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Vliv střevní mikrobioty na slizniční a systémovou imunitu při experimentální autoimunitní uveitidě

Modulation of the Mucosal and Systemic Immunity by Microbiota in Experimental Autoimmune Uveitis

Diplomová práce

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Prohlášení:

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Abstrakt

Využití probiotik pro léčbu zánětlivých onemocnění je slibnou strategií a předmětem výzkumu. Probiotika mohou přechodně ovlivnit složení střevní mikrobioty a s tím i komunikaci, kterou mikrobiota ovlivňuje imunitní systém svého hostitele. Podávání probiotik se prokázalo jako účinné na řadě zvířecích modelů zánětlivých onemocnění. Vliv probiotické léčby na uveitidu – zrak ohrožující onemocnění – však dosud nebyl podroben výzkumu.
V naší studii byly pro léčbu experimentální autoimunitní uveitidy (EAU) testována dvě komerčně prodávaná probiotika - *Escherichia coli* Nissle 1917 (EcN) a *Escherichia coli* O83:K24:H31 (EcO).

Závažnost očního zánětu byla stanovena oftalmoskopií a histologicky, zastoupení populací leukocytů a intracelulární exprese cytokinů byly testovány průtokovou cytometrií a střevní imunitní prostředí bylo prověřeno kultivací tkáně a metodou ELISA. Zjistili jsme, že profylaktické a časné orální podávání EcN snižuje závažnost uveitidy, zatímco EcO byla ve snížení zánětu neúspěšná. Léčba EcN byla, mimo jiné, provázena snížením produkce prozánětlivých cytokinů v Peyerových plátech, poklesem zastoupení populace prozánětlivých makrofágů a snížením Th1 a Th17 reaktivity $CD4^+$ T lymfocytů z lymfatických uzlin na specifický stimul imunizovaného IRBP antigenu. Pro hlubší poznání imunitního pozadí léčby EAU pomocí EcN cílila tato práce na popsání některých rozdílů mezi imunitním efektem těchto dvou bakterií, které by se mohly podílet na pozorovaném imunitním dopadu *in vivo*.

Pomocí RT-PCR vzorků stolice a střevních obsahů byla stanovena kolonizační schopnost obou bakterií. Mikroby se v kolonizaci myší nelišily. Délka přetrvání mikrobů ve stolici byla však závislá na původu zvířat. To naznačuje ovlivnění kontextem mikrobioty konkrétního zvěřince. Pro ověření, zda by se pozorovaného *in vivo* efektu mohly účastnit antigen prezentující buňky, byla stanovena odpověď makrofágů (BMDM) a dendritických buněk derivovaných z kostní dřeně (BMDDC) na obě bakterie. Produkce IL-1β, IL-12 a IL-10 byla stanovena metodou ELISA, respirační vzplanutí bylo zhodnocenou Griessovou reakcí a povrchové znaky účastnící se kostimulace a prezentace antigenu byly analyzovány průtokovou cytometrií. Lyzát EcN indukoval nižší odpověď BMDM, které reagovaly sníženou produkcí NO a IL-1β ve srovnání s reakcí na lyzát EcO. BMDDC projevily v reakci na EcN lyzát vyšší protizánětlivé vlastnosti zvýšenou expresí CD103 a produkcí IL-10.

Tato práce poskytuje data o rozdílech imunitního efektu dvou probiotik, které by se mohly podílet na jejich efektivitě v léčbě EAU. Tento mechanismus byl poprvé popsán a publikován naší skupinou.

Klíčová slova

Escherichia coli Nissle 1917; *Escherichia coli* O83:K24:H31; probiotika; probiotická kolonizace; makrofágy derivované z kostní dřeně; dendritické buňky derivované z kostní dřeně; bakteriální lyzáty; experimentální autoimunitní uveitida

Abstract

The use of probiotics has emerged in the last decades as a promising strategy when it comes to the treatment of inflammatory diseases. Through modulation of composition of the intestinal microbiota and the signalling it provides, probiotics can favourably tune the immune system. Beneficial effects of probiotic treatment have been documented in multiple animal inflammatory disease models. The effect of probiotic treatment on uveitis—a sight-threatening disease—has however not yet been described. In our study, we have tested two commercially available probiotics—*Escherichia coli* Nissle 1917 (EcN) and *Escherichia coli* O83:K24:H31 (EcO)—in the treatment of experimental autoimmune uveitis (EAU).

The disease severity was assessed by ophthalmoscopy and histology, proportions of leukocyte populations and intracellular expression of cytokines were evaluated by flow cytometry and the gut immune environment was analysed by tissue culture and ELISA. We found that prophylactic and early oral treatment with EcN reduces the severity of EAU. However, EcO treatment does not. The effects were accompanied by immune changes including a lowered production of inflammatory cytokines in Peyer´s patches, a shift in macrophage populations in ileum and mesenteric lymph nodes or a reduced IRBP-specific response of $CD4^+$ T cells in the lymph nodes. To describe the immune background further, this thesis has aimed to assess the differences in capacities of immunomodulation provided by EcO and EcN, which could determine their potential to reduce the severity of EAU.

Using RT-PCR we have assessed the colonization abilities of each microbe. We found, there are no differences in the time period microbes persist in the host. However, the data suggested that colonization could be affected by microbiota context determined by animal facility. To assess whether antigen-presenting cells could be involved in the immune effects induced by EcN *in vivo*, we tested the response of bone-marrow derived macrophages (BMDMs) and dendritic cells (BMDDCs) to lysates and cultivation filtrates derived from each microbe. The production of IL-1β, IL-10 and IL-12 was analysed by ELISA, respiratory burst was evaluated by Griess reaction and surface markers involved in co-stimulation and antigen-presentation were assessed by flow cytometry. EcN-derived lysate, compared to EcO lysate, induced lower production of NO and IL-1β by BMDMs and an increased expression of CD103 and an elevated production of IL-10 by BMDDCs.

Our study provides the first data documenting a reduction of EAU severity by treatment with EcN. This thesis offers additional data assessing the immune effects of EcN and EcO, which could determine their distinct effects in the treatment of EAU.

Key words

Escherichia coli Nissle 1917; *Escherichia coli* O83:K24:H31; probiotics; probiotic colonization; bone marrow-derived macrophages; bone marrow-derived dendritic cells; bacterial lysates; experimental autoimmune uveitis

List of Abbreviations

Ag	Antigen
APC	Antigen-presenting cell
ATB	Antibiotic
BMDDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumine
C5aR	Complement component 5a receptor
CAS	Czech Academy of Sciences
CD	Crohn´s disease
CFA	Complete Freund´s adjuvans
CFU	Colony-forming unit
cLN	Cervical lymph node
CN	Copy number
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco´s Modified Eagle Medium
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalitis
EAU	Experimental autoimmune uveitis
EcN	*Escherichia coli* Nissle 1917
EcO	*Escherichia coli* O83:K24:H31
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FAE	Follicle-associated epithelium
FM1	First Faculty of Medicine, Charles University
GALT	Gut-associated lymphoid tissue
GF	Germ free
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HDAC	Histone deacetylase
IBD	Inflammatory bowel disease
IC	Intracellular

I-CAM	Intercellular adhesion molecule 1
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iLN	Inguinal lymph nodes
IMMM	Immunomodulatory microbial molecule
iNOS	Inducible nitric oxide synthase
IRBP	Inter-photoreceptor retinoid-binding protein
IRF3	Interferon regulatory factor 3
IS	Immune system
LP	*Lamina propria*
LPS	Lipopolysaccharide
M cells	Microfold cells
MALT	Mucosa associated lymphoid tissue
MAM	Microbial anti-inflammatory molecule
MAMP	Microbial-associated molecular patterns
MCSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
mLN	Mesenteric lymph nodes
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NO	Nitric oxide
NS	Nervous system
OD	Optical density
OMV	Outer membrane vesicles
PAMP	Pathogen-associated molecular patterns
PB	Peripheral blood
PP	Peyer´s patches
PRR	Pattern recognition receptor
PSA	Polysaccharide A
PTx	Pertussis toxin
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction

SCFA	Short-chain fatty acid
SG	SYBR green
S-IgA	Secretory Immunoglobulin A
TCR	T cell receptor
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule 1
WT	Wild type

