded proteins and PGN fragments between the OM and the peptidoglycan leads to an increased turgor resulting in the bulging of the OM [142]. Degradation of covalent and non-covalent protein interaction of the OM and the PGN layer leads to an enlargement of the vesicle volume and ultimately to the formation of bottleneck shape constriction ending in the blebbing of the OMV in the extracellular milieu (Figure 1).

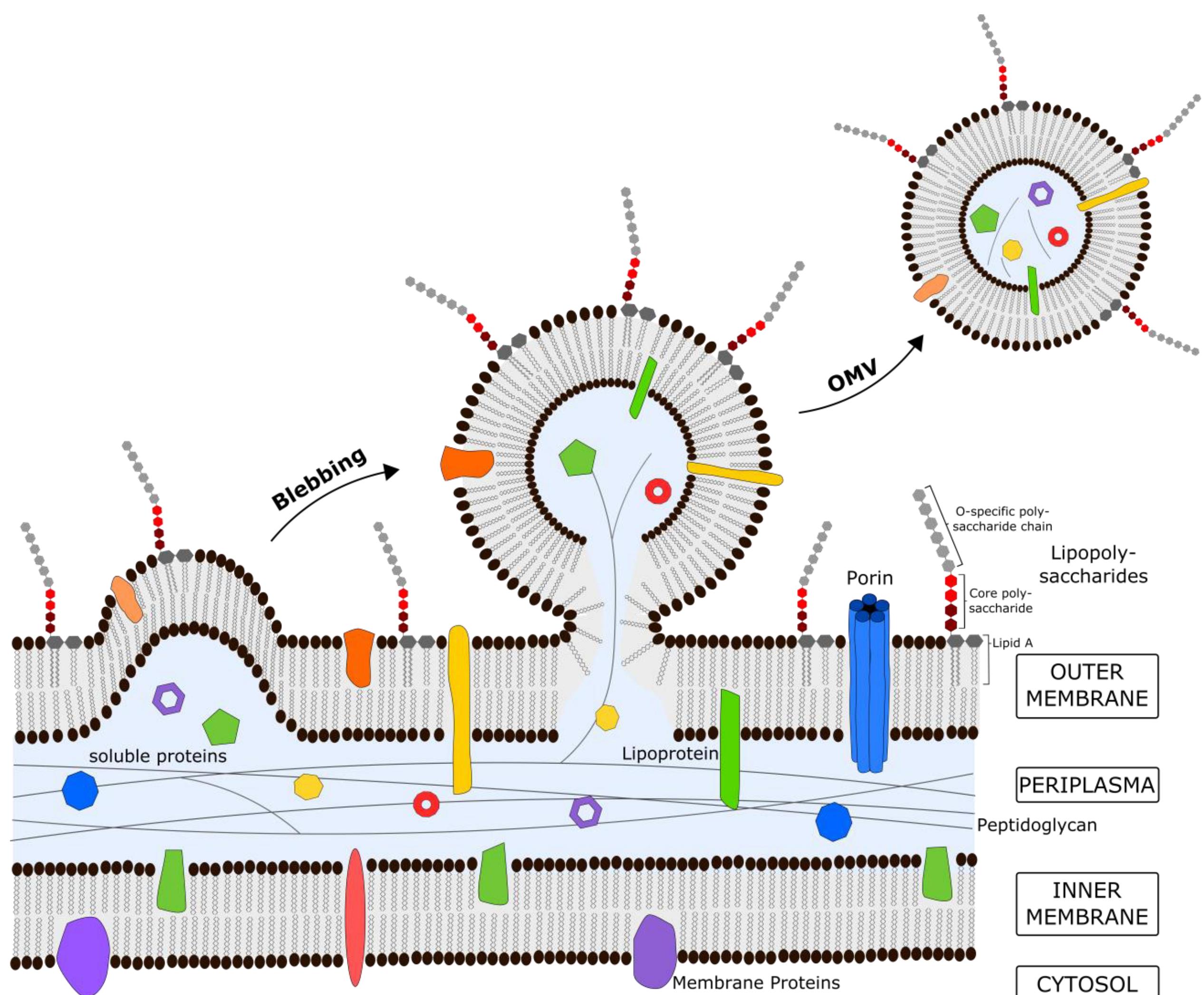

Figure 1. Biogenesis of bacterial outer membrane vesicles (OMVs). Due to a faster growth of the outer membrane (OM) in contrast to the peptidoglycan (PGN) layer and the inner membrane (IM) a bulge is formed. Concurrently, an increased turgor develops cause of the accumulation of periplasmic material such as misfolded proteins. The lateral transfer of proteins and the degradation of covalent and non-covalent protein interaction of the OM and the PGN layer lead to an enlargement of the vesicle volume and ultimately to the formation of bottleneck shape constriction ending in the blebbing of the OMV in the extracellular milieu.

The release of OMVs seems to be a strict regulated and conserved process and is subject to targeted functions [143] (Figure 2). Under alternating physical and chemical environmental conditions, the amount of produced and secreted OMVs by the bacteria cell also changes. During nutrition shortage, on the one hand OMVs are able to deliver in their lumen transported enzymes which degrade complex biomolecules in the extracellular surrounding to make them accessible for the uptake by viable bacteria and on the other hand, OMVs can collect and accumulate limited metal ions for the association with parental cells [143]. As stress response mechanisms during temperature or pH changes, the bacterial cell discharges misfolded or overexpressing proteins *via* OMVs [144]. Furthermore, OMVs act as decoys for antibacterial substances or bacteriophages and can consequently reduce the concentration of these substances in the surrounding milieu ("passive immunity") [145, 146]. Inactivation of antibiotics by OMV-localized proteases, peptidases and other enzymes has also been demonstrated [147]. The far-reaching influences on other organisms have OMVs as transport vehicles of different components. OMVs carry quorum sensing molecules for the long distance inter- and intraspecies communication. Containing small DNA fragments, OMVs are involved in the horizontal gene transfer between species [148]. Microorganisms competing for the same niche secret antimicrobial substances with bacteriolytic activities *via* OMVs to target specific killing of non-self-bacteria [149-151]. In addition to autolysins, virulence factors, cytotoxins and endotoxins are transported by OMVs which results in an immune-activating interaction with eukaryotic cells and thus leading to an modulation of the host immune system [152]. OMV production may also contribute to *biofilm formation* [144, 153-156].

The vesicle secretion is regarded as a separate secretion system with advantage for the bacterial cell over other systems: (1) bacterial lipids, membrane proteins and other hydrophobic molecules can be released from the parental cell; (2) via OMV secreted material is protected from active enzymes and components of the extracellular space; (3) OMVs enable the transport of luminal and membrane-embedded accumulated material in high concentrations; (4) MAMPs in the vesicle lumen and anchored in the OM of the vesicles are able to interact with the target cell and are accessible for the host immune system.

Therefore, the release of OMVs equipped with DNA fragments, autolysins, cytotoxins, virulence factors and a variety of other biomolecules constitutes a critical mechanism for intra- and inter-kingdom communication [24, 143, 157, 158].

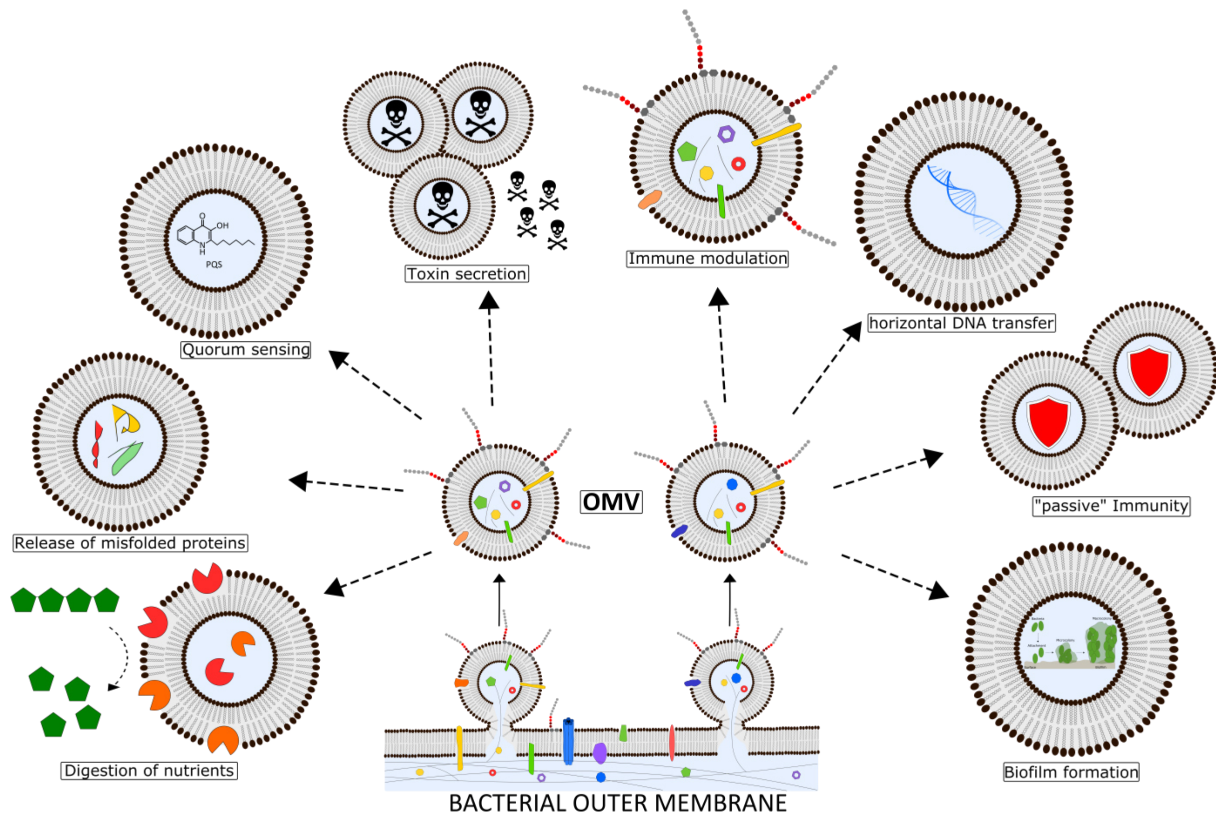

Figure 2. Potential functions of outer membrane vesicles (OMVs). OMVs deriving from different bacteria fulfill a cornucopia of specialised functions in inter- and intraspecies communication. The cargo of OMVs includes quorum sensing proteins, toxins, enzymes for the digestions of nutrients, misfolded proteins, DNA/RNA to initiate horizontal gene transfer and other immune-modulatory structures such as microbe-associated molecular patterns (MAMPs) which interacting with the host immune system. In addition, secreted OMVs are involved in the formation of biofilms and serve as a passive immune system for the bacteria by representing a decoy for antibacterial substances, bacteriophages and antibiotics.

4. The mucosal immune system

The mucosal immune system is comprised of a highly specialized and complex interplay between innate and adaptive immune mechanisms, which are activated and modulated by the interaction of colonizing bacteria with the gut-associated lymphoid tissues (GALT) consisting of the Peyer's patches (PP), mesenteric lymph nodes (mLN) isolated lymphoid follicles and cells residing in the lamina propria [159, 160].

As soon as commensal microbes surmount the microbial and chemical barrier (mucus layer, AMPs, sIgA) and translocate through the physical barrier (epithelial cell layer), they are either rapidly engulfed and neutralized by tissue-resident macrophages or recognized and captured by PRRs on dendritic cells (DCs) [57, 59]. In the latter, these antigen-loaded DCs implement a maturation and licensing and traffic from peripheral lymphatic tissue to the mLN to present processed commensal antigens *via* a peptide:MHC complex to naïve T cells and B cells which results in the differentiation of commensal-specific T helper (Th) cells and IgA-producing B cells. The strength of lymphocyte activation by DCs is dependent on the maturation state of the antigen-presenting cell (APC) [28, 91, 161]. The maturation in turn depends on the immunogenicity of the recognized microbial antigen and is characterized by a high and long-lasting expression of MHC class I or II and co-stimulatory proteins such as clusters of differentiation (CD) 40, CD80 and CD86 on the cell surface [24, 162, 163].
The re-trafficking of activated commensal-specific lymphocytes to the lamina propria and PPs and the prevailing microenvironmental cytokine milieu created by intraepithelial lymphocytes (IEL), mast cells (MCs) and macrophages, determine the outcome of the immune response. In this context, APC-activated $CD4^+$ T helper cells direct the type of immune response against commensals and pathogens: Th1 cells (key cytokine: IFNγ, transcription factor: T-bet) are developed due to the response to intracellular viral and bacterial pathogens; Th2 cells (key cytokine: IL-4, transcription factor: GATA-3) are required for the host defense against extracellular pathogens and induction of humoral immunity; Th17 cells (key cytokine: IL-17, transcription factor: RORγt) are involved in mucosal immunity and autoimmune disorders; regulatory T (Treg) cells (key cytokine: IL-10, transcription factor: FoxP3) are responsible for the maintenance of immune homeostasis through suppression of differentiation and activity of pro-inflammatory T helper cells [164, 165].

At steady state, evoked by constitutive sensing of commensal microbes, most IL-17 and IL-22-producing Th17 and IFNγ-producing Th1 cells are found in the GI tract fulfilling important homeostatic roles by inter alia influencing epithelial cell function [1]. IL-10-secreting Tregs can also accumulate in the intestine and complement immune homeostasis by exhibiting immunosuppressive functions [166]. Especially the differentiation of Tregs is initiated by certain commensal microbiota members and

their structural cell components as it was demonstrated for the polysaccharide A (PSA) of *Bacteroides fragilis* delivered by outer membrane vesicles [1, 86]. The provocation of the mucosal immune system caused by increased numbers of commensal and immunogenic bacteria translocating through a leaky epithelium resulting in a disturbed Th1/Th2/Th17/Treg balance are believed to be associated with pathogenesis of several local and systemic (autoimmune) diseases [164, 167-169].

Such a constellation is prevalent in IBD patients, where the epithelial barrier is leaky and innate immune responses are altered. In addition, the expressions of PRRs such as TLR4 are strongly upregulated leading to a disturbed antigen recognition and thus to the induction of Th1 and Th17 cell-driven pro-inflammatory responses [170, 171]. Further, IECs, which normally participate in balancing T cell responses, activate further effector T cells instead of inducing T cell anergy. The imbalance of activated effector and regulatory T cells results in the production and release of inflammatory cytokines such as IL-12, IL-18, TNF-like 1A and IFNγ which consequently stimulate the secretion of IL-1, TNFα and IL-6 from macrophages [171]. The changed cytokine profile in the lamina propria leads to the inhibition of anti-inflammatory cytokine secretion such as IL-10 by Tregs and to a mal-functioning counter-regulation of pro-inflammatory immune processes [1].

As described above, the activation and regulation of the host immune system requires the cooperative activities of various cell types that possess specialized functions during the response against microbes to either initiate a proper clearance of penetrating bacteria or to exhibit immune tolerance for the maintenance of immune homeostasis. Especially APCs are an important cell type connecting the innate and adaptive immune response by the recognition, transport and presentation of antigens to other immune cells and secretion of a certain set of soluble proteins.

4.1 Tolerant dendritic cells (DCs)

With the production of prominent TLRs, DCs are among the first PRR-expressing cells encountering components of the intestinal microbiota [172]. Originated in the bone marrow, DCs comprise a heterogeneous leukocyte population patrolling lymphoid and non-lymphoid organs [161, 172]. Activated DCs orchestrate the

induction of an adaptive immune response against pathogens while simultaneously maintaining tolerance to self-antigens and the commensal microbiota [161, 173]. In the process, the functionality of DCs is dependent on their maturation state. The maturation program in DCs is initiated by antigen sampling such as the binding of agonistic LPS to the MD-2/TLR4 complex and determines the efficiency of antigen processing and the subsequent antigen presentation [24, 161]. The mature phenotype in DCs is characterised by upregulated expression of MHC-II and co-stimulatory molecules CD40, CD80 and CD86 which are essential for a proper T cell activation and accompanied by the secretion of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-12 [162, 163, 174-176]. Depending on the origin of the recognized antigen, DCs can develop different maturation phenotypes which are strongly distinct from a classical mature state. It has been demonstrated that semi-mature or tolerant DCs play an crucial role for the prevention of intestinal inflammatory processes by maintaining intestinal immune homeostasis [24, 27, 28, 91, 177, 178]. This phenotype is hyporesponsive towards subsequent stimuli and possess tolerogenic properties by the mediation of unresponsiveness of T cells, the induction of Tregs, inhibition of pro-inflammatory Th1 and Th17 cell responses and the promotion of T cell apoptosis or anergy [161, 179].

4.2 Regulatory B cells (Bregs)

Besides DCs, B cells are involved in the establishment of a proper immune response. Thereby, B cells, as part of the humoral immune system, are commonly known as producers of antibodies augmenting immune responses and hence also contribute to the pathogenesis of autoimmune diseases [180]. In addition to the production of antibodies, B cells express PRRs such as TLRs and consequently serve as professional APCs after encountering MAMPs. They are capable of presenting antigens 10^3-fold to 10^4-fold more efficiently than non-professional APCs because of high expression levels of MHC-II and co-stimulatory proteins such as CD80 and CD86, which can induce optimal T cell activation [181, 182]. Additionally, B cells provide co-stimulatory signals and produce a multitude of cytokines to promote naïve $CD4^+$ T cell differentiation into Th1 or Th2 subsets either by direct interaction (surface molecules) or *via* soluble proteins (cytokine secretion) [183, 184]. A specific B cell phenotype with crucial effects on the proliferation and polarization of T helper cells

are regulatory B cells (Bregs) [185]. Certain Breg phenotypes are characterized and described, but they differ in their expression of surface proteins. However, these phenotypes possess similar functionalities including the potent suppression of Th1 cell differentiation, the inhibition of autoimmune pathogenesis and the maintenance of immune homoeostasis [186-193]. A common and most important feature of Bregs to perform these regulatory functions is the production and secretion of the anti-inflammatory cytokine IL-10. IL-10 fulfils regulatory functions by effectively suppressing cell-mediated inflammatory responses, thus restoring Th1/Th2 balance [194-197]. In addition to the production of IL-10, Bregs express and secrete suppressive molecules such as CD73, programmed death ligand 1 (PD-L1), Fas ligand (FasL), glucocorticoid-induced tumour necrosis factor receptor-related protein ligand (GITRL) and Epstein-Barr virus induced gene 3 (EBI3) and thus possess further mechanisms to regulate immune responses in an IL-10 independent manner [198-210]. The combination of these molecules perfect regulatory B cells to a strong immune-suppressive cell subset maintaining immune homeostasis and even attenuating inflammatory processes in autoimmune disease.