Outline

1. Introduction 12
1.1. Intestinal Mucosal Immune System 14
1.1.1. Anatomy and Function 15
1.2. Immunomodulatory Microbial Molecules 19
1.2.1. Structural Molecules 19
1.2.2. Released Microbial Products 22
1.3. Microbiota and Inflammatory Diseases 25
1.3.1. Inflammatory Bowel Disease 26
1.3.2. Extra-Intestinal Inflammatory Diseases 28
1.4. Probiotics 32
1.4.1. Escherichia coli Nissle 1917 32
1.4.2. Escherichia coli O83:K24:H31 35
2. Aims 37
3. Materials and Methods 38
3.1.1. Animals 38
3.1.2. Experimental Autoimmune Uveitis Induction 38
3.1.3. Probiotics 38
3.1.4. Clinical Evaluation 39
3.1.5. Histological Evaluation 40
3.1.6. DNA Isolation and RT-PCR 40
3.1.7. Preparation of Bacterial Lysates, Cultivation Filtrates and Controls 41
3.1.8. Generation and Culture of Bone Marrow-Derived Macrophages 41
3.1.9. Generation of Bone Marrow-Derived Dendritic Cells 42
3.1.10. Cell Preparation and Immunophenotyping by Flow Cytometry 42
3.1.11. Analysis of Cytokine and NO Production 43
3.1.12. Primary Intestinal Epithelial Cell Derivation and Cultivation 44

3.1.13. MODE-K Cultivation 45

3.1.14. Transepithelial Electrical Resistance Evaluation 46

4. Results 47

4.1. Initial in Vivo Experiments 47

4.1.1. Theory and Experimental Design 47

4.1.2. Results 47

4.2. Colonization Experiments 52

4.2.1. Theory and Experimental Design 52

4.2.2. Results 53

4.3. Stimulation of Bone Marrow-Derived Macrophages 54

4.3.1. Theory and Experimental Design 54

4.3.2. Results 55

4.4. Stimulation of Bone Marrow-Derived Dendritic Cells 58

4.4.1. Theory and Experimental Design 58

4.4.2. Results 59

5. Discussion 62

6. Conclusions 68

7. References 70

8. Supplementary Materials 86

1. Introduction

Human microbiota is an ecological community of microorganisms that colonises the human body. It consists of bacteria, fungi, viruses, and archea (Aggarwala, Liang, & Bushman, 2017; Hoffmann et al., 2013; Qin et al., 2010). Bacteria are the most extensively studied member of microbiota and are the main topic of this thesis. Microbiota colonises a vast array of different niches from the skin surface to the lumen of internal organs (Huttenhower et al., 2012). Microbes have been accompanying the mammalian evolution closely and have thus established a rich symbiosis with the hosts. They provide a continuous input of signals which fundamentally affect human physiological processes. The state of immune system (IS), nervous system (NS) or endocrine system are all strongly influenced microbiota (Clarke et al., 2014; Delgado, Sánchez, Margolles, Ruas-Madiedo, & Ruiz, 2020; Dinan & Cryan, 2017). Humans provide the bacteria with nutrients and a place to live. Counting cells, microbiota overtops the number of cells of human body 1.3-times and its collective genome is 150-fold longer (Gill et al., 2006; Sender, Fuchs, & Milo, 2016).

From an anthropocetric point of view we divide microbes to probiotics (beneficial), commensals (neutral) and pathogens (deleterious). It is, however, difficult to categorize microbes since their nature and favourableness are very context dependent and individual (Kverka & Tlaskalova-Hogenova, 2013). A probiotic to one individual could induce adverse effects to another (e.g. cases of sepsis induced by probiotic treatment in immunocompromised individuals (Land et al., 2005)) and classes are thus not fixed. Non-pathogenic, un-specified members of the microbiota are often generally referred to as commensals. Probiotics are, by their definition, microbes that, when administered in adequate amounts, confer a health benefit to the host. The term probiotic is usually used in context of using microbes as a treatment or a food supplement. A troublemaking group highlighting the importance of context are pathobionts which are common to healthy individuals but increase pathological susceptibility, additionally, there are species of microbes with commensal properties which can acquire pathogenic traits (Brandl et al., 2008; Proença, Barral, & Gordo, 2017; Wu et al., 2010).

Microbial niches of the host vary in conditions they offer. Accessibility to oxygen, humidity, mucus, microbial abundance and antimicrobial substances determine specific composition of each (Huttenhower et al., 2012). The most abundantly colonised niche is the gastrointestinal tract (GIT) with a 100 trillion bacteria from over a 1000 different species (Qin et al., 2010). Most abundant phyla are: *Firmicutes* (mainly class *Clostridia* and *Bacilli*

comprising 56 % and 1 % of the intestinal bacteria respectively), *Bacteroidetes* (mainly genera *Bacteroides*–12 % and *Prevotella*–10 %), Actinobacteria–9 % and Proteobacteria–2 %, as represented by stool samples from Czech donors (Bajer et al., 2017).

Composition of human intestinal microbiota differs among individuals as it is heavily shaped by environmental factors. Genetic heritability of the microbiota composition is low and it's the members of the same household who share similar traits in composition (Rothschild et al., 2018). Mode of delivery is the initial environmental factor. Ontogeny is probably a sterile process and first microbial colonisers which encounter an unoccupied environment mirror vaginal microbiota composition in case of vaginal delivery or more skin-like composition in case of caesarean section (Dominguez-Bello et al., 2010). During the first days of life, as gut is being rapidly colonised, the process might be driven more stochastically. Stochasticity of colonization was documented on *C. elegans,* when a simultaneous equivalent administration of two bacterial strains resulted in domination of either one in approximately a 50:50 ratio (Vega & Gore, 2017). Later, infant´s microbiota starts to depend on feeding and varies between formula-fed and breast milk-fed infants. Breast milk-fed infant gut is usually colonised by more *Bifidobacteria* and *Bacteroides* and fewer *Streptococci* and *Staphylococci* in comparison to formula-fed (Ma et al., 2020). That is partially caused by differed nutrient contents but also by direct transport of microbes in the breast milk (Moossavi et al., 2019). During childhood and adulthood microbiota composition gradually shapes under the influence of environmental factors. The main factors are diet, hygiene, stress and antibiotic treatment. Fat and fibre content shapes the composition significantly (Agus et al., 2016; Myhrstad, Tunsjø, Charnock, & Telle-Hansen, 2020). Treatment with antibiotics induces various changes dependent on the used substance. Some of these changes lasted up to 4 years and were often associated with disrupted microbial communication with the host (Zimmermann & Curtis, 2019). And stress was repeatedly shown to influence the composition too on various animal models (Murakami et al., 2017; Vlčková et al., 2018). Shared long-term environmental factors are reflected geographically in trends of composition within nations (Rehman et al., 2016). There are also temporary composition oscillations determined by diurnal cycle with significant changes in 15 % of intestinal operational taxonomical units and time-of-day-specific composition configurations (Thaiss et al., 2014). If, however, environmental factors remain stable than so does the adult long-term microbial composition.

Microbes differ in their capacity to colonise their host. While some species of intestinal microbes only pass through, others colonise transiently or, especially in adults, colonise for long-term. Colonization is dependent on the host, the composition context a

microbe occurs in and on microbial abilities to prevail and divide (Pedersen et al., 2018; Zmora et al., 2018).

Research of the human microbiota through cultivation-dependent methods was challenging as many of the microbes need specific conditions. However advanced techniques of metagenomics allowed the field to dramatically progress. Complete individual niche-specific analyses of microbial composition are possible now (Huttenhower et al., 2012). To define intestinal microbiota, usually, stool samples are analysed, which reflect about 70 % of long-term-colonising intestinal microbiota (Zmora et al., 2018). A more accurate method may represent biopsies, but for their invasiveness many studies still rely on stool analysis. Furthermore, there are concerns about the preparation procedure and its effects on composition as there are studies which document a short-term microbiota composition alteration upon the procedure (Nagata et al., 2019). Exciting models which highlight the importance of microbiota are germ-free (GF) models. GF animals are bred in isolators under sterile conditions. This allows us to assess how physiological and developmental processes are dependent on microbiota. GF mice exhibit serious pathophysiological traits and functional immune disturbances like: decreased size of lymph nodes and Peyer´s patches, fewer germinal centres, fewer plasma cells, intraepithelial cells and regulatory T cells. Production of antimicrobial peptides, imunoglobulins and nitric oxide is dramatically decreased and their general immune response balance is skewed toward Th2 (Round & Mazmanian, 2009). All these changes highlight the importance of crosstalk between the microbiota and the host for a development of a robust and balanced immune system.