Aim of this work

In previous studies our group could demonstrate that the composition of the intestinal microbiota has decisive influences on the induction or prevention of inflammatory immune responses affecting the development and progression of colitis. In detail, we uncovered the immune regulatory features of an intestinal commensal of the *Bacteroides* genus. The symbiont *Bacteroides vulgatus* mpk is competent to alleviate disease pathologies by inhibition of inflammatory immune processes and restoration of immune homeostasis in different mouse models of experimental colitis [27, 176, 177]. The maintenance of immune tolerance by *B. vulgatus* is mediated *via* the induction of a semi-mature phenotype in DCs [24, 28]. In contrast, pathobiotic *E. coli* mpk causes pro-inflammatory host responses and exhibits strong colitogenic potential in genetically predisposed host (deficient for Rag1 or IL-2) [178].

Here, we were interested to further decipher the interaction mechanisms of *B. vulgatus* and *E. coli* with the host and the resulting modulation of the immune system. We want to show that the secretion of outer membrane vesicles is a potent inter-kingdom communication mechanism to prime the host immune system by induction of semi-mature DCs. Further, we want to clarify which PRRs are involved in the recognition of *B. vulgatus* or *E. coli*-derived MAMPs and thus are crucial for the differentiation of DCs. To verify these immune-modulatory properties, we investigate the immunogenicity-dependent potential of symbiotic *B. vulgatus* (weak immunogenic) and pathobiotic *E. coli* (strong immunogenic) to regulate the immune system of the host *via* B cells with the main focus on the immune counter-regulative properties of regulatory B cells (Bregs).

Overall, we want show how different commensal members of the microbiota, represented by *B. vulgatus* and *E. coli,* immunogenicity-dependently activate, modulate and regulate the host immune system to maintain immune tolerance and even suppress intestinal inflammatory processes *in vitro* by interplay between DCs, B cells and T cells and *in vivo* in immune predisposed and immune competent host.

RESULTS AND DISCUSSION

Results of the following submitted or accepted publications and manuscripts are summarized and discussed in the individual “Results and discussion” sections:

a **Maerz J.K.**, Steimle A., Lange A., Bender A., Fehrenbacher B., Frick J.S.: *Outer membrane vesicles blebbing contributes to* B. vulgatus *mpk-mediated immune response silencing.* Gut Microbes 9 (1): 1-12 (2018)

b 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., Öz H.H., Pichler B.J., Autenrieth I.B., Molinaro A. and Frick J.S.: *Weak Agonistic LPS Restores Intestinal Immune Homeostasis.* Molecular Therapy pii: S1525-0016(19)30319-3. (2019)

c **Maerz J.K.**, Trostel C., Lange A., Parusel R., Michaelis L., Schäfer A., Yao H., Löw H. and Frick J.S.: *Bacterial immunogenicity is critical for the induction of regulatory B cells in suppressing inflammatory immune responses*. Front. Immunol. 10:3093.doi: 10.3389/fimmu.2019.03093 (2020)

1. Outer membrane vesicle secretion contributes to *B. vulgatus* – mediated immune response silencing

Bacteroides vulgatus belongs to one of the two predominant phyla of the intestinal microbiota: the *Bacteroidetes. Bacteroidetes* are contribute to host nutrient metabolism, maintenance of structural integrity of the gut mucosal barrier, protection against pathogens and immunomodulation of the immune response to mediate a healthy host-microorganism balance and prevent disease development [18, 211-213].

The immunomodulatory properties of some *Bacteroides* species have already been demonstrated [2, 27, 214]. Especially our group could show that *B. vulgatus* mpk exhibit inflammation-silencing effects leading to the prevention of intestinal inflammation in experimental mouse models for colitis [27, 176-178, 215, 216]. The anti-inflammatory capabilities of symbiotic *B. vulgatus* are mediated by the interaction with DCs and the resulting differentiation of a semi-mature phenotype maintaining immune tolerance [28, 162, 177, 178]. However, direct physical interaction between intestinal commensals and DCs in the colonic lamina propria (cLP) is limited due to the presence of a thick and almost sterile mucus layer covering the intestinal epithelium [24]. Therefore we were interested to identify and investigate the responsible interaction mechanisms between *B. vulgatus* mpk and host immune cells for the initiation of maturation programs in DCs [24]. In this context, the biogenesis and release of bacterial outer membrane vesicles (OMVs) by Gram-negative bacteria represent a possible communication mechanism with host target cells [24]. This secretion pathway combines the long-distance traveling ability of small soluble bacterial compounds which facilitated the transport of insoluble molecules by mimicking the characteristics of the whole bacterial cell and translocation not only through the mucus layer but also the intestinal epithelial cell barrier and entering the systemic circulation and affecting the permeability of the blood-brain barrier [24, 132, 133, 157, 158, 217-220]. Derived from the bacterial outer membrane, OMVs transport certain MAMPs which modulate the innate and adaptive immune responses by either directly or indirectly interacting with PRRs of host target cells and consequently regulate the course of inflammation in various disease models in mice [10, 217, 221-223].

These findings reveal *B. vulgatus*-derived OMVs as interesting immunomodulatory bacterial components that can function as potential inducers of tolerant, semi-mature DCs.

B. vulgatus produce *OMVs which are internalized by CD11c⁺ cells*

Secretion of OMVs by *B. vulgatus* (OMV_{BV}), the direct interaction with immature BMDCs and the capability to induce semi-mature DCs have to be assessed. Bacteria were incubated under different culture conditions and for different time periods to demonstrate the ubiquitous bulging of the outer membrane and the release of OMVs, which could be verified by transmission electron microscopy (TEM) (publication a, figure 1A). In addition, an internalisation timeline assay using $CD11c^+$ DCs in combination with different concentrations of fluorescein isothiocyanate (FITC)-labeled vesicles was established and performed (publication a, figure 1G). After an incubation time of 30 min with 5 µg μL^{-1} OMVs, the population of $CD11c^+OMV_{BV}^+$ cells increased with reaching a maximum of 86.3% after 180 min. Our results are in accordance with other studies demonstrating that OMVs derived from *B. fragilis*, *B. thetaiotaomicron* or *E. coli* Nissle 1917 promote their immunomodulatory properties *via* the uptake and entrance of epithelial cells and macrophages [217, 221, 224].

B. vulgatus OMVs induce tolerant $CD11c^+$ cells

To verify that the interaction of *B. vulgatus*-derived OMVs modulates the immune response *in vivo* and *in vitro* in an inflammation-silencing manner, the activation and maturation of $CD11c^+$ dendritic cells after 24 h stimulation with different vesicle concentrations compared to the challenge with viable, weak immunogenic *B. vulgatus* and strong immunogenic *E. coli* was determined (publication a, figure 1B) [24, 27, 225]. The stimulation of BMDCs with isolated OMV_{BV} leads to the comparable maturation state and semi-mature DCs phenotype like the stimulation with viable *B. vulgatus* since the analysis of MHC-II and T cell co-stimulatory proteins CD40, CD80 and CD86 was represented by a similar intermediate surface expression (publication a, figure 1D). These results are in line with the characteristics of a semi-mature or tolerant phenotype of $CD11c^+$ cells: the inability to promote a pro-inflammatory immune response by the induction and activation of Th1 or Th17 cells [161]. In addition to the lower expression of MHC-II, CD40, CD80 and CD86 in

tolerant CD11c$^+$ BMDC in comparison to the fully maturated cell phenotype (e.g. provoked by viable *E. coli*), the hyporesponsiveness towards subsequent maturation stimuli is part of the definition of semi-mature DCs [28, 176, 226]. Therefore further re-stimulation experiments were performed and could demonstrate that a first immune priming of CD11c$^+$ DCs with OMV_{BV} or the viable bacteria result in the preservation of the maturation state despite of a second immune challenge with strong immunogenic *E. coli*. Both MHC-II expression and secretion of the pro-inflammatory cytokine by BMDCs is lower and unaltered compared to PBS-primed and *E. coli*-challenged cells (publication a, figure 1F).

Thereby it was demonstrated that *B. vulgatus* secreted OMVs mimic the characteristics of the whole bacterial cell and were sufficient to induce tolerance in CD11c$^+$ cells by the prevention of further maturation processes and pro-inflammatory responses.

OMV-mediated immunomodulatory properties signal via host TLR2 and TLR4

The interaction of *B. vulgatus*-derived OMVs with CD11c$^+$ DCs and the resulting promotion of semi-mature DCs with tolerant immunomodulatory properties were proven. However for further experiments and possible therapeutical application, the exact mechanisms of bacteria-host recognition and the involved PPRs and MAMPs for signal transduction have to be identified. TLR4- as well as TLR2-agonists are prominent MAMPs exhibiting strong immunogenic properties and can be integrated into OMVs [76, 93, 227-229]. The stimulation of human embryonic kidney (HEK) cells overexpressing murine TLR2 and CD14/TLR4/MD-2 receptor complex with OMV_{BV} resulted in a concentration-dependent activation of the NF-κB signaling pathway and consequently to the secretion of IL-8. This confirmed the presence and transport of both TLR2- and TLR4- antigens by *B. vulgatus*-derived OMVs (publication a, figure 2A). For validation, BMDCs isolated from *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$x*Tlr4*$^{-/-}$ mice were stimulated with OMV_{BV}, viable *B. vulgatus* or *E. coli* (publication a, figure 2B). The deficiency of both TLRs on the cell surface leads to a complete impairment of antigen recognition independent of the used stimulus as implied by the low expression of MHC-II and CD40 and a marginal secretion of TNFα and IL-6 of *Tlr2*$^{-/-}$x*Tlr4*$^{-/-}$ CD11c$^+$ BMDCs (publication a, figure 2C) [24].

The still increased expression of MHC-II and CD40 as well as the secretion of TNFα and IL-6 in *Tlr2*$^{-/-}$ BMDCs underline the importance of a TLR4 agonist for the maturation of DCs since maturation signals are markedly diminished in *Tlr4*$^{-/-}$ CD11c^{+} BMDCs. However, to confirm that TLR4 ligands obtained by OMV_{BV} or viable *B. vulgatus* are solely sufficient to induce hyporesponsiveness and tolerant DCs and to investigate the involvement of TLR2 agonist, *Tlr2*$^{-/-}$ BMDCs were primed with OMV_{BV}, viable *B. vulgatus* or *E. coli* for 24 h and challenged for additional 16 h with *E. coli* (publication a, figure 2D). The deficiency of TLR2 on BMDCs leads to susceptibility to further stimuli characterized by a changed and increased expression of MHC-II and enhanced production of the cytokines TNFα and IL-6 in OMV_{BV} and viable *B. vulgatus*-primed and *E. coli*-challenged BMDCs compared to mock control (publication a, figure 2E). Consequently, an additional TLR2-dependent signaling is accessory required for adequate activation and differentiation of semi-mature BMDCs and suggests that both TLR4 and TLR2 ligands are requisite for the induction of tolerant BMDC to prevent pro-inflammatory responses.

These findings are in line with previously published results demonstrating the anti-inflammatory activity of OMV-transported Polysaccharide A (PSA) sensed *via* host TLR2 and TLR4 receptors [221, 230]. The binding of PSA to TLR2 leads to the induction of FoxP3^{+} regulatory T cells which regulates the activation of host immune system and maintenance of immune homeostasis [22, 24, 231]. In addition, the simultaneous interaction of different MAMPs with different host receptors might result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity [24, 232-235].

In conclusion, OMVs derived from symbiotic intestinal commensal *B. vulgatus* exhibit host immune system modulatory and regulatory properties by mimicking the parental bacterial cell. Furthermore, with their combined qualities of crossing physical barriers and the mediation of endotoxin tolerance and even cross-tolerance in DCs *via* the delivery of different MAMPs to target immune cells, the production of OMV represents an important key feature of this symbiotic strain to prevent from intestinal inflammation in the host [24].

B. vulgatus -mediated induction of tolerant DC phenotype and the resulting restoration of intestinal immune homoeostasis is dependent on the weak agonist LPS

As demonstrated above, the involvement of host TLR4 is mainly responsible for the activation and differentiation of host immune cells after antigen recognition. Lipopolysaccharides (LPSs), generated by Gram-negative bacteria, are one of the most abundant TLR4 agonists promoting potent immunomodulatory effects [91]. These effects ranging from strong immunogenic activation of the host immune system (agonistic) to a complete block of immune responses (antagonists), are dependent on the chemical structure and composition of lipid A which determine the biological activity of LPS [91, 93, 228, 236]. To avoid overstimulation of intestinal immune cells by the large load of strong agonistic LPS present in the GI content resulting in an overshoot of the immune response, the host developed and initiated endotoxin tolerance mechanisms [91]. The induction of endotoxin tolerance has been detected *in vitro* and *in vivo* both in animal models and humans but the underlying molecular mechanisms still remain incompletely understood [91, 237]. One mechanism is established by the semi-maturation of DCs characterized by hyporesponsiveness toward a second LPS (endotoxin) encounter through receptor desensitization as a result of a first LPS stimulus [24, 91, 161, 238].

As already demonstrated in previous experiments, OMV_{BV} and viable *B. vulgatus* induce tolerant $CD11c^+$ cells exhibiting immunomodulatory properties and consequently contributing to the prevention of inflammation processes in several mouse models for experimental colitis (publication a, figure 1F) [24, 27, 28, 216]. It is conceivable that this effect is mainly mediated by the interaction of LPS derived from OMV_{BV} and viable *B. vulgatus* (LPS_{BV}) with the respective PRR. To verify the hypothesis that LPS_{BV} is crucial for the induction of hyporesponsive $CD11c^+$ cells, LPS of *B. vulgatus* or *E. coli* (LPS_{EC}) were isolated and purified by the removing cell, growth medium and capsular contaminations and the enzymatic treatment to exclude proteins and nucleic acids [91]. Stimulation of $CD11c^+$ BMDCs with LPS_{BV} or LPS_{EC} resulted in the same BMDC phenotype as stimulation with the respective bacteria from which the LPS was isolated (publication b, figure 3A) [91]. To confirm the hyporesponsiveness of LPS_{BV}-primed BMDCs towards a secondary stimulus, cells were subsequently challenged with either *E. coli* or PBS as a negative control (publication b, figure 3B). The results demonstrate that isolated LPS of *B. vulgatus*

induces hyporesponsive semi-mature $CD11c^+$ cells (publication b, figure 3C). To confirm that isolated LPS_{BV} provides the same semi-maturation-inducing capacities and mediates prevention of intestinal inflammation in mice with established colitis comparable to viable *B. vulgatus*, T cell-transplanted *Rag1*$^{-/-}$ mice were administered with LPS_{BV} (publication b, figure 4A) [24, 28, 91, 178]. The treatment after 4 weeks of T cell transfer and colitis induction with LPS_{BV} and *B. vulgatus* led to a significantly lower intestinal inflammation compared to non-treated mice elucidated by H&E-stained colonic sections and histological colitis score (publication b, figure 4B+C). This result infers that administration of purified LPS_{BV} reduces established intestinal inflammation in a mouse model of experimental colitis [91].

Detailed studies on the breakdown of the exact structure of *B. vulgatus*-derived LPS and the binding to host PRR are ongoing; however, it is suggested that the weak agonistic activity of LPS_{BV} for the interaction with the MD-2/TLR4 receptor complex ameliorate inflammation processes through the induction of semi-mature $CD11c^+$ cells and consequently through the mediation of endotoxin tolerance toward strong immunogenic endotoxins [24, 91].

2. Bacterial immunogenicity is critical for the induction of tolerant and regulatory immune cell phenotypes resulting in the suppression of inflammatory immune responses

In preceding experiments, the immune regulatory features of the intestinal commensal *Bacteroides vulgatus* mpk were investigated. By the induction of a tolerant semi-mature phenotype in dendritic cells, *B. vulgatus* is competent to inhibit inflammation development and maintain immune homeostasis in immune compromised host [24, 27, 28, 176, 177]. In contrast, the stimulation with pathobiotic *E. coli* induces fully mature DCs characterized by a high expression of T cell activation markers and strong production of pro-inflammatory proteins, concluding that a potent TLR activation provided by strong immunogenic bacteria leads to enhanced immune responses, aggravating the course of disease [24, 27, 28, 161, 176-178]. Thus, the different immunogenic properties of commensal bacteria affect the differentiation and maturation of certain immune cells. 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 (publication a, publication b) [24, 91].

To investigate the immunogenicity-dependent potential of weak immunogenic *B. vulgatus* and strong immunogenic *E. coli* on the activation and differentiation of other immune cells (in addition to DCs) and consequently the modulation and regulation of host immune system, the direct interaction of bacteria with naïve B cells was analysed.

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 mouse and humans e.g. multiple sclerosis (MS) [239-241]. However, B cells are not only the producer of antibodies but also fulfill multifaceted functions within the immune system. B cells are capable of efficiently present recognized antigens by the high expression of MHC-II and, in combination with costimulatory proteins such as CD80 and CD86 and the secretion of soluble proteins, induce optimal T cell activation [242, 243].

Therefore, 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) [244]. 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 [245-249]. 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 [250-252]. 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) [185, 186]. The main features of Bregs include the potent suppression of Th1 cell differentiation, the inhibition of autoimmune pathogenesis and the maintenance of immune homoeostasis [187]. Only few studies have been published that investigate the direct interaction of B cells 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 (publication c) [210, 253-256].

Therefore, the focus is on the different activation and induction of Bregs by commensal bacteria with low immunogenicity (*B. vulgatus*) and strong immunogenicity *(E. coli*) *in vitro*, the cellular interplay between B cells, DCs and T cells to mimic *in vivo* like condition and the emerging potential to maintain immune tolerance and even suppress intestinal inflammation processes *in vivo.*

B cell activation and maturation via strong immunogenic E. coli are TLR-dependent

To show the direct interaction of B cells with *B. vulgatus* and *E. coli* and the recognition of MAMPs *via* B cell-expressed PRRs, viability and proliferation of wild type (WT) and *Tlr2*$^{-/-}$x*Tlr4*$^{-/-}$ isolated naïve B cells after stimulation for several time periods were analyzed. The longevity and proliferation of naïve B cells is strongly dependent on a potent stimulus provided by strong immunogenic *E. coli* since survival of B cells decreased significantly 24 h after stimulation with PBS (mock) or *B.*

vulgatus (publication c, figure 1A+B). In addition, TLR2xTLR4-deficiency on naïve B cells caused a hyporesponsiveness of B cells toward *E. coli* and *B. vulgatus* resulting in an increased mortality and lack of proliferation, indicating that the activation of naïve B cells depends on a robust bacterial interaction with TLR2 or TLR4 on the surface of B cells. Interestingly, maturation and differentiation of naïve WT B cells, characterized by the increased production and expression of MHC-II, CD80, CD86 and IgM, were significantly upregulated in response to strong immunogenic *E. coli* (publication c, figure 1C). In addition, the maturation of B cells was TLR2/4 dependent since *Tlr2*$^{-/-}$x*Tlr4*$^{-/-}$ splenic B cells did not express high levels of activation markers, regardless of the used bacterial stimulus.

Strong immunogenic E. coli induces differentiation of regulatory B cell phenotypes

Similar to the differentiation and maturation of CD11c^{+} DCs, the strong immunogenic properties of *E. coli* led to a profound proliferation, activation and maturation of naïve B cells whereas the stimulation with *B. vulgatus* resulted in semi-mature cell phenotypes. However, no definitive tolerant phenotype exhibiting immune regulatory capabilities has been identified and characterized for B cells. Though, by the expression of suppressive molecules and the secretion of anti-inflammatory cytokine (e.g. IL-10), a subset of activated B cells named regulatory B cells possess regulatory properties to modulate immune responses. Therefore, 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 unstimulated B cells was determined [257]. The induction of all three analyzed Breg phenotypes and the secretion of the anti-inflammatory cytokine IL-10 were significantly enhanced after stimulation with *E. coli* in comparison to *B. vulgatus* or PBS, whereas only low levels of pro-inflammatory TNFα were detectable (publication c, figure 2B+C). Furthermore, Bregs mediate their immune regulatory properties through the expression of membrane-bound and secretion of soluble suppressive molecules CD73, PD-L1, FasL, GITRL and EBI3. *E. coli*, but not *B. vulgatus* stimulation of naïve splenic WT B cells resulted in a significantly elevated productions of these immune suppressive proteins (publication c, figure 2D).

As interim conclusion, it has been demonstrated that, in contrast to the induction of tolerant DCs, the activation and differentiation of immune regulatory B cells requires a potent immunogenic stimulus provided by *E. coli*.

E. coli-stimulated B cells inhibit DC activation and maturation

To investigate the influences of immunomodulatory features exhibited by *E.coli*-induced Bregs on other immune cells and the host immune system, the maturation of immature CD11c$^+$ BMDCs in co-culture with isolated B cells in direct contact or separated *via* Transwell membranes after stimulation with *B. vulgatus* or *E. coli* was determined (publication c, figure 3A). The presence of naïve B cells during the stimulation of DCs with *E. coli* led to an inhibition of DC maturation indicated by a significantly reduced expression of MHC-II, CD40, CD80 and CD86 and significantly diminished secretion of pro-inflammatory TNFα in comparison to the maturation level of *E. coli* activated DCs in single cell culture (publication c, figure 3B+C). This effect was observed in both direct and indirect DC-B cell co-cultures stimulated with *E. coli.* However, the maturation of DCs was slightly more suppressed in direct interaction with B cells stimulated with *E. coli.* Fittingly, the concentration of IL-10 in co-culture supernatant was highest in *E. coli*-stimulated samples (publication c, figure 3C).

The interaction of DCs with *E. coli*-stimulated B cells significantly inhibited antigen-presentation function and consequently T cell activation ability of CD11c$^+$ DCs, 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. 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 previous 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 [206, 210, 258].

E. coli-primed B cells inhibit T cell activation and induce Treg differentiation

It has been demonstrated in previous experiments, that *E. coli*- induced Bregs have crucial influences on the antigen-presentation activities of other APCs. For this

reason, it is important to elucidate the impact of B cell-driven regulatory mechanisms 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* [259]. Therefore, a proliferation and polarization assay with PBS, *B. vulgatus* or *E. coli* -primed WT B cells pulsed with Ova-peptide and subsequently co-cultured with naïve CFSE-labelled CD4^{+}CD44^{-} T cells isolated from OT-II mice, which express an Ova peptide-specific TCR were implemented (publication c, figure 4A). The proliferation of naïve CD4^{+} T cells was significantly lower in co-cultures with *E. coli*-primed B cells (publication c, figure 4B). The cultivation of T cells with naïve unstimulated B cells led to an intense T cell proliferation and a polarization towards Th1 and TH17 cells. In contrast, T cells incubated with *E. coli*-primed B cells favored a polarization shifted towards Th2 cells and Tregs (publication c, figure 4B+C). *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 [167-169].

<u>Strong immunogenic E. coli counter-regulates inflammation via activated B cells during DSS-induced inflammation</u>

In the *in vitro* experiments, 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 to the hypothesis that in a host having a 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 maintain immune homeostasis.

To verify this hypothesis, germfree WT mice were colonized with *B. vulgatus* or *E. coli* for 4 weeks *via* drinking water 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 (publication c, 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 (publication c, figure 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 animals (publication c, 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. Interestingly, the influx of neutrophils was also reduced in *B. vulgatus*-colonized mice as compared to germfree mice (publication c, figure 5E). 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 (publication c, 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 (publication c, figure 6B). In addition, we analyzed the polarization of $CD4^{+}$ T cells in all three DSS-administered groups. No significant differences were observed in the Th2 cell differentiation between the groups. 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 reduced in the mLN in comparison to germfree or *B. vulgatus*-colonized DSS-treated mice (publication c, figure 6C).

Here it was demonstrated: (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, activated 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.

CONCLUSION

In this work, two regulation-mechanisms of the host immune system to prevent inflammatory responses and maintain immune homeostasis in healthy and immune compromised host have been elucidated. The induction of these mechanisms is dependent on the immunogenicity of commensal bacteria and the resulting strength of cell activation after the interaction between MAMPs delivered *via* OMVs or viable bacteria with TLRs of target immune cells.

On the one hand, weak immunogenic signal provided by *B. vulgatus* is beneficial in genetically predisposed host (deficient for Rag1 or IL-2) in the course of inflammation whereas in these genetic backgrounds 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 [27, 91, 178]. These findings may be attributed to the induction of tolerant DCs by weak immunogenic *B. vulgatus* preventing pro-inflammatory immune responses by the mediation of endotoxin tolerance and to the induction of fully maturated DCs by strong immunogenic *E. coli* triggering an excessive immune response, aggravating the course of disease in immune compromised host (Figure 3) (publication c).

Thus, 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 [260]. *H. hepaticus* thus induces an anti-inflammatory immune response through the activation of regulatory macrophages to maintain immune homeostasis [261]. 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 [262-264]. 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.

On the other hand and in contrast, in this comparative study including *B. vulgatus* (low immunogenic) and *E. coli* (strong immunogenic), it was demonstrated that the potent stimulation of naïve B cells with *E. coli* resulted in a strong activation and differentiation of Breg phenotypes *in vitro* and *in vivo* leading to an anti-inflammatory balance of Th1/Th2/Th17/Treg cells, marked by a pronounced differentiation of Tregs, which is important for the maintenance of immune homeostasis in a healthy host and decisive for the prevention of inflammation development and consequently the possible reason for the extenuated inflammation in *E. coli*-colonized and DSS treated mice [167-169]. It has already been published in mouse models for T1D that strong immunogenic bacteria have inflammation-suppressive properties and can even prevent the onset of disease, on the condition that the host provides a functional immune system [265].

In summary, 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. These findings may contribute in developing therapeutical approaches for treating inflammatory bowel disease and other microbiota-dependent autoimmune diseases.

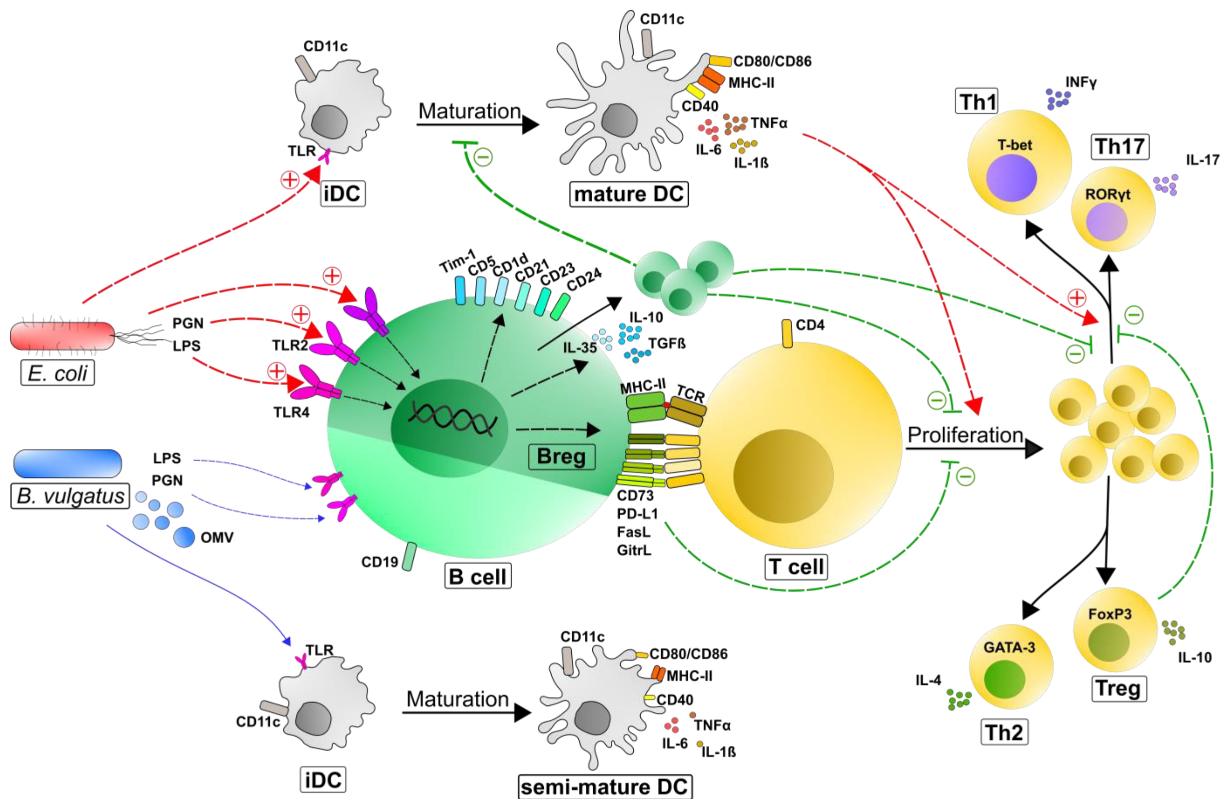

Figure 3. Immune modulation mechanisms via activation of regulatory cell phenotypes induced by immunogenic bacteria. Depending on the immunogenicity of a bacterial antigen, APCs (immature DCs and naïve B cells) are activated differently through the recognition of MAMPs (e.g. LPS or PGN) through Toll-like receptors. Interaction of weak agonistic viable *B. vulgatus*, OMV_{BV} or LPS_{BV} leads to semi-maturation of $CD11c^{+}$ DCs exhibiting tolerant properties. Binding of strong agonistic *E. coli* results in the fully maturation of DCs expressing high amount of MHC-II and T cell co-stimulatory proteins and secreting increased levels of immune-modulatory cytokines. In contrast, 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 [266]. 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); OMV (outer membrane vesicle); TLR (toll-like receptor); iDC (immature dendritic cell); CD (cluster of differentiation); PD-L1 (programmed death ligand 1); FasL (Fas ligand); GitrL (glucocorticoid-induced tumour 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 (tumour-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) (Adapted from publication c).

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List of posters and presentations

“Intestinal microbiota counter-regulates inflammatory immune responses by the induction of regulatory B”
10th Seeon Conference “Microbiota, Probiotics and Host”, Seeon, Germany, July 2017

“Induction of regulatory B cells by members of the intestinal microbiota to counter-regulate inflammatory disorders”
9th Seeon Conference “Microbiota, Probiotics and Host”, Seeon, Germany, June 2016

“Induction of tolerogenic APCs with regulatory functions via symbiotic bacteria for maintaining intestinal homeostasis and prevention of chronic inflammation”
IRTG “Immunotherapy", Molecular Basis and Clinical Application, SFB685, Tübingen, Germany, June 2016

“Outer membrane vesicles derived from B. vulgatus mpk as an acellular therapeutical agent for treatment of inflammatory disorders”
Cytokines 2015 – Symphonies in Health and disease, 3rd annual meeting of the International Cytokine and Interferon Society (ICIS), Bamberg, Germany, October 2015

“Outer membrane vesicles derived from *B. vulgatus* mpk as a novel acellular therapeutical agent for treatment of colitis”
10th Network Meeting of the DFG training and graduate programs in Life Sciences of the University of Würzburg, Erlangen and Tübingen, Blaubeuren, Germany, July 2015

“Outer membrane vesicles – a novel bacterial cell wall structure that prevents from colitis?”
7th Seeon Conference “Microbiota, Probiotics and Host”, Seeon, Germany, July 2014
Poster-Slam-Award received

“Binding of mesenchymal stem cells and periosteal stem cells to distinct peptides differs significantly from binding patterns observed with dermal fibroblast”
Meeting of the “ESOU – ESFFU – ESGURS”, Tübingen, Germany, October 2013

“Attachment of mesenchymal stromal cells from bone marrow and term placenta to distinct peptides”
65. Kongress der Deutschen Gesellschaft für Urologie e.V. (DGU), Dresden, Germany, September 2013

“Adhesion to extracellular matrix-derived peptides can differentiate between bone marrow derived mesenchymal stem cells and MSC-like pericytes
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Appendix: publications

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Maerz J.K., Steimle A., Lange A., Bender A., Fehrenbacher B., Frick J.S.: Outer membrane vesicles blebbing contributes to *B. vulgatus* mpk-mediated immune response silencing. Gut Microbes 9: 1-12 (2018)

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RESEARCH PAPER/REPORT

Outer membrane vesicles blebbing contributes to *B. vulgatus* mpk-mediated immune response silencing

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ABSTRACT
The Gram negative intestinal symbiont *Bacteroides vulgatus* mpk is able to prevent from induction of colonic inflammation in *Rag1*$^{-/-}$ mice and promotes immune balance in *Il2*$^{-/-}$ mice. These inflammation-silencing effects are associated with *B. vulgatus* mpk-mediated induction of semi-mature dendritic cells, especially in the colonic lamina propria (cLP). However the beneficial interaction of bacteria with host immune cells is limited due to the existence of a large mucus layer covering the intestinal epithelium. How can intestinal bacteria overcome this physical barrier and contact the host immune system?

One mechanism is the production of outer membrane vesicles (OMVs) via ubiquitous blebbing of the outer membrane. These proteoliposomes have the ability to traverse the mucus layer. Hence, OMVs play an important role in immunomodulation and the maintenance of a balanced gut microbiota. Here we demonstrate that the stimulation of bone marrow derived dendritic cells (BMDCs) with isolated OMVs originated from *B. vulgatus* mpk leads to the induction of a tolerant semi-mature phenotype. Thereby, microbe- associated molecular patterns (MAMPs) delivered by OMVs are crucial for the interaction and the resulting maturation of immune cells. Additional to the binding to host TLR4, a yet unknown ligand to TLR2 is indispensable for the conversion of immature BMDCs into a semi-mature state. Thus, crossing the epithelial mucus layer and directly contact host cells, OMV mediate cross-tolerance via the transport of various Toll-like receptor antigens. These features make OMVs to a key attribute of *B. vulgatus* mpk for a vigorous acellular prevention and treatment of systemic diseases.