1.1. Intestinal Mucosal Immune System

The intestinal mucosa is, along with all other mucosal surfaces, used by pathogens to enter the tissues of the body. While host has to prevent bacterial translocation and infection, it is essential to tolerate the continuous input of innocuous microbial and food-borne antigens. The intestinal mucosal immune system carries out both of these tenets through complex mechanisms that enable trillions of microbes to live and divide inside the human intestine.

The intestinal mucosal immune system consists of cells and their humoral products which are supported by general defence means like mucus, hydrochloric acid, bile and peristalsis. The cellular component is generally referred to as the mucosa-associated lymphoid tissue (MALT) which can be specified to gut-associated lymphoid tissue (GALT). GALT consists of both the innate and the adaptive branch of the immune system, both of

leukocytes and non-leukocytes. It is the largest lymphatic organ of the human body containing majority of body´s lymphocytes.

1.1.1. *Anatomy and Function*

The intestinal wall is built of intestinal epithelial cells (IECs). While apical side of IECs faces the lumen, basal side is bound to basement membrane and the *lamina propria mucosae* (LP) which collectively with underlying *muscularis mucosae* comprise the intestinal mucosa. Underneath the mucosa are layers of *submucosa*, *muscularis externa* and *serosa* with blood vessels. Under the epithelium, scattered throughout the intestinal LP*,* are organised regions of immune cells—lymphoid follicles. Clustered lymphoid follicles in the intestine create macroscopic structures—Peyer´s patches (PPs) which are covered with follicle-associated epithelium (FAE) with specialised microfold cells (M cells). PPs harbour dendritic cells, macrophages, T cells and B cells placed in subepithelial dome, T cell areas and B-cell follicles. Among villous absorptive IECs, in the intestinal wall, there are also other specialised cell types with distinct functions—Paneth cells, Goblet cells, enteric endocrine cells and stem cells. The LP and epithelium also harbours many scattered leukocytes outside the lymphoid follicles. Majority of the T cells scattered among IECs are $CD8^{+}$, while under the epithelium, in LP, there are $CD4^{+}$ T cells, plasma cells, macrophages and dendritic cells which can project dendrites into the intestinal lumen for direct antigen acquisition (Hořejší, Bartůňková, Brdička, & Špíšek, 2017; Mowat, 2003; Tokuhara et al., 2019).

The intestinal wall is covered with a layer of mucus secreted by Goblet cells. While the upper layer is composed of mainly mucin glycoproteins the lower layer forms a denser glycocalyx form. Mucus is enriched with antimicrobial substances secreted by Paneth cells which reside in the intestinal crypts. They produce: α-defensin, lysozyme C, phospholipase A2 and ribonuclease. Plasma cells produce high amounts of secretory IgA and to a lesser degree IgM which are transported into the intestinal lumen and act as neutralising agents. Under the mucus layer, intestinal epithelial cells form a solid wall. Epithelial cells are connected by desmosomes, gap junctions and tight junctions which limit paracellular transport to around 30-60 Å (Buschmann et al., 2013; Zihni, Mills, Matter, & Balda, 2016) . Nutrients and antigens thus need to be transported selectively—through cells. Collectively, the items, which participate in prevention of bacterial translocation, realize the intestinal barrier function (Hořejší et al., 2017; Mowat, 2003; Tokuhara et al., 2019). Interestingly, microbiota affects the barrier function too. Partially through stimulation of barrier fortification and repressing activities towards other microbes but also simply through its presence which

ultimately lowers the chance of pathogenic adherence and translocation (see chapter 1.2. Immunomodulatory Microbial Molecules) (Zyrek et al., 2007).

Luminal antigens are extensively transported by M cells, which pass them on to the antigen-presenting cells (APCs) underneath—dendritic cells, macrophages or B cells. APCs then either present the antigen in lower region of the follicle or migrate to present via efferent lymphatics to the local lymph nodes which drain antigens from the intestine—the mesenteric lymph nodes (mLNs). An alternative way of antigen source, represent snorkelling DCs, which reside under IECs in LP. They sample luminal antigens directly and then migrate to mLNs to present. Additionally, IECs also transport antigens through Ag-shuttling receptors and pass them on to phagocytes residing in LP which then mediate presentation. After presentation of the intestinal antigen, primed effector lymphocytes either migrate to the intestinal mucosa or distribute systematically to propagate corresponding effects. Migration to the intestinal mucosa is then mediated by expression of specific chemokine receptor CCR9 (Hořejší et al., 2017; Mowat, 2003; Tokuhara et al., 2019).

It is noteworthy that new antigen-acquisition mechanisms, involving many cells from GALT mediating the process directly or indirectly, are being documented. We only have limited knowledge about the process and other cellular roles and strategies in luminal antigen processing are probably involved (Schulz & Pabst, 2013). The process has been elegantly reviewed and depicted in a simplified manner (**Figure 1**) (Zgair, Wong, & Gershkovich, 2016).

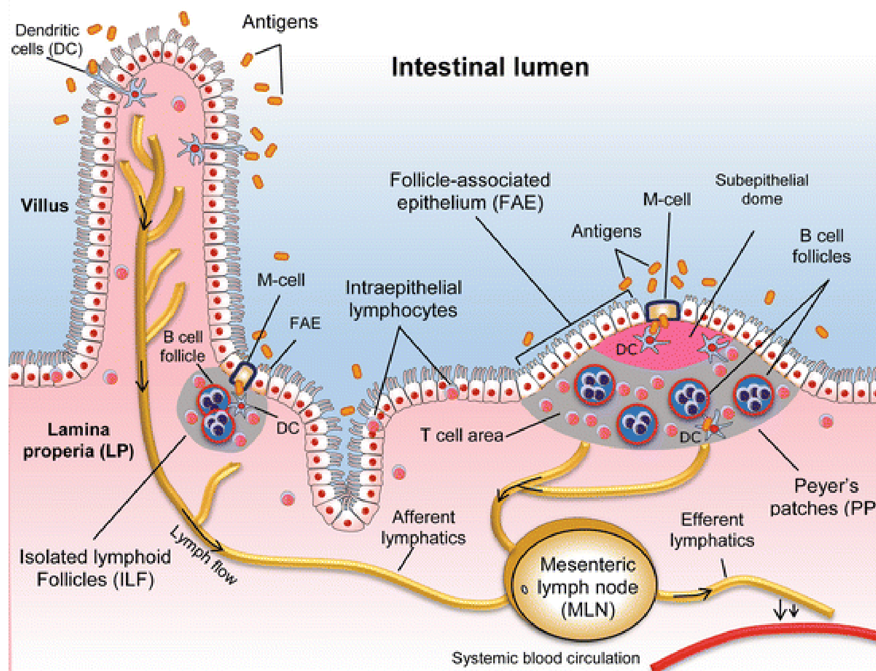

Figure 1. Schematic representation of the gut-associated lymphoid tissue (Zgair et al., 2016).

According to the place a cell takes in the process of immune response, its function can be either inductive (which sets up the immune response) or effector (which takes corresponding effects). Typical cell with inductive function are dendritic cells which engulf and present antigen, on the other hand primed T cells represent effector type which, upon priming, migrate to the intestinal mucosa or spread systematically to propagate the effects. According to the localisation of these functional groups, GALT can be differentiated to induction sites, represented by PPs, smaller isolated lymphoid follicles and mLNs and effector sites—epithelial and sub-epithelial regions of the intestinal mucosa. The localisation is, however, not exclusive as the effector site of intestinal mucosa is interlaced with cells from the induction group as well (e.g. dendritic cells) (Mowat, 2003). In experimental practice, Peyer´s patches and mLNs can be isolated to undergo analyses and delineate the environment at the intestinal immune induction site. The initial stimulus and environmental context at the induction site determine the immune response which can be either inflammatory or tolerant (Hořejší et al., 2017; Mowat, 2003; Tokuhara et al., 2019).

Without a pathogenic insult, the intestinal mucosal immune system is primed to induce tolerance through a process of oral tolerance. By this process the IS remains to be tolerant to harmless food antigens it encounters in GALT. However, simultaneously, microbial antigens get transported as well. Such antigens include inflammatory stimuli like lipopolysaccharide (LPS). If, however, the barrier retains its integrity, GALT does not sense

infection and microbiota provides its healthy balanced stimuli, no inflammation is triggered (Mowat, 2003).