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Introduction

The intestinal microbiota provides important features which are considered to be beneficial for the host organism, such as (1) the maintenance of the structural integrity of the intestinal mucosal barrier, (2) the development of epithelial cell function, (3) host nutrient metabolism, (4) modulation of the immune system and (5) prevention of bacterial overgrowth.[1,2] Hereby, a certain microbial diversity is considered to be required for the maintenance of the immune homeostasis and crucial for the optimal functionality and interaction with the host. Concerning the interplay between microbiota and the host immune system, a disturbed or altered composition of the gut microbiota, called “dysbiosis,” is associated with the pathogenesis of both intestinal and systemic immunological disorders.[3] Such a dysbiosis of the intestinal microbiota, can also influence the disease onset and progress in a variety of mouse models for autoimmune diseases (AID) such as rheumatoid arthritis (RA), type 1 diabetes (T1D) and inflammatory bowel disease (IBD).[4-6] This highlights the importance of a deeper knowledge of the interaction between the host immune system and the intestinal microbes. Based on this cross talk, specific modulation of the intestinal microbiota composition is considered to be used as therapy for the treatment of microbiota-associated AID.[7,8] Hereby, intestinal commensals are decisive for the induction (pathobionts) or prevention (symbionts) of a pathological immune response in a certain predisposed host.[9] In this context, members of the genus *Bacteroides* were already demonstrated to exhibit symbiotic properties concerning the modulation of the intestinal immune system.[10]

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Accordingly, *Bacteroides vulgatus* mpk was already depicted to mediate inflammation-silencing effects finally preventing from colitis induction in various moue models for IBD.[1-13] These immune regulatory properties of *B. vulgatus* mpk are traced back to the induction of a tolerant dendritic cell (DC) phenotype in the colonic lamina propria (cLP) termed semi-mature.[12-14] However, direct physical interaction between intestinal commensals and cLP DCs is limited due to the presence of a thick and almost sterile mucus layer covering the intestinal epithelium. Therefore, we were interested in how this symbiotic commensal manages to enhance the communication with host cells to modulate the immune system.

Instead of direct physically interacting with target cells, bacteria can communicate with more distant host environments via secretion of signaling molecules, such as toxins, quorum sensing molecules and DNA.[15] These mechanisms involving non-viable bacteria provide distinct advantages for bacteria, since secreted soluble material is smaller and non-viable.[16,17] Therefore, this material can gain access to environments usually being inaccessible for living bacteria. In this context, the biogenesis and release of bacterial outer membrane vesicles (OMVs) by Gram-negative bacteria represent a secretion pathway combining the long-distance traveling ability of small soluble bacterial products with mimicking the characteristics of the whole bacterial cell by facilitating the transport of insoluble molecules.[18,19] OMV release therefore constitutes a critical mechanism for intra- and inter-kingdom communication.[20,21] Derived from the outer membrane of bacteria, OMVs harbour a broad variety of microbe-associated molecular patterns (MAMPs) which interact with pattern recognition receptors (PRRs) of host target cells, leading to innate and adaptive immune responses.[22,23] It was shown that the application of OMVs critically modulates the course of disease in animal models of intestinal inflammation by either a direct or an indirect interaction with the intestinal immune system.[24,25] Hickey et al. even showed that OMVs of *B. thetaiotaomicron* can be found in macrophages of the intestinal mucosa. This implicates a passaging through not only the mucus layer but also through the intestinal epithelial cell layer.

In this study, we provide evidence that the symbiotic commensal *B. vulgatus* mpk produces OMVs. This generation of OMVs contribute to the mediation of the immune-system silencing properties provoked by this strain via the induction of DC semi-maturation. Thereby, both TLR2- and MD-2/TLR4-mediated signaling is crucial for this OMV-induced generation of semi-mature DCs, indicating that TLR2 and TLR4 ligands carried by OMVs are indispensable for the manifestation of this tolerant DC phenotype. Therefore, B. vulgatus mpk-caused OMV blebbing seems to contribute to host-microbe communication at intestinal sites which are hardly accessible for the living bacterium.

Results

Bacteroides vulgatus mpk outer membrane vesicles induce tolerance in CD11c$^+$ cells

It is well known that components of the intestinal microbiota have widespread effects on the mucosal immune system in the intestine.[14,26] Hereby, commensal bacteria exhibit important functions for the priming of immune cells underlying the mucosal epithelial barrier. However, physical contact between bacteria and such immune cells is restricted due to the presence of a large mucus layer covering the intestinal epithelium. Almost all bacteria produce outer membrane vesicles (OMVs) through bulging of the outer bacterial membrane.[27] These vesicles are able to cross the mucin layer, finally increasing the probability of interaction of mucosal immune cells with bacterial surface structures such as MAMPs.[28] The mechanism of OMV production is a key characteristic of Gram negative bacteria and commensal-derived OMVs seem to be important for the modulation of the host immune system.[29]

In previous work, we could demonstrate that *B. vulgatus* mpk modulate the immune response *in vivo* and *in vitro* in an inflammation-silencing manner. This is mainly mediated by the induction of tolerant CD11c$^+$ cells in the colonic lamina propria, which seem to be responsible for the maintenance of homeostatic conditions in the intestine.[12,13] Several molecular mechanisms for tolerance induction have already been identified in CD11c$^+$ bone marrow derived dendritic cells (BMDCs).[30] Until now, not all immune system-modulating surface structures of *B. vulgatus* mpk were already identified. Therefore, we were interested if this symbiotic commensal produces OMVs and if this vesicle production contributes to the observed immuno-

modulatory properties of this commensal strain. The secretion of OMVs can be a response due to environmental stress of the prokaryotic cell.[28,31] Therefore, we incubated the bacteria under different culture conditions and for different periods of time, to elucidate, if this strain produces OMVs and if this is dependent on the culturing conditions. We could demonstrate that *B. vulgatus* produces vesicles ubiquitarily under non-detrimental conditions through bulging of the outer membrane as demonstrated in TEM pictures (Fig. 1a). To gain information on the immune system modulating features of OMVs, CD11c$^+$ BMDCs were generated as described and stimulated with *B. vulgatus* mpk-derived OMVs (OMV$_{BV}$) for 24 h (Fig. 1b). After end of incubation time, BMDCs were checked for surface expression of MHC-II, CD40, CD80 and CD86 by flow cytometry (Fig. 1c, gating strategy) since these surface markers indicate the maturation status of CD11c$^+$ BMDCs.[11,12,14,32] PBS-stimulated BMDCs were used as a negative control (mock), representing completely immature CD11c$^+$ cells. BMDCs stimulated with pathobiotic *E. coli* mpk were used as positive controls, representing mature CD11c$^+$ cells, since CD11c$^+$ BMDC stimulation with this strain was demonstrated to induce complete maturation.[11] *B. vulgatus* mpk was already demonstrated to induce CD11c$^+$ cell semi-maturation as indicated by intermediate expression of MHC-II, CD40, CD80 and CD86 which was significantly lower compared with the expression of these respective surface proteins of mature CD11c$^+$ BMDCs.[11] We could demonstrate that OMV$_{BV}$ provide the same properties like live *B. vulgatus* mpk when used at a concentration of 50 ng mL^{-1}, since we could not detect any difference in the expression of MHC-II, CD40, CD80 and CD86 between CD11c$^+$ cells stimulated with either OMV$_{BV}$ or live *B. vulgatus* mpk, respectively (Fig. 1d). Additionally and as confirmed for live *B. vulgatus* mpk, CD11c$^+$ cells stimulated with OMV$_{BV}$ provided significantly lower surface expression of these proteins compared with *E. coli* mpk stimulated CD11c$^+$ BMDCs. However, the surface expression of MHC-II and T cell co-stimulatory surface proteins of CD11c$^+$ BMDCs represents only a first hint on the maturation of these cells. A key feature of semi-mature CD11c$^+$ cells is tolerance toward a secondary bacterial stimulus after priming with *B. vulgatus* mpk.[11] Therefore, we primed CD11c$^+$ BMDCs with either OMV$_{BV}$, live *B. vulgatus* mpk, *E. coli* mpk or PBS (mock), incubated these cells for 24 h, changed cell culture medium and challenged them with either PBS or *E. coli* mpk (Fig. 1e). We analyzed the surface expression of MHC-II and the secretion of the pro-inflammatory cytokines TNF and IL-6. Immature PBS-stimulated CD11c$^+$ cells showed a significantly increased surface expression of MHC-II as well as secretion of TNF and IL-6 after challenge with *E. coli* mpk. Already mature *E. coli* mpk primed CD11c$^+$ cells maintained their high expression level of MHC-II and IL-6 after challenge with *E. coli* mpk. Due to the high secretion of TNF during the first 24 h, indicating a maturation of the BMDCs, secreted TNF was not detectable in the supernatant of mature CD11c$^+$ cells upon a second challenge with *E.coli* mpk after exchange of media.[33,34] This is in line with the literature reporting on a non-responsiveness of mature BMDCs to additional stimuli.[11,32,35] Semi-mature CD11c$^+$ cells stimulated with either live *B. vulgatus* mpk or OMV$_{BV}$ were non-responsive toward subsequent challenge with *E. coli* mpk concerning the surface expression of MHC-II and the secretion of TNF in the supernatant (Fig. 1f). Furthermore, to provide evidence for a direct interaction of *B. vulgatus*-derived OMVs with antigen-presenting cells, we performed an internalisation timeline assay using CD11c$^+$ BMDCs in combination with different concentration of FITC-labeled vesicles. OMV$_{BV}$ were taken up by cultured BMDCs, leading to an extensive increase of CD11c$^+$OMV$_{BV}$$^+$ cell population after 30 minutes as shown in Figure 1g. As shown by Shen et al., the rapid internalization of vesicles can be achieved in an actin-dependent manner directing the expression of surface markers and inflammatory cytokine secretion in BMDCs.[24]

OMV$_{BV}$-mediated immunomodulatory properties are sensed via host TLR2 and TLR4 receptor complexes

We demonstrated that *B. vulgatus* mpk and its outer membrane vesicles interact with innate immune cells and modulate the host immune system in an anti-inflammatory sense. Though, the exact mechanisms and involved pattern recognition receptors for signal transduction were not yet accurately identified. Therefore, we wanted to describe the responsible receptor on host target cells for the interaction with microbial ligands on bacterial cell surface and outer membrane vesicles, respectively. In this context, Toll-like receptors (TLRs) are the most pivotal pattern-recognition

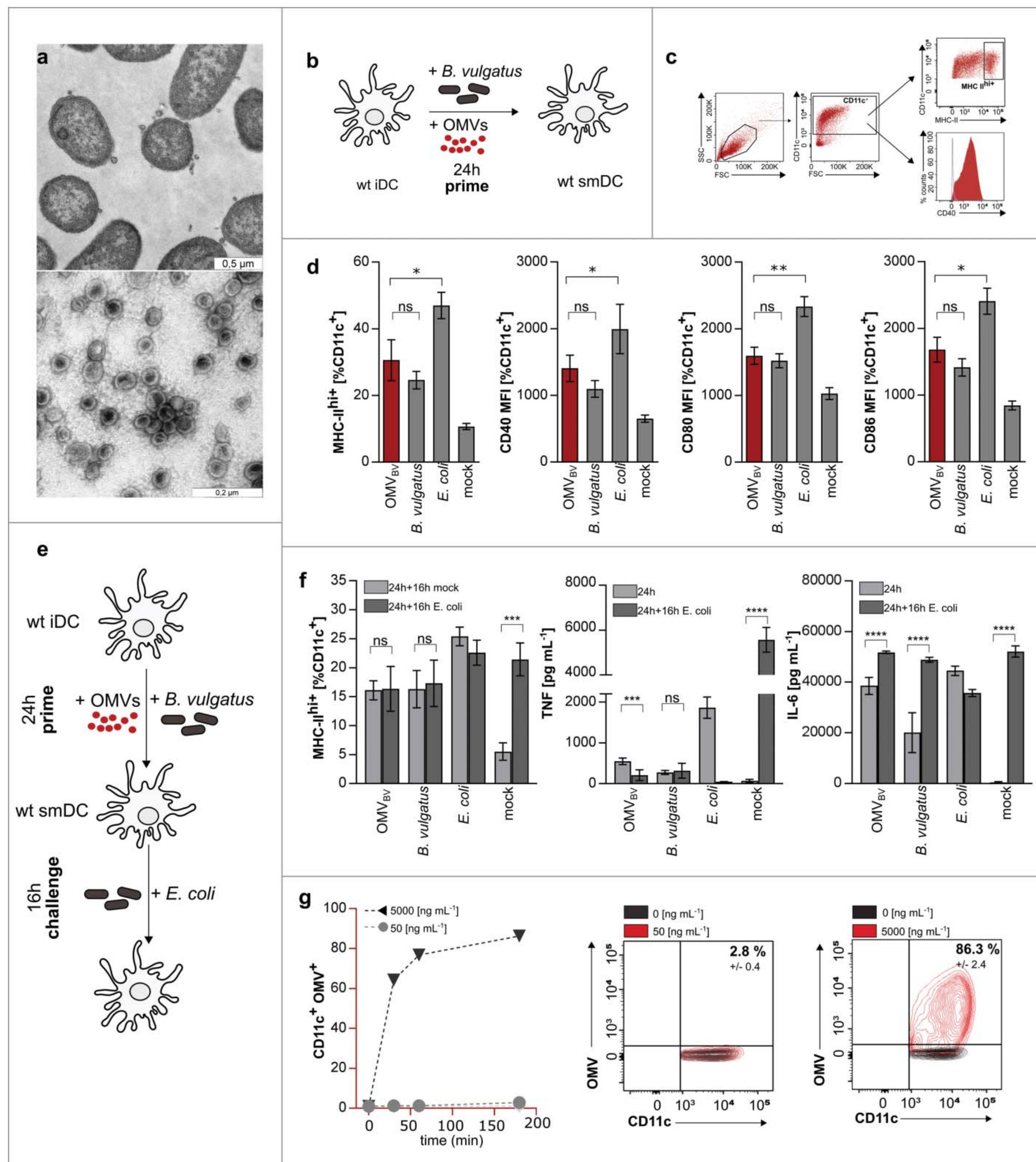

Figure 1. Induction of dendritic cell semi-maturation via *B. vulgatus* OMVs (OMV_{BV}). (a) Evidence for vesicle production derived from the outer membrane of cultivated *Bacteroides vulgatus* cell. Black arrows indicate single secreted vesicles of fixed bacterial cells after toluidine blue staining captured with Transmission Electron Microscope (upper picture). Native OMV_{BV} after isolation and purification stained negative with uranyl acetate (lower picture). (b) Experimental setting for the analysis of surface maturation marker expression on dendritic cells after stimulation with OMVs. BMDCs are primed for 24 h with either PBS (mock), *B. vulgatus* or *E. coli* as control and additionally with 50 ng mL^{-1} OMV_{BV}. (c) Gating strategy for the determination of MHC class II high positive (MHC-II $^{hi+}$), CD40, CD80 or CD86 BMDCs. CD11c-negative cells were excluded and the proportion of MHC-II $^{hi+}$ (dot plot), CD40, CD80 or CD86 (histogram) DCs within the population of $CD11c^{+}$ cells was determined as shown. Dashed line shows negative control. (d) MHC class II $^{hi+}$, CD40, CD80 or CD86 population $CD11c^{+}$ BMDCs that were primed with PBS (mock), *B. vulgatus* mpk, 50 ng mL^{-1} OMV_{BV} or *E. coli* mpk for 24 h (n = 4). (e) Experimental setting for investigation of dendritic cell tolerance after induction of semi-maturation. BMDCs are primed for 24 h with either PBS (mock) to preserve an immature phenotype, *B. vulgatus* mpk or 50 ng mL^{-1} OMV_{BV} to induce semi-maturation or *E. coli* to induce BMDC maturation. After medium change, these cells were secondarily challenged for 16 h with either PBS (mock) as controls or *E. coli* to proof non-responsiveness of tolerant cells. (f) Expression of analyzed maturation markers MHC class II, TNF and IL-6 of $CD11c^{+}$ BMDCs after priming with PBS (mock), *B. vulgatus* mpk, 50 ng mL^{-1} OMV_{BV} or *E. coli* mpk for 16 h and subsequently challenge with either PBS (mock) or *E. coli* mpk for 16 h (n = 4). (g) Flow cytometry analysis of OMV_{BV} internalization by BMDCs. Different concentrations of vesicles were labeled with fluorescein isothiocyanate (FITC) and incubated with cultured DCs for various times. Percentages show $CD11c^{+}OMV_{BV}^{+}$ cell populations. Unlabeled OMVs served as negative control (black population in dot plot graph). All statistical analyses were performed using student's t test. Error bars represent SD.

receptors (PRRs) for the detection of MAMPs.[36-39] The most abundant MAMPs exhibiting strong immunogenic properties are lipopolysaccharides (LPS), which are recognized by CD14 and the TLR4/MD-2 receptor complex.[14,40-42] Besides TLR4 agonists, TLR2 ligands like bacterial lipopeptides, play a prominent role regarding target activation of antigen presenting cells.[43-45] In general, TLR4- as well as TLR2- agonists can be integrated into OMVs and therefore be transported by these vesicles.[46] To characterize the *B. vulgatus* mpk- and OMV_{BV}- associated ligands which might be relevant for the induction of semi-mature BMDCs, we used Human Embryonic Kidney cells (HEK) overexpressing mouse TLR2 or the mouse CD14/TLR4/MD-2 receptor complex. The resulting IL-8 secretion into cell supernatant was detected to determine receptor activation. PBS treated cells served as negative control, whereas Pam_3CSK_4 was used as TLR2 ligand. As demonstrated in Fig. 2a, OMV_{BV} induced both activation of the TLR2 receptor as well as CD14/TLR4/MD-2 receptor complex. To validate these results, we used $CD11c^+$ BMDCs derived from mice deficient for Toll-like receptor 2 ($Tlr2^{-/-}$), TLR4 ($Tlr4^{-/-}$) and for both receptors ($Tlr2^{-/-}xTlr4^{-/-}$). $CD11c^+$ BMDCs of *wt* and $Tlr2^{-/-}$, $Tlr4^{-/-}$ and $Tlr2^{-/-}xTlr4^{-/-}$ mice were generated as described and primed with OMV_{BV} for 24 h (Fig. 2b). $CD11c^+$ cells were harvested and analyzed for surface expression of MHC-II and CD40 (Fig. 1c, gating strategy). Additionally, the amount of secreted pro-inflammatory TNF and IL-6 was detected. The deficiency of both TLRs on the cell surface leads to a complete loss of antigen recognition independent of the used stimulus as indicated by the low expression of MHC-II and an abolished secretion of TNF of $Tlr2^{-/-}xTLR4^{-/-}$ $CD11c^+$ BMDCs. In accordance with the findings generated with HEK cells (Fig. 2a), the activation of the TLR4/MD-2 receptor complex via *B. vulgatus* mpk- secreted OMVs is decisive for the main outcoming signal. In addition, the expression of MHC-II, the costimulatory proteins and the secretion of the inflammatory cytokines in *Tlr2−/−* stimulated BMDCs is nearly as strong as in the *wt* cells after challenging with *E.coli* mpk, *B. vulgatus* mpk or high concentration of OMV_{BV}. Nevertheless, we also observed a dose-dependent increase of all analyzed proteins upon stimulation of $Tlr4^{-/-}$ $CD11c^+$ BMDCs with purified OMV_{BV}, pointing out that the TLR2 receptor is competent to recognize agonistic structures present in OMV_{BV}. Furthermore, TLR2-dependent signaling is also involved in vesicle- induced BMDC activation and maturation (Fig. 2c). This observation prompted us to elucidate whether the contact of host immune cells with a bacterial TLR4 agonist such as LPS alone is sufficient for the activation and differentiation of tolerant BMDCs or whether an additional TLR2 activation is accessory required for adequate induction of BMDC semi-maturation. Therefore, BMDCs were generated from $Tlr2^{-/-}$ mice, primed with OMV_{BV} for 24 h and challenged with *E. coli* mpk for additional 16 h (Fig. 2d). As mentioned before, this experimental setting is required to check for tolerance in semi-mature BMDCs. We could verify that a TLR2-dependent signaling is necessary for tolerance induction in BMDCs, since $Tlr2^{-/-}$ failed to become tolerant upon priming with *B. vulgatus* mpk or OMV_{BV} as demonstrated by the high expression of MHC class II and the enhanced secretion of TNFα upon *E. coli* mpk challenge (Fig. 2e).

Discussion

In this study, we demonstrated that the Gram negative symbiotic commensal *B. vulgatus* mpk produces outer membrane vesicles (OMV). This OMV blebbing mechanism seems to contribute to the immune response modulating properties of this strain which was demonstrated in several previous studies. *B. vulgatus* mpk-derived OMVs (OMV_{BV}) induced tolerance in $CD11c^+$ BMDCs in a TLR4 and TLR2 dependent manner. However, the defined vesicle-associated receptor ligands were not identified. Nevertheless, simultaneous activation of both receptors was required to induce effective DC tolerance. In line with Shen et al. and Hickey et al.[24,25,27] this feature indicates an additional mechanism how *B. vulgatus* mpk as a symbiotic commensal, modulates the immune system at mucosal interfaces. As Bacteria are separated from the host intestinal mucosa by a thick mucus layer that is thought to impede a direct contact between luminal bacteria and the intestinal mucosa,[47] the shedding of OMVs by *B. vulgatus* is of special importance since OMVs can diffuse through the mucin layer.[25,48] This underlines the role of OMVs for the modulation of intestinal immune cells. These characteristics make OMV_{BV} to an important attribute of *B. vulgatus* mpk for an effective passive protection and treatment of systemic diseases.

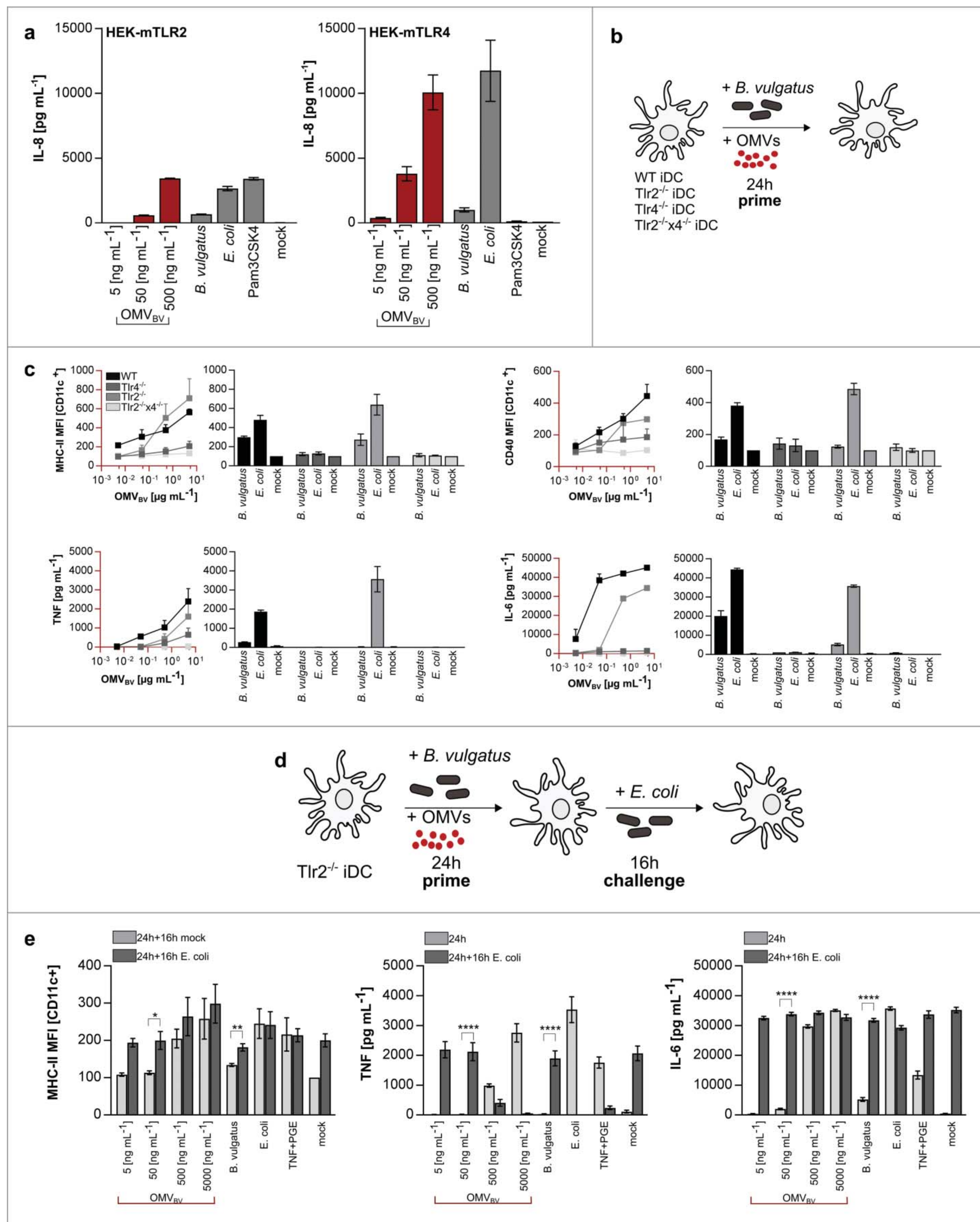

Figure 2. Induction of dendritic cell tolerance via OMV_{BV} is TLR2 and TLR4 dependent. (a) Stimulation of Human embryonic kidney (HEK) cells overexpressing murine TLR 2 or 4. HEK cells are primed with PBS (mock), *B. vulgatus* mpk, various concentrations of OMV_{BV} or *E. coli* mpk for 24 h. Pam3CSK4 served as TLR2 specific control. (b) Experimental setting for the analysis of surface maturation marker expression on dendritic cells after stimulation with OMVs. *wt, Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$*xTlr4*$^{-/-}$ BMDCs are primed for 24 h with either PBS (mock), *B. vulgatus* or *E. coli* as control and additionally with increasing concentrations of OMV_{BV}. (c) MHC class II^{hi+} or CD40 population ($CD11c^{+}$) (normalized to mock) and secreted concentration of TNF or IL-6 of *wt* and TLR-deficient BMDCs that were primed with PBS (mock), *B. vulgatus* mpk, different concentrations of OMV_{BV} or *E. coli* mpk for 24 h (n = 4). (d) Experimental setting for investigation of dendritic cell tolerance after induction of semi-maturation in Tlr2$^{-/-}$ DCs.BMDCs are primed for 24 h with either PBS (mock) to preserve an immature phenotype, *B. vulgatus* or OMV_{BV} to induce semi-maturation or *E. coli* to induce BMDC maturation. After medium change, these cells were secondarily challenged for 16 h with either PBS (mock) as controls or *E. coli* to proof non-responsiveness of tolerant cells. (e) Expression of analyzed maturation markers MHC class II, TNF and IL-6 of $CD11c^{+}$ BMDCs of TLR2$^{-/-}$ mice after priming with PBS (mock), *B. vulgatus* mpk, OMV_{BV} or *E. coli* mpk for 16 h and subsequently challenge with either PBS (mock) or *E. coli* mpk for 16 h (n = 4). All statistical analyses were performed using student's t test. Error bars represent SD.

Gram negative bacteria are able to deliver a chemically diverse spectrum of cargo protected from the external environment over long distances by the help of outer membrane vesicles.[49] Though, OMVs can cross physical barriers which are impermeable for the whole bacterium such as the intestinal mucus layer, gain access to the intestinal epithelium and therefore to the underlying immune cells present in the intestinal lamina propria.[24,25,28,48] This makes OMV blebbing to an important immune modulating mechanism. Considering pathogens, vesicles of *Salmonella enterica* or *Helicobacter pylori* harbour virulence factors to enhance bacterial survival and the ability to colonize host mucosal tissues.[50,51] This leads to a stringent immune reaction and local inflammation.[52] However, OMVs derived from commensal symbiotic bacteria were demonstrated to be involved in maintaining or restoring immune homeostasis in the intestine.[53,54] For example, *Bacteroides fragilis*, representing an intestinal commensal, produces OMVs and interacts with the host immune system by delivering a capsular polysaccharide (PSA) to dendritic cells.[21,55] The release of PSA in OMVs induces immune-modulatory effects, activates regulatory T cells and prevents from experimental colitis.[24] However, a general role for OMVs in mediating immune responses, accounting for all OMV-producing commensal, was not yet demonstrated.[31,56]

Findings using live *B. vulgatus* mpk cells indicate that this symbiotic bacterium prevents from disease induction in different mouse models for experimental colitis.[12,13] In this context, *B. vulgatus* mpk promotes the maintenance of the immune equilibrium via induction of tolerant semi-mature dendritic cells in the intestine and the resulting regulation of host Cathepsin S activity and secretion in these antigen-presenting cells.[32] In this study, we demonstrated that OMVs derived from *B. vulgatus* mpk contribute to these observed effects since they interact with bone marrow derived dendritic cells, leading to a semi-mature phenotype characterized by a low expression of T cell activation and maturation markers as well as pro-inflammatory cytokines.

Innate immune cells recognize conserved microbial ligands through pattern recognition receptors (PRRs) leading to immunologic responses. For example, Polysaccharide A (PSA) from *Bacteroides fragilis* is recognized by TLR2. In consequence, the binding of PSA to TLR2 leads to the induction of FoxP3^{+} regulatory T cells which affect both the development and homeostasis of the host immune system.[57,58] Our study now reveals that OMV_{BV} also contain TLR2 activating ligands which are, in consequence, necessary for the induction of tolerant BMDCs. Since these tolerance-inducing effects are abolished in TLR2- deficient BMDCs, a TLR2-dependent intracellular signaling is indispensable for OMV_{BV}-mediated induction of tolerance in BMDCs. These observations indicate that both receptors, MD-2/TLR4 and TLR2, are required for the induction of DC tolerance. In addition to the LPS in the outer membrane, OMVs of *B. vulgatus* mpk contain and a yet unidentified TLR2 agonist, as it is e.g. the case for PSA in vesicles secreted by *B. fragilis*. Interestingly, the genome of *B. vulgatus* mpk contains 9 different loci coding for capsular polysaccharides (e.g., glycosyltransferases and polysaccharide export outer membrane proteins), indicating the presence of a TLR2-interacting polysaccharide on the outer membrane of the bacterium cell and on vesicles derived from *B. vulgatus* mpk.[46] But the comparison of the various loci in *B. fragilis* and *B. vulgatus* revealed different arrangements of the genes necessary for the biosynthesis of capsular polysaccharides (data not shown). This implicates, that polysaccharides present in *B. vulgatus* mpk has a different structure as compared with the capsular polysaccharide produce by *B. fragilis*.

The presence of a TLR4- and TLR2- ligand on the outer membrane indicates that the mechanism by which OMV_{BV} induce tolerance in DCs is not originated in classical endotoxin tolerance. Usually, low doses of one TLR agonist desensitize the host to subsequent stimulation with a related agonist or endotoxin.[59-61] However, the addition of a Toll-like receptor (TLR) ligand can also induces tolerance to subsequent challenges with other stimuli that signal through one or more different TLRs (cross-tolerance). Since outer membrane vesicles harbor various different TLR ligands, OMV_{BV} might even mediate TLR-cross-tolerance. The simultaneous interaction of different MAMPs with different receptors might therefore result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity.[62-64]

In summary, outer membrane vesicles derived from the symbiotic intestinal commensal of *B. vulgatus* mpk play a pivotal role in modulating and regulating immune responses of the host. With their combined

qualities of crossing physically barriers and the mediation of endotoxin tolerance and even cross-tolerance in dendritic cells via the delivery of different microbial ligands to immune cells, the production of OMV represents an important key feature of this symbiotic strain, to prevent from intestinal inflammation in the host. Due to these properties, OMV_{BV} might even represent a potential therapeutical tool to hamper or recover from inflammatory bowel diseases (IBD) and other systemic inflammatory disorders.

Materials and methods

Cultivation of bone marrow derived dendritic cells (BMDCs)

Bone marrow cells were isolated and cultivated for differentiation as described previously.[65] At day 7 after isolation, resulting CD11c positive, bone marrow derived dendritic cells (BMDCs) were harvested and used for stimulation.

Cultivation of human embryonic kidney cells (HEK cells)

Before stimulation, cryoconserved cell stocks were thawed and cultured in DMEM media supplemented with 10% FCS, 1% Penicillin/Streptomycin up to passage 7.

Stimulation of bone marrow derived dendritic cells

Two $\times$ 10^6 BMDCs were stimulated with either PBS or *B. vulgatus* mpk or *E. coli* mpk at a MOI of 1 or various concentrations of *B. vulgatus*-derived OMVs (5, 50, 500, 5000 ng mL^{-1}). Cells were stimulated for a maximum of 24 hours. If a second challenge was used, media were changed and cells were restimulated with either PBS or *E. coli* mpk MOI 1 for a maximum of 16 hours. Additionally, exogenous TGF (Sigma-Aldrich) 20 ng mL^{-1} and prostaglandin E (Sigma-Aldrich) 1 μM were used as positive and Toll-like receptor- independent control.

Stimulation of human embryonic kidney cells

Two $\times$ 10^4 HEK cells were stimulated with either PBS or *B. vulgatus* mpk or *E. coli* mpk at a MOI of 1 or various concentrations of *B. vulgatus*-derived OMVs (5, 50, 500 ng mL^{-1}). Cells were stimulated for a maximum of 24 hours. FSL-1 (10, 100 ng mL^{-1}) and Pam3CSK4 (30, 300 ng mL^{-1}) (both InvivoGen) were used as positive and TLR2-specific agonist.

Flow cytometrical analysis

Multi-color FCM analyses were performed on a FACS LSRII (BD Biosciences). All fluorochrome-coupled antibodies were purchased from BD Biosciences. Data were analyzed using the FlowJo software (Tree Star Inc., USA).

Cytokine analysis by ELISA

For analysis of IL6, TNF and IL1ß concentrations in cell culture supernatants, ELISA Kits purchased from BD Bioscience were used according to the manufacturer's instructions.

Bacteria cultivation

The bacteria used for stimulation of the murine dendritic cells were *Escherichia coli* mpk and *Bacteroides vulgatus* mpk. The *E. coli* mpk strain was grown in Luria-Bertani (LB) medium under aerobic conditions at 37°C. *Bacteroides vulgatus* was grown in Brain-Heart-Infusion (BHI) medium and anaerobic conditions at 37°C.

Detection of secreted OMVs

Part of the cultured bacteria suspension was sedimented for 2 h and fixed with Paraformaldehyde-Glutaraldehyde Solution (Karnovsky's Fixative) in a rate of 7:2 (A:B) for 1 h at room temperature after removal of excess fluids. Following, for the stabilization of proteins and lipids samples were post-fixed in osmium tetroxide. Fixed cells are then embedded in agar and washed in 0.1 M cacodylate buffer. Addition of uranyl acetate for 1 h enhanced membrane stabilization and improved overall contrast. Dehydration was performed with an ETOH dilution row, followed by transfer in epoxide solution. The polymerization of the agarose pad was achieved via incubation for 20 h at 45°C and plus 48 h at 60°C. For visualization, the semi-thin sections were treated with toluidine blue staining. Images were recorded with Zeiss Libra 120 Transmission Electron Microscope.

Isolation protocol and microscopy of purified OMVs

Cultured *B. vulgatus* mpk suspension was slightly vortexed and centrifuged for 10 min at 10,000 x g. Supernatant was vacuum sterile filtrated and vesicles were pelletized for 2 h at 38,000 x g. Pellets were resuspended in sterile PBS and mixed with Iodixanol (OptiPrep) density gradient solution to prepare a 40% working solution. Subsequently, a dilutions serial with decreasing densities of 40%, 35%, 30%, 25%, 20% and 5% OptiPrep was assembled and stacked up in an appropriate ultracentrifugation tube. The samples were fractionated for 3 h at 280,000 x g and the developed, vesicle containing layers were collected. The isolated vesicles were natively held via the grid-on-drop technic and stained for negative imaging via TEM with uranyl acetate.