Microbes exhibit various mechanisms by which they facilitate colonization and maintain tolerance from the host. For instance, there are variances in the common microbial associated molecular patterns (MAMPs) that determine the immune response. While the basic structures are common and shared, there are specific modifications of MAMPs that characterise their bearer. Some commensal MAMPs therefore trigger lower response after recognition by pattern-recognition receptors (PRRs), than MAMPs produced by pathogens which trigger robust pro-inflammatory signalling (Hajjar, Ernst, Tsai, Wilson, & Miller, 2002). Another mechanism that differentiates the response to pathogens and commensals is the expression of toxins and virulence factors associated with pathogenic traits which often induce tissue damage which leads to release of danger-associated molecular patterns (DAMP) and induction of inflammation. Furthermore virulence factors are also recognised by PRRs which propagates the inflammation. For instance, a pathogenic strain of *E. coli* expresses P fimbriae which help it to adhere to the epithelium. This microbe triggers TLR4 signalling and inflammatory response of epithelial cells. However, a mutant of this strain which lacks the P fimbriae does not trigger this response (Fischer, Yamamoto, Akira, Beutler, & Svanborg, 2006). Lastly, specific commensals produce distinct immunomodulatory molecules that tune host´s immune system to lower the inflammatory response, induce tolerant and suppressive phenotypes, fortify intestinal barrier or induce other mechanisms which help to maintain balanced colonization (see chapter 1.2. Immunomodulatory Microbial Molecules) (Delgado et al., 2020).

According to the context of the initial encounter with an antigen the IS decides how to react. Either it finds commensal-type MAMPs and antigens a balanced, non-interrupted environment, with accompanying tolerant signals and imprints APCs to prime lymphocytes into a tolerant phenotype, or it finds a translocated pathogen-associated molecular patterns (PAMPs) and antigens in an infected environment and triggers inflammation. The former scenario involves signalling from IECs and macrophages which secrete anti-inflammatory factors like TGF-β or PGE-2. Under the influence of these signals DCs engulf antigens and migrate to present the antigens. They accompany the presentation with IL-10. This presentation induces the differentiation of regulatory T lymphocytes (Tregs) which then migrate back to the intestinal mucosa (i.e. effector site) to provide their immunosuppressive effects or spread systematically. On the other hand the scenario initiated from an infected site starts with innate immune cells and IEC sensing translocated pathogenic antigens through

PRRs. That leads to pro-inflammatory cytokine secretion (IL-1β, TNF-α, IL-6, IL-8), maturation of DCs, presentation of an antigen with IL-12 being secreted simultaneously and priming of Th1 response which then takes effects in the mucosa, enhancing the inflammation by IFN-γ production and CD40L expression, further activating macrophage microbicidal activities like NO and ROS production to block further spreading of infection. Alternatively Th2 response may be triggered if the infection is primarily recognised differently (Mowat, 2003). [1]

1.2. Immunomodulatory Microbial Molecules

As already mentioned, some human microbiota members produce substances with immunomodulatory properties. These immunomodulatory microbial molecules (IMMMs) are produced in the intestinal lumen and can reach not only epithelial cells lining the gut lumen, but also the immune cells underneath. The impacted cells of GALT then drive both local and systemic immunity. Generally, IMMMs are structural molecules, products of metabolism or other distinct released factors. They can be common to a broader phylum or unique for specific species. Collectively IMMMs provide a wide range of impulses which induce complex immune tuning (Delgado et al., 2020). This chapter will be dedicated to examples of specific IMMMs and the immune responses they provoke.

1.2.1. *Structural Molecules*

IMMMs which form microbial structure include fragments of the bacterial wall, capsule or other organ components like flagellum, pili proteins or nucleic acids (Ottman et al., 2017). An abundant widely shared IMMM is LPS. It is anchored in the top layer of Gramm-negative cell wall. There are two main types of LPS: S-form and R-form. Most wild-type (WT) Gramm-negative bacteria express S-form, which consists of lipid A, core polysaccharide and repeating units of O-polysaccharide (O-antigen). R-form is produced by "rough" mutant bacterial strains and lacks the O-polysaccharide units. LPS (or more specifically lipid A) is recognised by Toll-like receptor 4 (TLR). Recognition of S-form LPS is dependent on LPS-binding protein and CD14 while R-form can stimulate TLR4 independently of the helper proteins (Huber et al., 2006). Binding of lipid A to TLR4 can result in two distinct signalling pathways. First is MyD88-dependent which leads mainly to

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[1] Information regarding the anatomy and function of the intestinal mucosal immune system were acquired from reviews and textbooks (Hořejší et al., 2017; Mowat, 2003; Tokuhara et al., 2019).

IL-12, TNF-α and CCL3 (an inflammatory chemokine) production whereas the second MyD88-independent pathway activates interferon regulatory factor 3 (IRF3) and leads to type I interferon production. The latter pathway is generally considered as anti-inflammatory as it is dominant in LPS-tolerant response (Biswas et al., 2007).

WT bacterial LPS is highly heterogeneous. O-polysaccharide repetition ranges from 1 to 50 units and R mutants are often also present. R mutants exhibit variances in degrees of core-polysaccharide completion and are further classified into Re and Ra types. These major differences, along with many other modifications, determine the response LPS triggers. R-form triggers a stronger inflammatory response. Bone-marrow derived mast cells react to R-form with high IL-6 and TNF production. In comparison S-form induces virtually no response (Huber et al., 2006). Acylation of lipid A affects the intensity of TLR4 signalling and of the response too. Hexa-acylated lipid A is a strong agonist a triggers a robust response whereas penta-acylated lipid A binds the receptor complex differently and induces much weaker response. Other factor determining the response to LPS is the number of phosphate groups. The fewer groups there are the lower the response is. Combination of modifications can make LPS (or lipid A) be a strong agonist, weak agonist or an antagonist which blocks TLR4 signalling (Steimle, Autenrieth, & Frick, 2016). By this differentiation, the reaction to LPS is dependent on the original bearer. The response corresponds not only with the species LPS is derived from but also with the current state of the bearer. Pathogenic adaptation of *Pseudomonas aeruginosa* is associated with a switch in lipid A acylation and becoming a strong agonist. On the other hand *P. aeruginosa* counterpart missing this pathogenic adaptation does not induce this robust pro-inflammatory signalling (Hajjar et al., 2002). The process of LPS editing is called phase-shift and describes editing of LPS to be associated with adherence, pathogenicity and inevitably visibility to the IS. Commensals are thus proposed to have types of LPS which trigger lower responses than pathogens. Downregulated response of TLR4 to LPS from commensal microbiota may be a supporting mechanism which blocks overstimulation by the microbial load. An additional protective mechanism is carried out by luminal alkaline phosphatase which dephosphorilates lipid A and turns it into a non-toxic form (Bates, Akerlund, Mittge, & Guillemin, 2007).

Polysaccharide A (PSA) is an IMMM produced by *Bacteriodes fragilis*. It is one of eight polysaccharides comprising its bacterial capsule. PSA is the most highly expressed and most immunomodulatory potent. *B. fragilis* belongs to the *Bacteroidetes* phylum and *Bacteroides* genus. It is a common member of the human intestinal microbiota and creates up to 1 % of the intestinal microbial load. PSA-induced immunomodulation is an archetypal

example of an IMMM influence on the IS. PSA mediates its effects through TLR2 and MHCII presentation. That is allowed by its unique structure. It consists of hundreds of repeating four-sugar subunits which carry negative charge on carboxyl group and positive charge on amino groups. This makes PSA a zwitterionic polysaccharide, which is an uncommon capsule-saccharide characteristic and a means allowing PSA to be presented on MHCII. TLR2 binding, however, seems to be crucial for propagating its effects as it induces a transcriptional switch essential for a successful presentation (Sharma, Erickson, & Troutman, 2017).