FITC-labeling of isolated vesicles

Isolated and purified OMVs were incubated in the dark for 1 h with 1 mg mL^{-1} Fluorescein isothiocyanate (FITC) at room temperature. Suspension was washed twice with HEPES buffer and centrifuged for 30 min at 52,000 x g. The pellet containing fluorescence-labeled OMVs was resuspended in sterile PBS.

Determination of OMVs concentration for stimulation experiments

To ensure a standardized application of vesicles and invariant stimulation of BMDCs and HEK cells, the exact concentration of every OMV batch was calculated and defined by the Pierce^{TM} BCA Protein Assay Kit using the manufacturer's instructions.

Mice

C57BL/6J mice were purchased from Charles River Laboratories. Toll-like receptor 2 and 4 deficient ($Tlr2^{-/-}$, $Tlr4^{-/-}$) mice were provided by Jackson Laboratory. All animals were kept and bred under SPF conditions. For isolation of bone marrow, only female mice aged 6–12 weeks were used. Animal experiments were reviewed and approved by the responsible institutional review committee and the local authorities.

Statistics

Statistical analyses were performed using the unpaired student's t test. For comparing *in vitro* results, samples were considered to be biologically independent if the samples were generated from BMDCs from different mice. Differences were considered to be statistically significant if $p < 0.05$. Error bars, if shown, represent ± standard deviation (SD).

Disclosure of potential conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Therapy
Original Article

Weak Agonistic LPS Restores Intestinal Immune Homeostasis

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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]

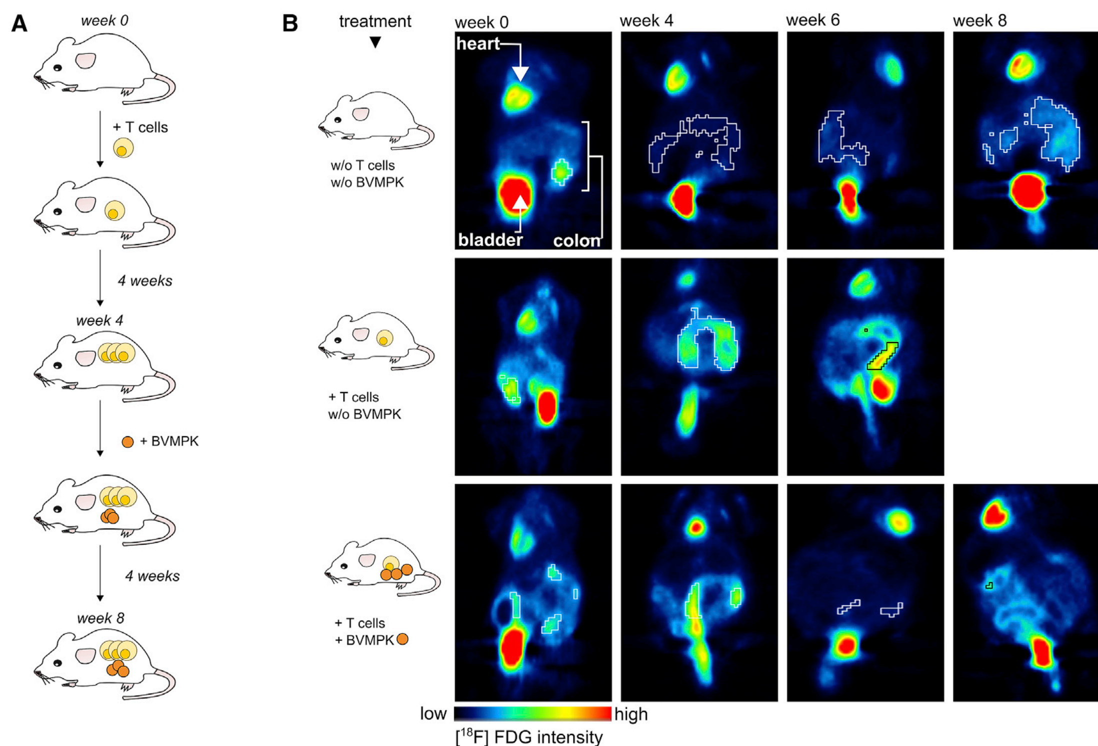

Figure 1. Administration of Live *B. vulgatus* mpk Reduces Intestinal Inflammation in *Rag1*$^{-/-}$ Mice with Established Colonic Inflammation, as Confirmed by Non-invasive *In Vivo* PET Imaging

(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

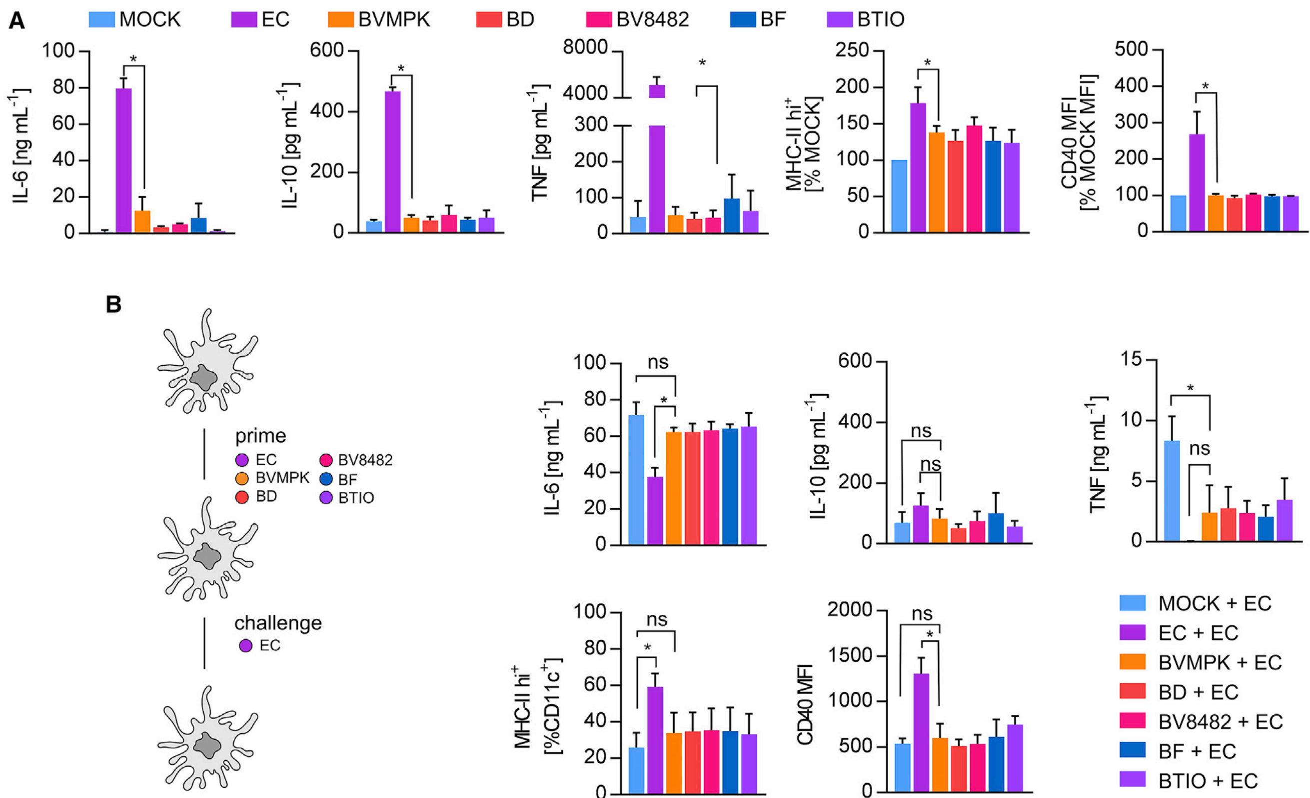

Figure 2. Induction of Hyporesponsive CD11c+ Cells Is a Common Feature of Various *Bacteroides* Species

(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

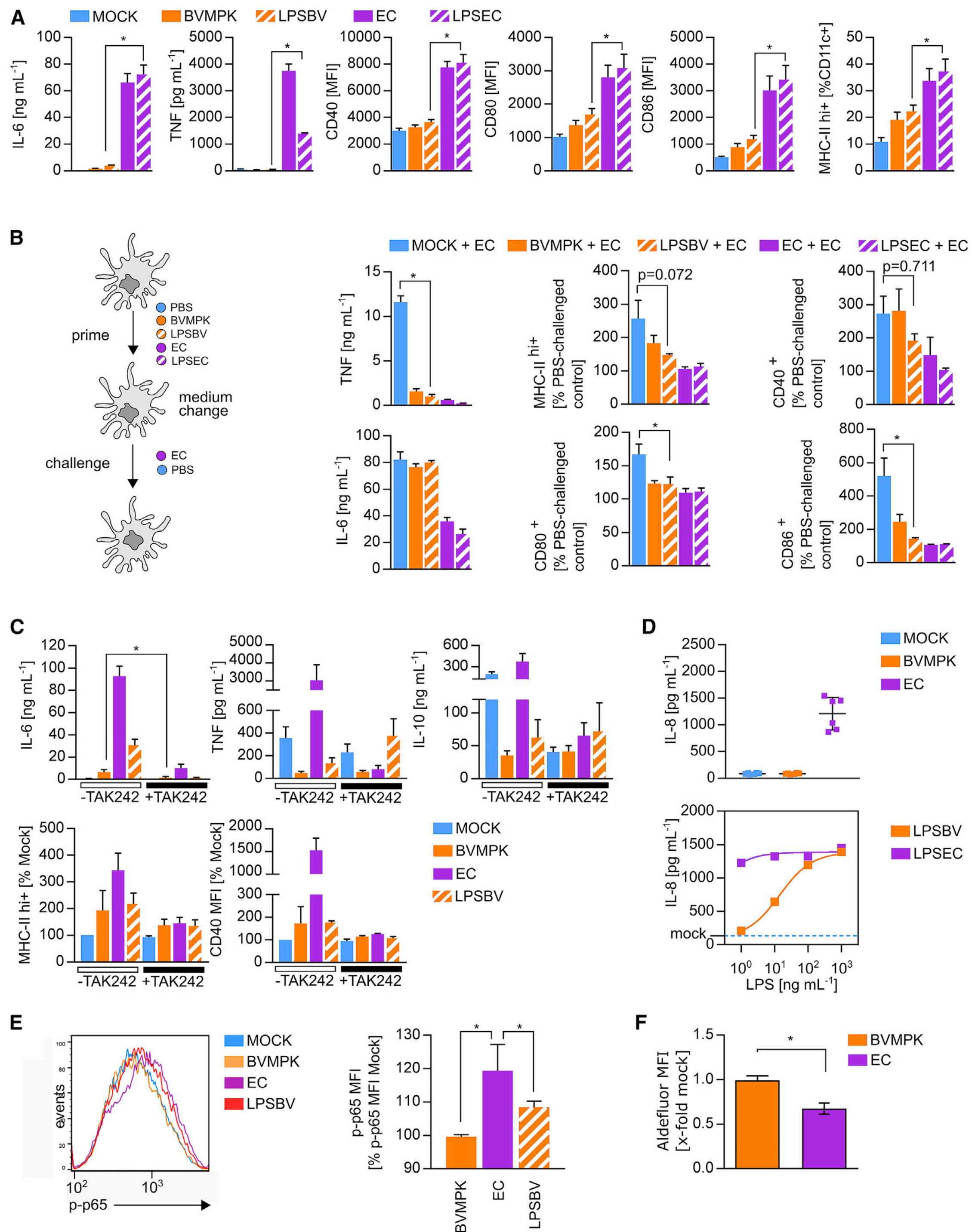

Figure 3. Isolated LPS of BVMPK Induces Hyporesponsive Semi-mature CD11c+ Cells

(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.

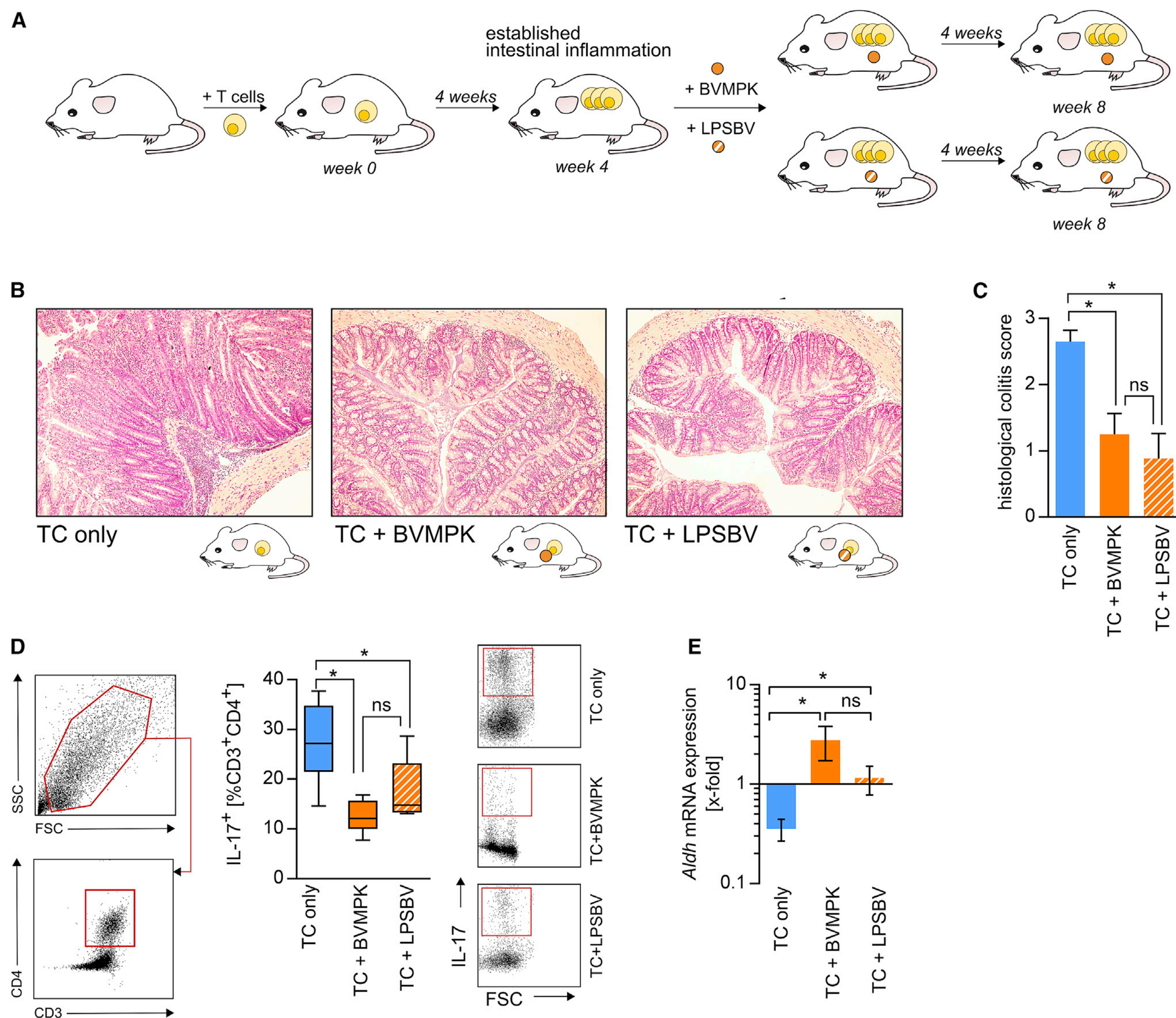

Figure 4. Isolated *B. vulgatus* mpk LPS Provides the Same Capability to Reduce Intestinal Inflammation in *Rag1*$^{-/-}$ Mice with Established Colonic Inflammation as Live *B. vulgatus* mpk

(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}

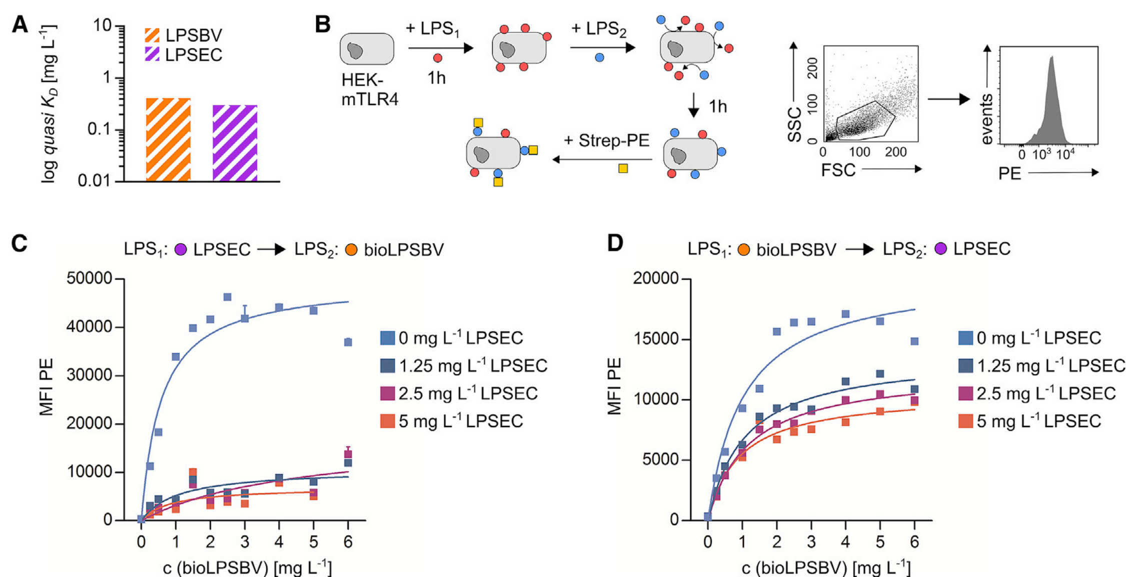

Figure 5. Weak Agonistic LPSBV Is Not a Competitive Inhibitor of Strong Agonistic LPS