B. fragilis induces complex effects in the IS as is demonstrated by monocolonization studies of germ-free mice. Most germ free-related immune-pathologies were corrected by monocolonization with *B. fragilis* but not a with PSA mutant of *B. fragilis* (Mazmanian, Liu, Tzianabos, & Kasper, 2005). Main immunomodulatory effects are probably mediated through dendritic cells or more specifically plasmacytoid dendritic cells which present PSA to T lymphocytes and prime them into a Foxp3^{+}, CD25^{+}, IL-10-producing Tregs (Dasgupta, Erturk-Hasdemir, & Ochoa-Reparaz, 2015). Induced Tregs then, propagate suppressive effects, restrain Th17 activities and enable for increased tissue-associated colonization of *B. fragilis* (Round et al., 2011; Telesford et al., 2015). These effects were found to be very beneficial in inflammatory diseases. Treatment with *B. fragilis* reduced severity of colitis in multiple animal models (Dasgupta et al., 2015). Furthermore, *B. fragilis* also reduced tumorgenesis during colorectal carcinoma (Y. K. Lee et al., 2018). Strikingly, PSA effects exceed beyond local. *B. fragilis* was able to reduce brainstem inflammation in viral encephalitis. The effects were accompanied by two-fold increase in Treg population in spleen and cervical lymph nodes (cLNs) and an increased production of IL-10 and IFN-γ. Interestingly, the effects were not dependent on Treg population but rather on the IL-10 and IFN-γ cytokines produced by several cell types (Ochoa-Repáraz et al., 2010; Ramakrishna et al., 2019). Notably, treatment of viral encephalitis with PSA was associated with a switch in DC populations. DCs with enhanced tolerogenic abilities were isolated from cervical LN, outside of local intestinal-draining LN. This suggest that systemic effects of IMMM are not limited to lymphocyte redistribution and that tolerogenic DCs induced by other IMMMs could propagate its effects on peripheries too. CD103^{+} DC are particularly of note in this process as they were significantly more effective in Treg conversion of CD4^{+} T cells than CD103^{-} DCs.

Bifidobacterium bifidum represents another microbe producing IMMMs of a structural origin, although not yet fully defined. The IMMM was assigned to exopolysaccharides transferable by membrane vesicles. This was presented by a study where

DCs stimulated with *B. bifidum*-derived vesicles were subsequently co-cultivated with naïve $CD4^+$ T cells which exhibited increased suppressive activities demonstrated by cytokine production and differentiation (López et al., 2012). However, besides the induction of anti-inflammatory cytokine production and reduction of pro-inflammatory cytokines, *B. bifidum* also fortifies the intestinal barrier by increasing tight junction protein production and increases reactive oxygen species scavenging enzyme production. Some of these effects are suggested to be mediated by translocation of NF-kB subunit, a component of a fundamental transcription factor responsible for inflammatory reactions (Din et al., 2020). Furthermore, *B. bifidum* is also a producer of another important type of IMMMs—short chain fatty acids (LeBlanc et al., 2017).

1.2.2. *Released Microbial Products*

A different branch of IMMMs are products of energetic metabolism. The most well covered and thoroughly studied are short-chain fatty acids (SCFAs). SCFAs are derived mainly from plant-cell wall components and energy-storing saccharides in a process of fermentation mediated by members of human intestinal microbiota. Polysaccharides like cellulose, hemicellulose, xylan, pectin or inulin are compounds of plant-based diet indigestible to humans. However some *Clostridia* and *Lactobacilli* (for instance) possess membrane-bound enzymes which are capable of breaking these components down and providing them to humans as an energy source and an immunomodulating mediator (Chassard, Goumy, Leclerc, Del'homme, & Bernalier-Donadille, 2007; Robert & Bernalier-Donadille, 2003).

The process is characterised by a rich microbial cooperation and cross-feeding of metabolised intermediates (Freilich et al., 2011; Turroni et al., 2015). Degrading of cell wall components is mediated by cellulosome—an enzymatic complex that harbours subunits with specific hydrolytic activities (Murashima, Kosugi, & Doi, 2002). Degradation intermediates are then processed by (the same or other) fermenting microbes to be metabolised into SCFAs. The three main SCFAs with immunomodulatory characteristics are acetate, propionate and butyrate (butyrate being the most potent) (Waldecker, Kautenburger, Daumann, Busch, & Schrenk, 2008). Their concentration in the intestinal lumen is usually around 70–140 mM while acetate has about three-fold higher concentration then butyrate and propionate (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). The capacity of producing individual SCFAs is species-dependent. The most prominent butyrate producers are *Eubacterium rectale* and *Faecalibacterium prausnitzii* which are simultaneously also inulin

degraders. About 70 % of SCFAs is consumed by colonocytes as an energy source, the rest proceeds further, reaches other cell types and ultimately is released into the bloodstream (Serpa et al., 2010). Since production of these substances relies mainly on a continuous feed of plant-originated substrates, SCFAs are an example of immunomodulation dependent on an individual´s diet.

SCFAs affect cells as IMMMs in two major ways. First, they are histone deacetylase (HDAC) inhibitors (Waldecker et al., 2008). Second, they are ligands of Fatty-acid-binding G-protein-coupled receptors (GPCRs) (Le Poul et al., 2003). HDACs mediate deacetylation of histone N-termini. Deacetylated histones form denser chromatin unavailable for transcription. Thus inhibition of these enzymes leads to acetylated euchromatin formation and actively expressed genes. Furthermore, HDAC activities are not limited only to histones. Their substrates include transcription factors as well. (De)Acetylation of transcription factors affects their activities by altering their capacities to bind DNA or to bind their essential subunits. There are 18 known HDACs each with specific activities. Cellular effects of HDAC inhibition are thus specific to the SCFA and the HDAC it targets (Schulthess et al., 2019). It is, however, often challenging to assign a specific molecular pathway to a SCFA since GPCRs can affect HDACs too. There are, however, pathways dependent on transporters to mediate their effects which suggests their direct HDAC effects rather than mediation by GPCRs (N. Singh et al., 2010).

SCFAs are generally associated with anti-inflammatory effects. They mediate the effects through several different mechanisms. Relaxing of chromatin can be targeted to regions coding transcription inhibitors. Inhibitors then target expression of pro-inflammatory genes which are then downregulated (Roger et al., 2011). Or, on the other hand, increased expression may target essential anti-inflammatory genes like Foxp3 (Schwarz, Philippsen, & Schwarz, 2021; Tao et al., 2007). Elevated Foxp3 expression leads to an increase in colonic Treg populations. In fact, intestinal luminal concentration of SCFAs positively correlates with colonic Treg populations (Furusawa et al., 2013). Direct HDAC-mediated modulation of transcription factor activities takes effects on NF-kB subunit—ReIA. Acetylated ReIA is unable to associate and form a functioning complex thus leading to lowered expression of pro-inflammatory genes (L. -f. Chen, Wolfgang, Verdin, & Greene, 2001; Schulthess et al., 2019). This is supported by multiple studies documenting decreased NF-κB activities upon SCFA treatment (Huang et al., 2020; Lührs et al., 2001; Qiao, Qian, Wang, Ma, & Wang, 2014). Acetylation can, however, also increase binding activities of transcription factors as it

does with Foxp3 (Tao et al., 2007). SCFAs thus enhance regulatory activities of T cells by increased Foxp3 expression as well as the efficiency.

Interestingly, the effects of SCFA on leukocytes are not only anti-inflammatory. SCFA play in important role in mediation of rapid $CD8^+$ memory response. Systemic infection leads to a five-fold increase in blood acetate concentration which then induces a boost in GAPDH activity through its acetylation leading to augmented glycolysis and prompted reactivity demonstrated by enhanced IFN-γ production (Balmer et al., 2016). Furthermore, SCFAs affect more cell types. Through lowered expression of adhesion molecules VCAM-1 and I-CAM on endothelial cells fewer cells migrate to sites of inflammation (Maslowski et al., 2009). By activation of MUC-2-coding regions in goblet cells and modulation of hypoxia-inducible factor in IECs, SCFA can also fortify intestinal barrier through increased mucin and tight junction production (Burger-van Paassen et al., 2009; Kelly et al., 2015; Peng, Li, Green, Holzman, & Lin, 2009). This fortifying factor is also enhanced by intestinal sub-epithelial macrophages which produce increased amounts of anti-microbial peptides in response to SCFA-mediated HDAC inhibition (Schulthess et al., 2019).

The last type of an IMMM to be described here is the microbial anti-inflammatory molecule (MAM) produced by *Faecalibacterium prausnitzii* a member of Clostridia class (phylum *Firmicutes*). MAM, interestingly, serves as a communication mediator without having a primary structural or energetic function. *F. prausnitzii* is a common human microbiota member that usually accounts for 5—10 % of total intestinal bacteria (H. Sokol et al., 2008). In Crohn´s disease and colitis patients´ *F. prausnitzii* abundance is, however, significantly reduced. This decrease is matched with a MAM decline in stool samples. In fact MAM is now proposed to be used as a biomarker of Crohn´s disease (McLellan et al., 2020). MAM is actively released into the environment as proved by studies where cultivation supernatant without the cells was capable of prompting its immunological effects. MAM is described to block NF-kB activation in a dose dependent manner in colitis studies (Breyner et al., 2017; Harry Sokol et al., 2008). MAM-transfected intestinal epithelial cells rescued their inflammatory tune of the cells both *in vitro* and *in vivo*. *In vivo*, this led to a decreased production of Th1 and Th17 cytokines IFN-γ and IL-17 in mLN and increased TGF-β production by colon tissue (Breyner et al., 2017). Furthermore transfected intestinal epithelial cells also increase their zona occludes-1 protein expression and improve their barrier integrity (Xu et al., 2020).

The effects of MAM are, however, blurred by the fact that *F. prausnitzii* is also a producer of butyrate. And while SCFAs may add up to the effect, butyrate itself was not able to reproduce equivalent anti-inflammatory effects as *F. prausnitzii* did (Harry Sokol et al., 2008). *F. prausnitzii* is, along with other common microbes, a microbe which promotes a complex immunomodulation mediated by multiple IMMMs (Machiels et al., 2014).

These are several examples of the continuously increasing knowledge about IMMMs. Some groups have recently reviewed this topic (Delgado et al., 2020). There are many more IMMMs and only few have well-described molecular and cellular signalling background. Many of these immunomodulatory mechanisms may have probably evolved as a protective measure to the host and a means for a close colonization of a host with a complex immune system. This symbiosis was to be favourable for both the microbes receiving nutrition and a niche and for the host who receives nutritional and protective benefits. Being accompanied by microbes throughout the evolution, humans have become dependent on the signals microbes provide to develop a healthy immune system. If the communication is disrupted, individuals may develop diseases.

1.3. Microbiota and Inflammatory Diseases

Considering microbiota´s immunomodulatory abilities, microbiota represents a susceptibility factor and a potential therapeutic target in various diseases. The composition of microbiota is also altered in many diseases. Broadly defined, any change of residential commensal communities relative to the community found in healthy individuals is referred to as dysbiosis (Petersen & Round, 2014). There are three main types of (or rather hallmarks) of dysbiosis: first, loss of beneficial microbial organisms, second, expansion of pathobionts or potential harmful organisms and third, loss of overall microbial diversity (Petersen & Round, 2014). During dysbiosis, one receives disrupted signals from an imbalanced microbiota which then impairs physiological processes under microbial influence. An individual could acquire dysbiosis by a poor combination of environmental factors like diet or antibiotic treatment (H. J. Kim, Lee, & Hong, 2020). Furthermore, diseases themselves can induce or enhance dysbiosis (Machiels et al., 2014). For example inflammatory bowel disease (IBD) is associated with pathological changes which boost the microbial imbalance. It is thus often difficult to differentiate whether the dysbiosis is a cause or a consequence.

Specific microbes with anti-inflammatory effects represent a promising therapeutic agent of inflammatory diseases and an alternative to pharmacotherapy. Probiotic supplementation may also promote a return to a balanced microbial composition which would

provide the correct, favourable signals an individual needs (H. Sokol et al., 2008). Alternatively, patients could benefit from other probitotic effects like colonization resistance or intestinal barrier fortification. These microbial properties made probiotics extensively studied in context of many diseases.

1.3.1. *Inflammatory Bowel Disease*

IBD is a good example of the microbial influence during an inflammatory disease. It is characterised by a low-diversity type dysbiosis and microbes appear both in the prevention phase as well as in the phase of development and progress of the disease (Machiels et al., 2014). IBD is an inflammatory disease of the intestine which can be categorized in two types: ulcerative colitis (UC) and Crohn´s disease (CD). UC affects mainly the rectal and sygmiod colon although in more severe cases of extensive colitis the inflammation may spread into more proximal sections as well and the inflammation is usually limited to the mucosal layer of the intestine. On the other hand CD affects mainly terminal ileum and proximal colon, although it can spread to virtually any part of the gastrointestinal tract and the inflammation is transmural affecting all the intestinal layers. Symptoms and complications of IBD include rectal bleeding, diarrhoea, abdominal pain, nausea, ulcerations, rupture of the bowel and colorectal cancer. Both IBD types share similar immune-cell background. Bacterial translocation activates innate immune cells like macrophages and dendritic cells which produce IL-1b, TNF-a, IL-12 and IL-6. T cells are polarised into Th17 and Th1. In the case of UC, Th2 cells may play a more important role too. T helper cells then produce corresponding cytokines like IL-17 and IFN-y and fuel the inflammation. Tissue is infiltrated with macrophages, eosinophils, plasmatic cells, neutrophils or lymphocytes. A common trait is also a reduced number of Tregs and reduced IL-10 production (S. H. Lee, Kwon, & Cho, 2018).

IBD pathogenesis is complex but it is believed to be caused by an abnormal reaction to the intestinal microbiota in genetically predisposed individuals. The occurrence of IBD is influenced by diet and antibiotic treatment (Amre et al., 2007; Jantchou, Morois, Clavel-Chapelon, Boutron-Ruault, & Carbonnel, 2010), two factors which heavily modulate microbial composition too. A scenario of IBD pathogenesis could be following: composition of microbiota may be influenced by a combination of environmental factors (like disturbances in diet or ATB treatment) forcing it into a dysbiotic state all in a genetically predisposed individual. Dysbiosis decreases the number of favourable microbes which provide anti-inflammatory IMMMs and the number of microbes with favourable antagonistic activities (H.

J. Kim et al., 2020; Machiels et al., 2014). The intestinal tissue slowly outweighs to inflammation as is simultaneously challenged by more infectious pathogens. Persisting inflammation changes the intestinal environment which boosts dysbiosis as other species are favoured. Also, previously commensal microbes start expressing pathogenic traits since they are more challenged by the intestinal immune system (Proença et al., 2017). Epithelium gets damaged under the influence of inflammation and pathogens, which enables more frequent bacterial translocation. Translocated bacteria trigger robust immune responses whether pathogenic or not and trigger systemic manifestations (Thaiss et al., 2018). Production of ROS and carcinogenic substances in the inflamed intestine may lead to DNA damage and mutations. Mutated cells accumulate and ultimately form adenoma or later carcinoma.

Faecal microbiota transplantation offers an insight into the role of microbiota during IBD. Transfer of faecal microbiota from IBD patients into GF mice led to a pro-inflammatory tuning of the intestinal mucosal immune system associated with Th17 and Th2 population increase and Treg decrease. This shift was deleterious in a colitis model with IBD patient faecal microbiota transplant which exacerbated the disease. The intensity of Th17/Treg shift the transplant induced also correlated with the severity of the disease the donor suffered from with more Th17 and less Treg induced by more severe cases (Britton et al., 2019). Transplantation of mucosa-associated IBD microbiota itself, however, cannot induce the disease and an additional insult is needed (Du et al., 2015). Interestingly, the immune state of GF mice colonised with faecal microbiota derived from IBD patients was improved after a treatment with a defined microbiota transplant derived from healthy patients. This treatment reshaped the intestinal immune system, increased Treg counts and decreased Th17 cell counts. Treatment was also associated with microbiota density restoration. The Th17 population decrease was associated with one specific strain which induces Th17 response and whose relative abundance was significantly decreased by the treatment (Britton et al., 2020).