(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

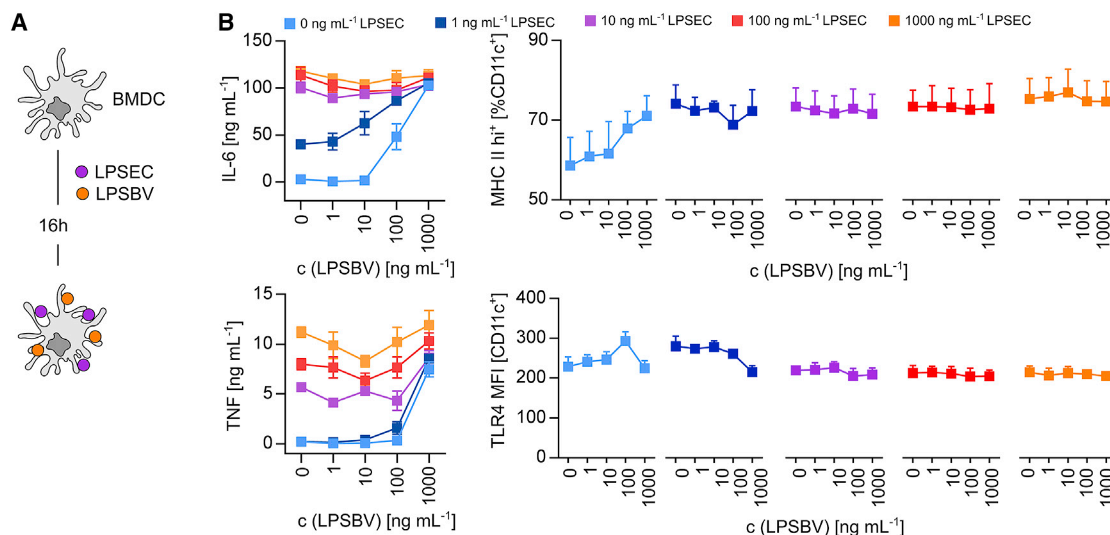

Figure 6. LPSEC-Induced BMDC Maturation Can Not Be Anticipated by the Simultaneous Presence of LPSBV *In Vitro*

(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

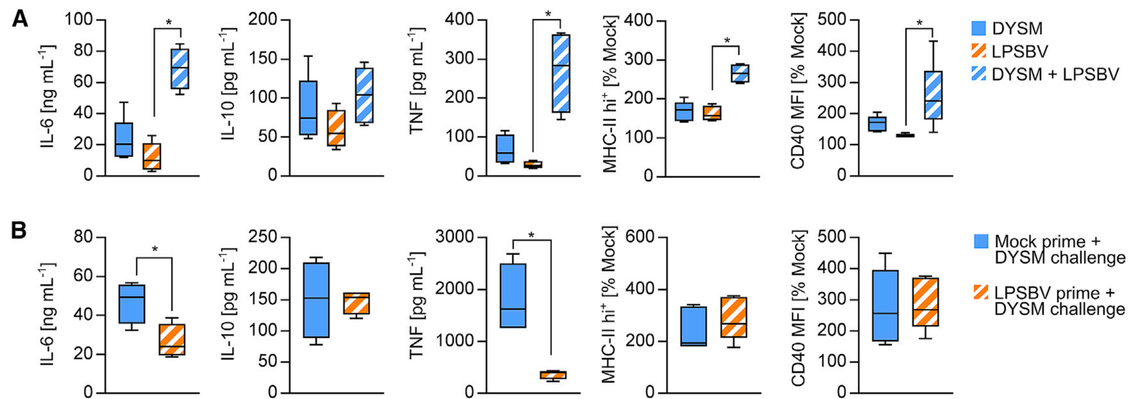

Figure 7. LPSBV-Mediated BMDC Tolerance Is Induced in the Absence of Agonistic LPS and Protects from Pro-inflammatory Response to a Dysbiotic Microbiota Composition

(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

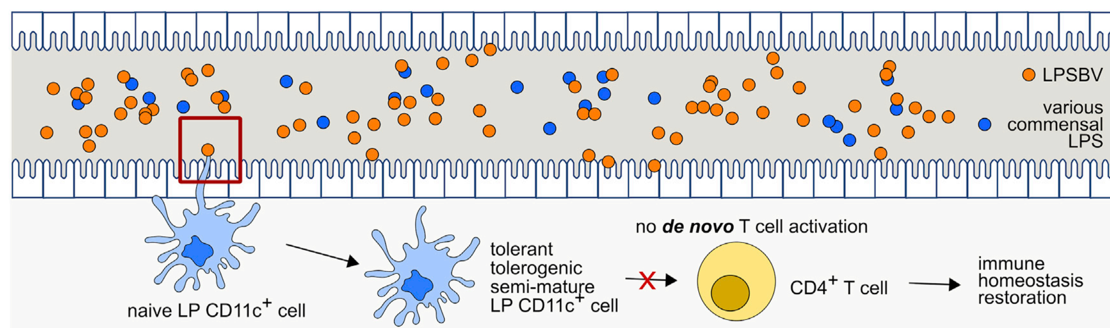

Figure 8. Proposed Mechanism of How Weakly Agonistic LPSBV Influences MD-2/TLR4 Receptor Complex Activation and Prevents the Initiation of a CD4$^+$ T Cell-Mediated Immune Response via Modulation of the Intestinal CD11c$^+$ Cells

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 $\mu g\ \mu L^{-1}$. 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 $\times$ *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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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 $TLR2^{-/-} \times TLR4^{-/-}$ 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 $CD4^+CD44^-CD8a^-CD11b^-CD11c^-CD19^-CD25^-CD45R^-CD49b^-CD105^-MHCII^-Ter\text{-}119^-TCR\gamma/\delta^-$ 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 $Tim\text{-}1^{+}$ 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)

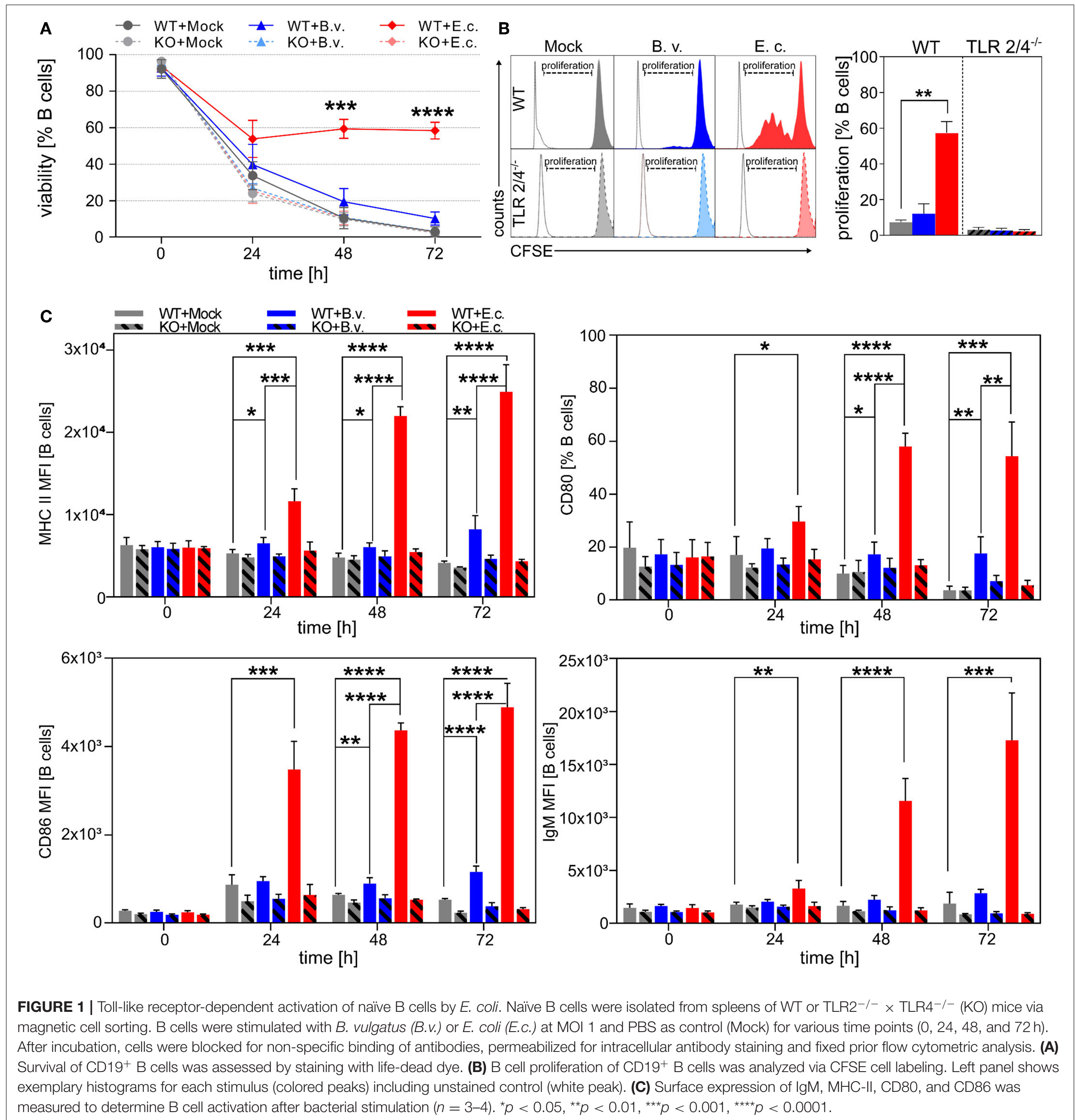

FIGURE 1 | Toll-like receptor-dependent activation of naïve B cells by *E. coli*. Naïve B cells were isolated from spleens of WT or TLR2$^{-/-}$ × TLR4$^{-/-}$ (KO) mice via magnetic cell sorting. B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) 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

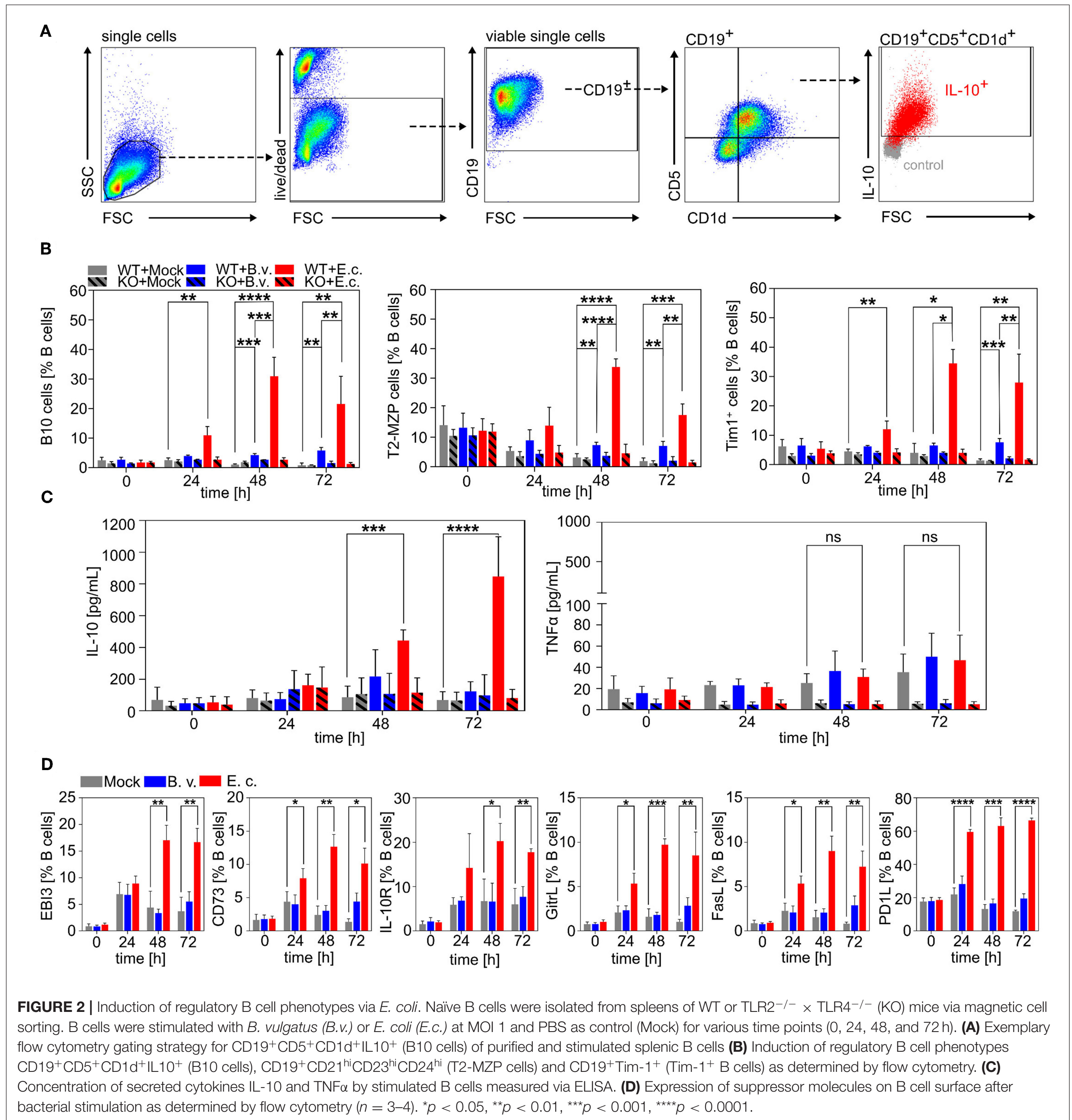

FIGURE 2 | Induction of regulatory B cell phenotypes via *E. coli*. Naïve B cells were isolated from spleens of WT or TLR2$^{-/-}$ × TLR4$^{-/-}$ (KO) mice via magnetic cell sorting. B cells were stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 and PBS as control (Mock) for various time 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$^+$IFNγ$^+$), Th2 (CD4$^+$IL4$^+$), Th17 (CD4$^+$IL17A$^+$IL4$^-$IFNγ$^-$) 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

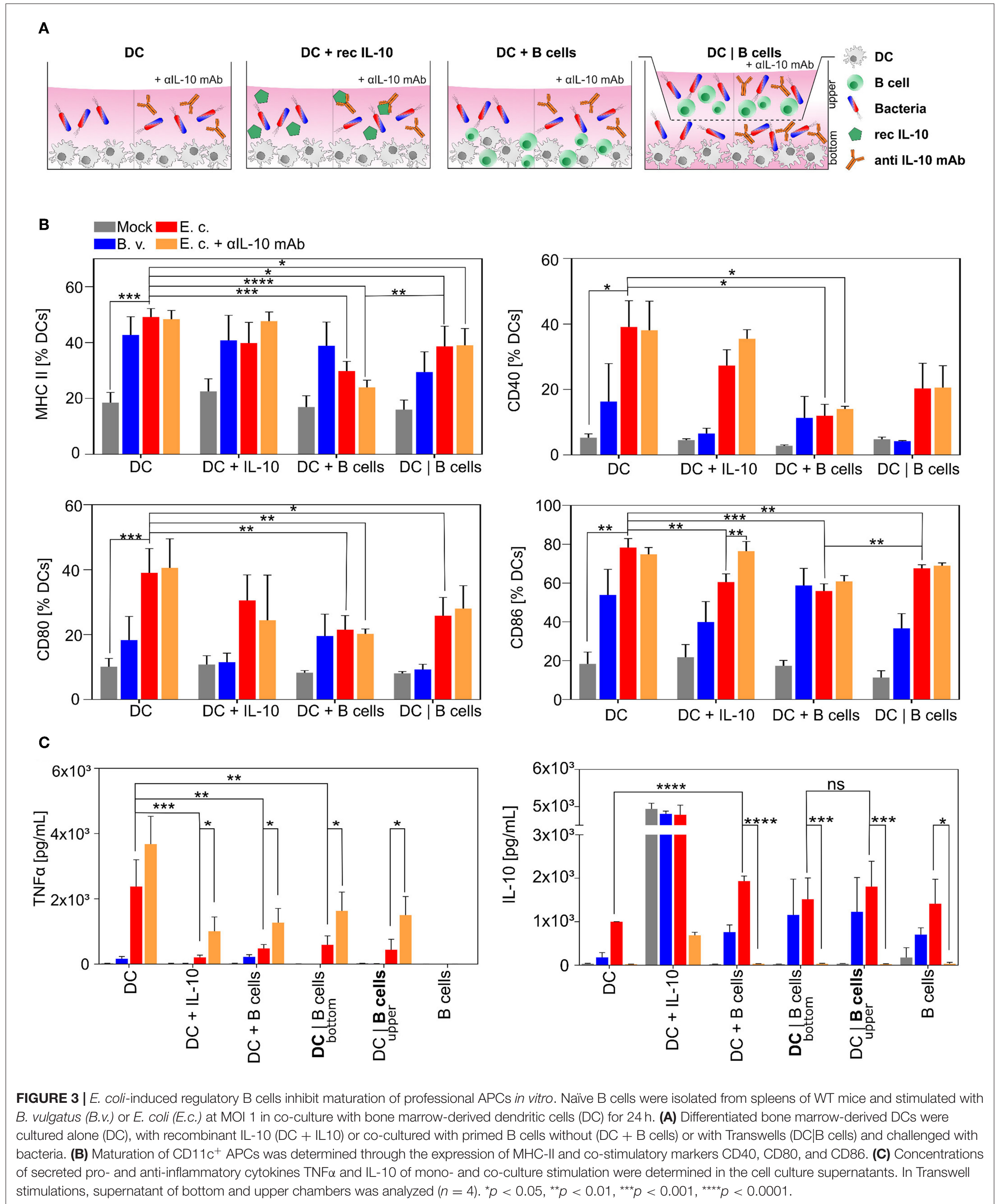

FIGURE 3 | *E. coli*-induced regulatory B cells inhibit maturation of professional APCs *in vitro*. Naïve B cells were isolated from spleens of WT mice and stimulated with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 in co-culture with bone marrow-derived dendritic cells (DC) for 24 h. **(A)** Differentiated 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 ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