Supplementation of specific probiotics or IMMMs was, however, proved to be beneficial in IBD treatment as well. SCFA-producing microbes are extensively studied for their anti-inflammatory and barrier-supportive properties. IBD dysbiosis is associated with reduced numbers of SCFA-producing microbes. Impaired SCFA-mediated signalling was found to be a negative factor in animal IBD models and vice versa SCFA signalling supplementation led to amelioration of the disease (Glauben et al., 2006; Masui et al., 2013). Furthermore, human studies reviled a significant decrease in disease severity index after 8 weeks of butyrate treatment in IBD patients. This improvement was demonstrated by a decrease in macrophage NF-kB activity, reduced numbers of neutrophils in the intestinal

crypts as well as reduced lymphocyte and plasma cell numbers in LP (Lührs et al., 2002). More recent research of colitis modelled on mice has identified a shift in macrophage population associated with an attenuation of the inflammation following butyrate treatment. Colonic macrophages increased the expression of Arg1—a protein marker of M2 macrophages, which exhibit tolerogenic properties and are associated with inflammation resolution and tissue reparation. Exploring the effects of SCFAs on macrophages further, the team then stimulated bone marrow-derived macrophages (BMDM) with butyrate *in vitro* which confirmed the facilitated polarisation towards M2 phenotype in a simplified environment and helped to uncover the molecular (H3K9 histon acetylation) basis underlying the process. Macrophages also enhanced their migrating and wound-healing abilities. Furthermore an adoptive transfer of M2 differentiated BMDM to colitic mice ameliorated the disease and reduced weight loss. These changes were accompanied by reduced levels of IL-6, TNF and IL-1β (Ji et al., 2016).

1.3.2. *Extra-Intestinal Inflammatory Diseases*

The influence of microbiota is even more fascinating in the context of diseases localised outside the intestine and it highlights the systemic immune impact of microbiota-mediated immunomodulation.

An interesting example is multiple sclerosis (MS) and its model—experimental autoimmune encephalitis (EAE). MS, in the context of microbiota, is interesting not only for its extra-intestinal localisation but also for being a disease affecting an immune-privileged site. It is an inflammatory disease of the central nervous system affecting the white matter. It is characterised by inflammation, demyelination, axonal loss/damage and gliosis. The pathogenesis is unclear and multifactorial. EAE features a disrupted blood brain barrier and leukocytes infiltrated in the nervous tissue. The disease is mediated mainly by Th1 and Th17 cells targeting the antigens of myelin creating cells—the oligodendrocytes. Later stages of the disease are characterised by infiltration of Th1 and Th17 lymphocytes boosting the inflammation, CD8+ T cells mediating the oligodendrocyte destruction, activated macrophages with engulfed myelin fragments, plasma cells producing autoreactive antibodies as well as activated local phagocytes. MS occurs in three forms: relapse-remitting (characterised by attacks of CNS infiltration manifested by neurological impairments interspaced with stable periods), primary progressive and secondary progressive (characterised by gradually increasing disabilities with attacks). The common experimental form is induced by immunisation with subcutaneously injected myelin oligodendrocyte

glycoprotein peptide (MOG) in complete Freud´s adjuvans, followed by intraperitoneal administration of pertussis toxin (Chapel, Haeney, Misbah, & Snowden, 2014; C. S. Constantinescu, Farooqi, O'Brien, & Gran, 2011). However, there are also other methods of induction using different antigens or transgenic mice (Zgair et al., 2016). The form of EAE progression is dependent on the animals and the method of induction.

MS, similarly as IBD, features a shift in intestinal microbiota composition. Transplantation of faecal microbiota derived from MS patients into GF mice induced hypersensitivity and cognitive impairment accompanied by T lymphocytes changes in the lamina propria and spleen with increased Th17 numbers (H. Chen et al., 2020). This suggests that MS-associated microbiota itself can actually drive neurological changes as is supported by the fact that each form of MS has its signature traits in the microbiota changes (H. Chen et al., 2020). EAE was experimentally treated with SCFAs which reduced the clinical score as well as the subsequent histological grading which revealed decreased infiltration and demyelination. These changes were accompanied with increased Treg numbers in the draining LNs. Interestingly, in this study even increase in fibre content in the diet resulted in a clinical improvement and was represented by increased acetate concentrations in caecum (Mizuno, Noto, Kaga, Chiba, & Miyake, 2017). Ketogenic diet was found to have beneficial effects too. It has improved results in behavioural tests, reversed tissue atrophy and perivascular lesions and reduced production of reactive oxygen species and inflammatory cytokines in LNs and CNS, suggesting diet adjustments to be a promising therapeutic approach (D. Y. Kim et al., 2012). Probiotic treatment also had positive effects on the disease progression. Supplementation of *Lactobacillus reuteri* reduced Th1 and Th17 absolute numbers and percentage in peripheral blood mononuclear cells and spleen as well as their cytokine levels in plasma. Additionally, the treatment also reduced proliferation of MOG-stimulated splenocytes derived from mice 12 days post induction. Notably, these improvements were matched by a restored intestinal microbiota diversity (He et al., 2019).

As the beneficial immunomodulatory systemic effects of microbes are being described, more potential disease candidates to be treated via microbiota or IMMMs are being proposed. One of the recently added diseases is uveitis—a sight-threatening inflammatory disease with increasing prevalence.

Just like other mucosal surfaces, the ocular mucosa can be a site of infection. However, since an inflammation could be destructive for the organ, some structures of the eye were established immune-privileged. The human eye can thus be immunologically divided into two segments. First is *saccus conjuctivae* which is protected by IgA, lysozyme and mast

cells and the second is the bulbus which is isolated from the immune system by hemato-ocular barrier, lack of lymphatics and a variety of mechanisms which suppress immune reactions through production of suppressive cytokines and active induction of apoptosis via Fas ligand. However, in case of a disturbance, the eye, being a partial immune-privileged organ, can be a target of inflammation (Chapel et al., 2014).

Uveitis affects the uveal tract of the human eye. The uveal tract comprises the vascularised part of the bulbus consisting of anterior structures (iris and ciliar body) and posterior structures (choroid and retina). Being richly vascularised the uveal tract is more susceptible to deposit antigens and immunocomplexes which may induce inflammation and damage to the barrier and tissue. Uveitis can affect any of the uveal structures both in an acute and chronic manner. The disease can be localised solely in the eye or be a part of a systemic disorder. According to the localisation uveitis is categorized into anterior (represented by choroiditis and cyklitis) and posterior (choroidits and retinitis). While the anterior form is often manifested by pain, redness, photophobia and pupil deformities the posterior form manifests mainly by vision impairment and may not be otherwise apparent. Uveitis can be induced by an injury of the eye which leads to autosensitisation to uveal antigens, it can be a part of a systemic disorder like ankylosing spondylitis, systemic lupus erythematosus, juvenile arthritis or multiple sclerosis, it can be induced by an infection, especially in immune-impaired individuals or it can be idiopathic. In fact, up to 50 % of patients develop uveitis from an unknown cause. The disease is mediated mainly by Th1 and Th17 cells and macrophages. Despite the treatment (consisting mainly from steroids and mydriatics), about 10–20% of patients become blind and uveitis makes about 20 % of all blindness causes (Chapel et al., 2014).

The animal model of uveitis is experimental autoimmune uveitis (EAU). EAU has given us insight into how uveitis is mediated on a cellular level and how it could be treated. Uveitis can be developed spontaneously in transgenic models, through viral infection of immune-deficient animals, by endotoxin injection, or by immunisation with an uveal antigen like rhodopsin, recoverin or in our case inter-photoreceptor retinoid-binding protein (IRBP). Antigen is injected subcutaneously in complete Freud´s adjuvans which is followed by intraperitoneal Pertussis toxin injection (Bansal, Barathi, Iwata, & Agrawal, 2015).

EAU manifests in common uveitic traits of retinal or choroidal inflammation, retinal vasculitis, photoreceptor destruction and vision impairment. Cellular immune background resembles that of human endogenous uveitis with Th1 cells being the main effector population. This notion was supported by the fact that non-IFN-γ-producing cells are non-

uveitogenic (Tarrant, Silver, Chan, Wiggert, & Caspi, 1998), also re-stimulation of T cells participating in the inflammation triggered mainly IFN-γ release as is supported by IFN-γ being the most prominently produced cytokine in the impaired organ. Th1 responding animals are also generally more susceptible to the disease (Caspi et al., 1996). Th17 cells however take part as well. Although IL-17 is not as abundantly produced, if it is neutralised, the disease can be prevented or reversed (Luger et al., 2008). This suggests that while IFN-γ may be important for the development of the disease, IL-17 is critical for its maintenance.

Recently, microbiota has been suggested to be an important immunomodulator of the eye homeostasis and gut-eye axis was proposed. Similarly as in the case of MS, uveitis was associated with dysbiotic microbiota states with patients having shifted microbiota composition (Kalyana Chakravarthy et al., 2018). This was supported by EAU studies which demonstrated a reduced intestinal microbial diversity with a specific decrease of anti-inflammatory species after the immunisation but prior to the development of the ocular inflammation (Janowitz et al., 2019; Kalyana Chakravarthy et al., 2018). Furthermore, uveitis onset was reduced in germ-free conditions and manifestations were ameliorated after antibiotic treatment pointing out the involvement of microbiota in the pathological process too (Nakamura et al., 2016). Microbiota thus may be involved in aggravation of the disease by providing inflammatory signals or lacking anti-inflammatory signals during dysbiosis and possibly also in development of the disease through molecular mimicry (V. K. Singh, Yamaki, Abe, & Shinohara, 1989).