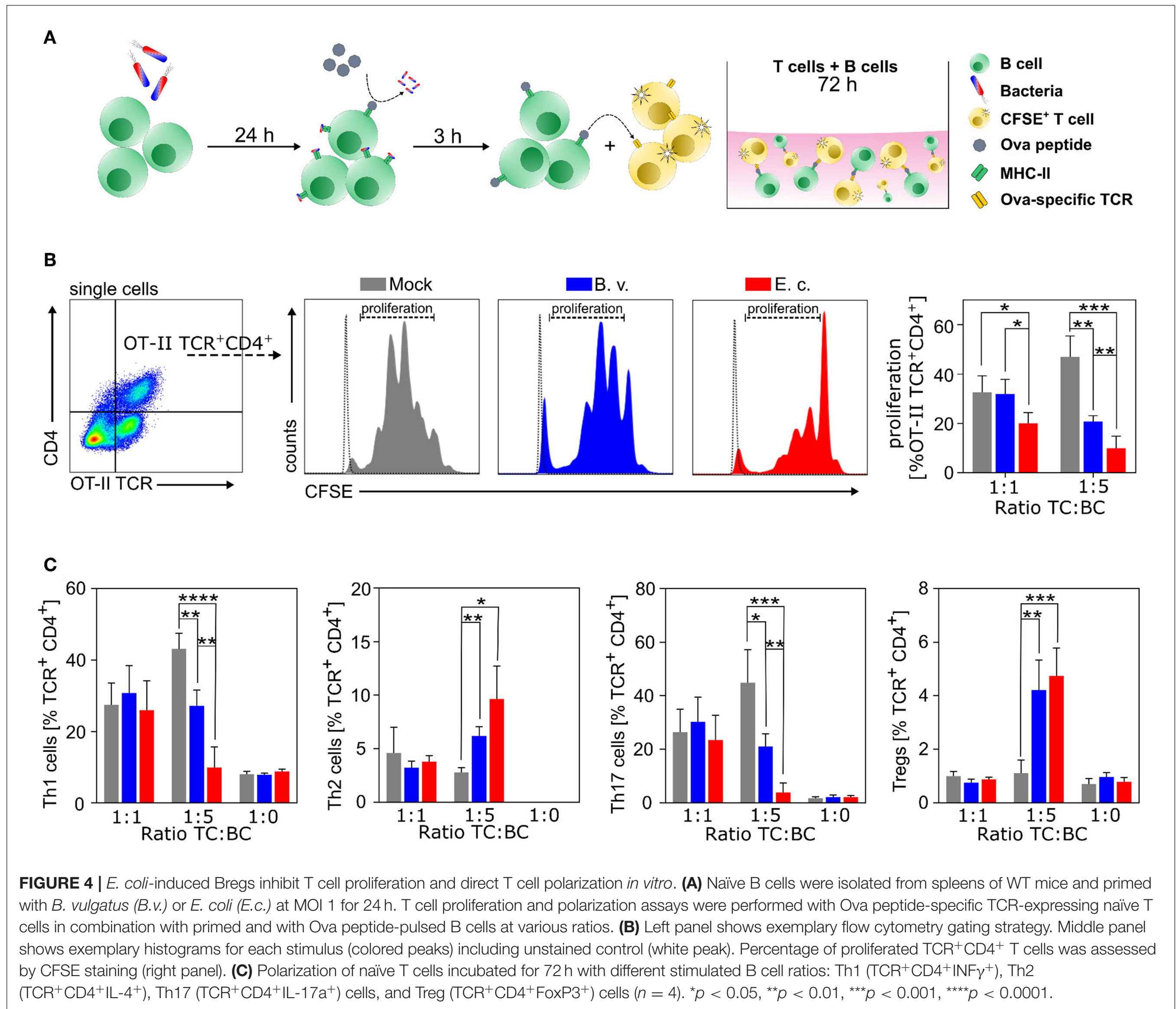

FIGURE 4 | *E. coli*-induced Bregs inhibit T cell proliferation and direct T cell polarization *in vitro*. **(A)** Naïve B cells were isolated from spleens of WT mice and primed with *B. vulgatus (B.v.)* or *E. coli (E.c.)* at MOI 1 for 24 h. T cell proliferation and polarization assays were performed 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

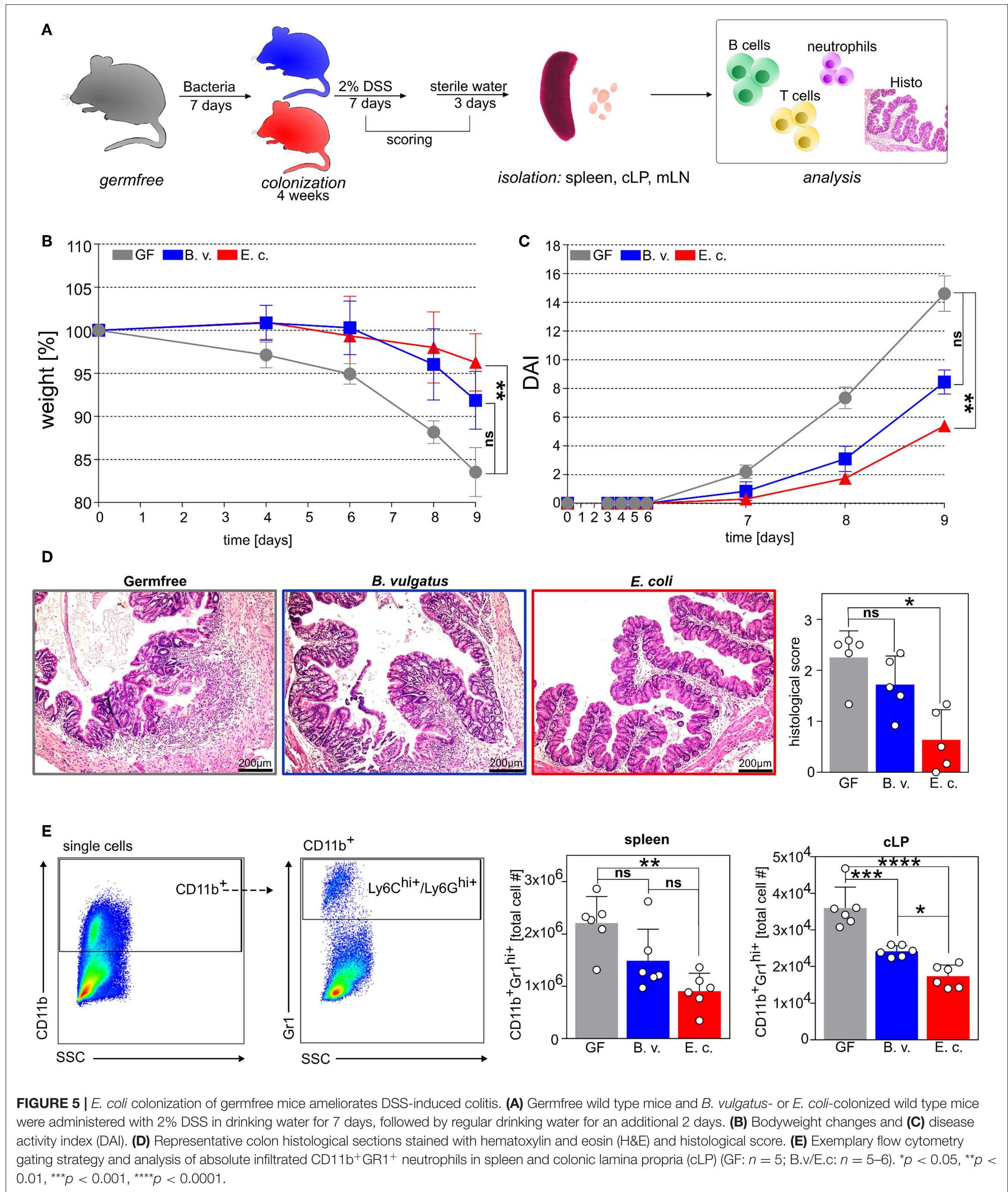

FIGURE 5 | *E. coli* colonization of germfree mice ameliorates DSS-induced colitis. **(A)** Germfree wild type mice and *B. vulgatus*- or *E. coli*-colonized wild type mice were administered with 2% DSS in drinking water for 7 days, followed by regular drinking water for an additional 2 days. **(B)** 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^{+}IFNγ^{+}) and Th17 (CD19^{-}CD3^{+}CD4^{+}IL-17^{+}) cells was significantly

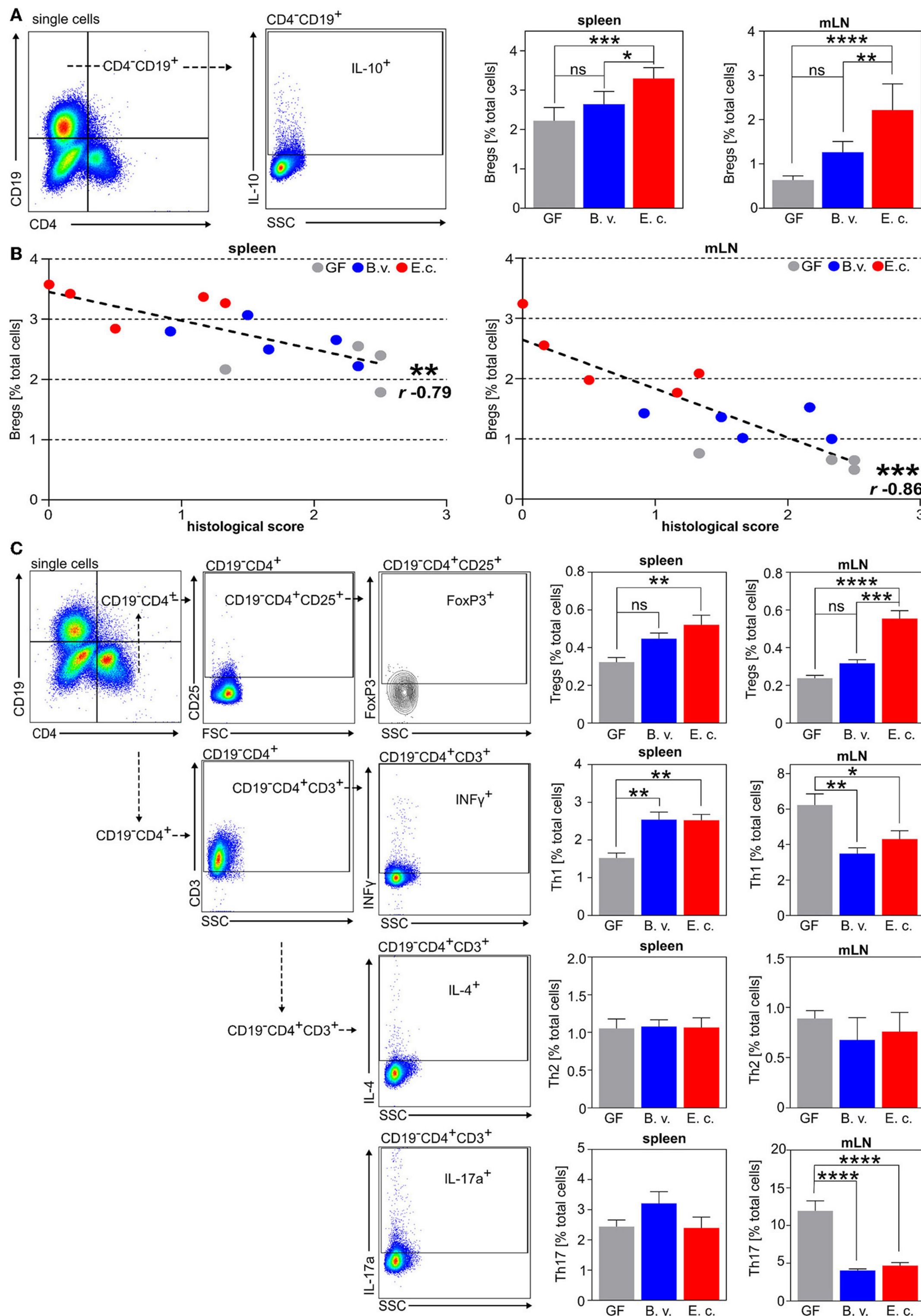

FIGURE 6 | *E. coli* colonization of germfree mice triggers the induction of regulatory B cells accompanied by an increase of Treg cells during DSS-induced colitis. **(A)** Exemplary flow cytometry gating strategy and analysis of $CD4^-CD19^+IL\text{-}10^+$ regulatory B cells (Bregs) in the spleen and 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.

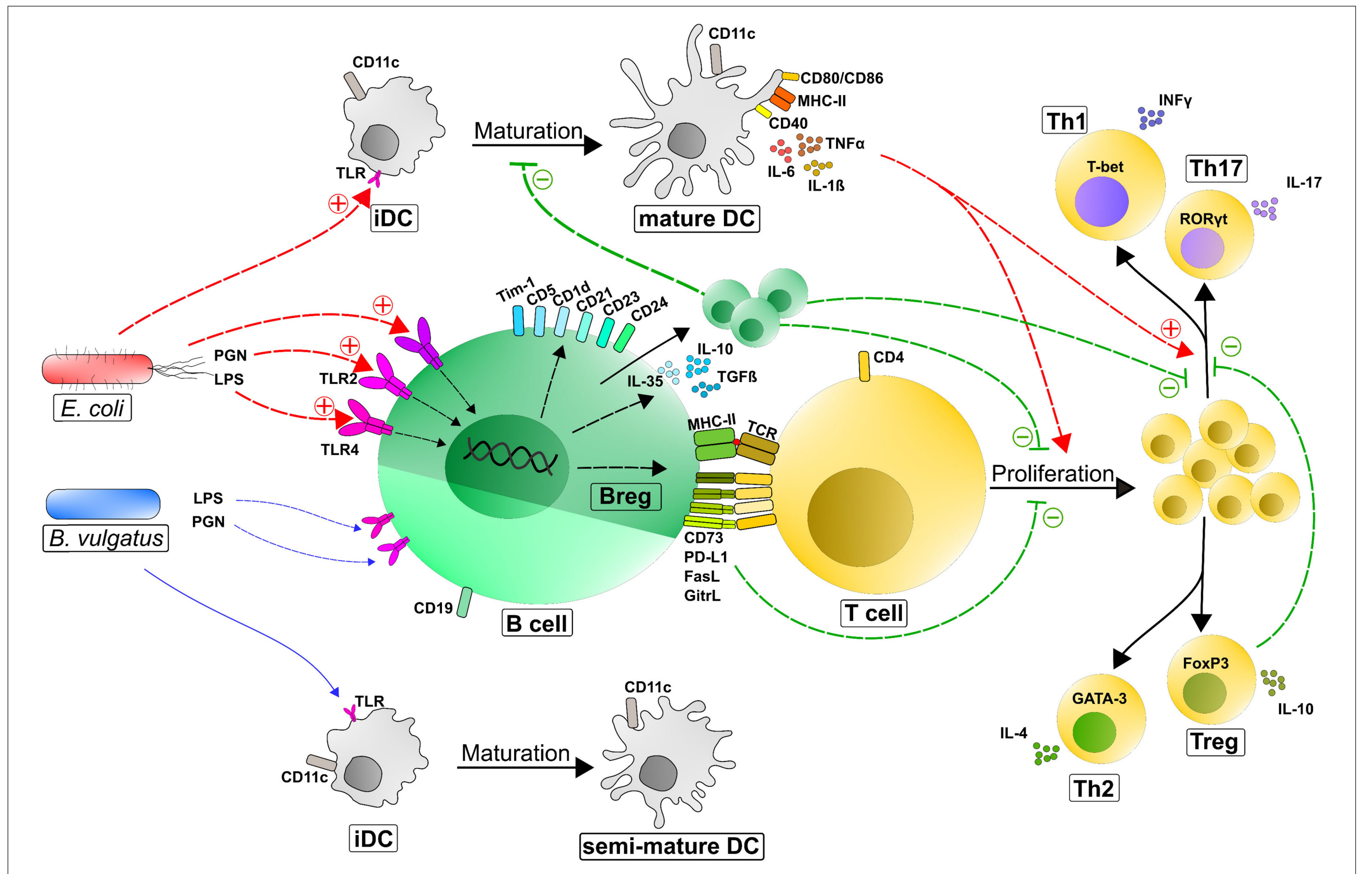

FIGURE 7 | Counter-regulatory mechanisms of Bregs after the induction of immune responses via immunogenic bacteria. Depending on the immunogenicity of a bacterial antigen, APCs (immature DCs and naïve B cells) are activated through the recognition of MAMPs (e.g., LPS or PGN) via Toll-like receptors to 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 $Tim\text{-}1^+$ B cells ($CD19^+$ $Tim\text{-}1^+$) 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.

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