Similarly as in the case of MS, the anti-inflammatory aspects of the microbiota become a means for experimental treatment of EAU. Although far less research has been done on this topic compared to MS treatment, some studies have already shown promising results. SCFA treatment was successful in EAU studies as it reduced uveitic traits like uveal infiltration and excessive inflammatory cytokine production in bulbus. It also balanced T cell populations by decreasing Th17 proportion and increasing Treg numbers. These lymphocytic changes were apparent in LP of the intestine, gut-draining mLNs but also in spleen and eye-draining cervical lymph nodes (cLN). Excessive trafficking of leukocytes from gut to peripheries was also decreased by the treatment (X. Chen et al., 2017; Nakamura et al., 2017). Oral treatment with a mixture of five probiotic species from *Bifidobacterium* and *Lactobacillus* genera reduced uveitic traits and was accompanied by an increase of Treg population and a reduction of IFN-γ^{hi} and IL-17^{hi} lymphocytes (J. Kim et al., 2017). The treatment of EAU using probiotics has thus shown positive results and potential. Most of the yet known immunomodulatory mechanisms participating in this process involve regulation of

Th17 and Treg balance. Whether other mechanisms could be involved too is discussed in the following chapters.

1.4. Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organisation; World Health Organisation, 2001). Probiotics are extensively studied for their well-being effects as well as preventive or therapeutic potential in a variety of diseases: eradication and prevention of infections (Antibiotic-associated *Clostridium difficile*-associated diarrhoea, *Helicobacter pilori* infection, respiratory infections), GIT disorders (irritable bowel syndrome, necrotising enterocolitis) dermatitis and depression, just to name a few aside the already discussed (Gao, Mubasher, Fang, Reifer, & Miller, 2010; Goderska, Agudo Pena, & Alarcon, 2018; Guyonnet et al., 2007; Hao, Dong, & Wu, 2015; Kaikiri et al., 2017; Lin et al., 2005; Nikolova, Zaidi, Young, Cleare, & Stone, 2019). Generally, probiotics can mediate their effects through: competition with pathogens (for nutrition and adhesion to the epithelium), antagonism (production of antimicrobial substances), inhibition of toxin production by pathogens and by immunomodulation of the host (Markowiak & Śliżewska, 2017). However, the beneficial effect in many disorders still remains debated as high-quality studies still face opposing results from others leading to ambiguous conclusions supported by methodology variability and individual/context-dependent microbial effects (Suez, Zmora, Segal, & Elinav, 2019). As a result only a few probiotics are prescribed as a disease therapy.

The dominant commercially sold probiotic genera and species include mainly members of *Lactobacillus* and *Bifidobacterium* genera, *Lactococcus* species, *Streptococcus Thermophilus*, *Escherichia coli* Nissle 1917 and *Escherichia coli* O83:K24:H31. For the purposes of this study two microbes with immunomodulatory abilities were tested: *Escherichia coli* Nissle 1917 (EcN) and *E. coli* O83:K24:H31 (EcO). Even though they both belong to the same species, they exhibit distinct characteristics.

1.4.1. *Escherichia coli Nissle 1917*

EcN is a microbe recognised for over a hundred years. It was originally found by a German medical doctor and bacteriologist prof. Alfred Nissle. During a *Shigella* outbreak among soldiers in the First World War Alfred Nissle noticed that some soldiers were immune to the infection and so, being a bacteriologist, he subjected their stool samples to cultivation analyses. He found out that protected soldiers were colonised by an antagonistic bacterium

which was able to block growth of pathogenic species. Since this was in pre-antibiotic era, having a tool to fight bacterial infections was very sought-after. The potential of EcN was thus quickly recognised and in 1917 the bacterium started to be mass-produced and sold under the name of Mutaflor. Mutaflor was prescribed both prophylactically and as a treatment of infectious diarrhoea. After introduction of antibiotics the interest in EcN faded. However with increasing knowledge of other microbial immunomodulatory abilities and global antibiotic resistance, EcN become a point of interest once again (Sonnenborn, 2016).

EcN has several documented immunologically-relevant activities. Antagonism—inhibition of growth or killing of pathogenic bacteria, was recognised the first. This process is mediated via production of colicins and microcins which are usually aimed towards near, often pathogenic, relatives from the *Enterobacteriaceae* family. These substances include sidopherons and other iron ion acquisition microcins or colibactin—a genotoxic virulence factor (Massip et al., 2019). Suppression of pathogen growth is, however, mediated also indirectly through signalling to the intestinal epithelium which responds by increased beta-defensin 2 production. This facilitates the barrier function and helps to neutralise pathogens (Schlee et al., 2007). Support of the barrier function is also improved on cellular level. EcN induced arrangement of tight junctions in intestinal epithelium models after barrier injuries caused both by puncture and by pro-inflammatory cytokine damage (Guo et al., 2019). This is supported by multiple studies documenting improved barrier electrical resistance values, decreased FITC-Dextran flux and increased zona ocludin-1 and claudin-1 expression (Zyrek et al., 2007). These mechanisms led to reduced pathogen invasion in co-infection experiments by up to 99 % and abolished pathogen-mediated barrier disruption (Boudeau, Glasser, Julien, Colombel, & Darfeuille-Michaud, 2003). EcN has thus multiple abilities by which it handicaps pathogens and improves its own position and colonization outlook. Interestingly, it does so without exhibiting pathogenic activities. Injection of human organoids with pathogenic *E. coli* leads to barrier destruction however injection of EcN does not even though the bacterium proliferates (Pradhan & Weiss, 2020).

EcN, also induces anti-inflammatory effects. In sepsis models and colitis models, oral EcN treatment decreased excessive production of TNF in plasma and colon. It also reduced responsiveness of extra-intestinal lymphocytes. Activated splenocytes decreased their production of IL-2 and IL-5 and conversely increased the production of IL-10. (Arribas et al., 2009). Interestingly, even direct stimulation of human peripheral blood (PB) T cells supports these results. If CD3 stimulation is accompanied with EcN cultivation supernatant, not only do PB T cells increase their IL-10 production but also reduce IL-2, IFN-γ and TNF production

in comparison to CD3-stimulated cells only. Furthermore, the EcN supernatant also inhibited cell cycling and expansion of the PB T cells (Sturm et al., 2005).

From the prevention of acute diarrhoea EcN has, for its immunomodulatory abilities, emerged as a point of interest in therapy of intestinal inflammatory and barrier damage diseases as well. IBD is one of the main targets of EcN therapy research. A meta-analysis addressing aspects of remission and relapse which compared treatment of UC patients with EcN and the common IBD drug—mesalazine—has assessed their equivalent efficiency (Losurdo et al., 2015). Mesalazine, however, has a list of unwanted side effects so an alternative would be advantageous. Models of colitis have shown the changes of IBD progress under influence of EcN treatment. Improvements in disease activity index are associated with decreased expression of IL-1β and IL-12, increased mucin production in the colon, reduced inflammatory cell infiltrate supported by lowered expression of ICAM-1 and counteraction of colitis-associated dysbiosis (Rodríguez-Nogales et al., 2018). Recently, progress in research of treatment of extra-intestinal disease with EcN has been made too. EcN-treated mice with EAE have reduced the disease severity. Clinical improvement was associated with decreased pro-inflammatory cytokine secretion and increased IL-10 secretion in peripheral LN and in CNS. Furthermore, EAE-associated intestinal barrier impairment was repaired. Interestingly, this was associated with an increase of MOG-specific CD4 T cells in peripheries and a decrease in the affected organ (Secher et al., 2017). This suggests that EcN may affect migration of lymphocytes as was indicated in the case of IBD too.

Many of these immunomodulatory activities have been described more than ten years ago, however a complete understanding of the molecular signalling involved is still lacking. One of the key structures mediating some of the EcN´s effects is its flagellum. It is not only important for the adhesion to the mucus of the epithelium but it is also responsible for the induction of defensin production as demonstrated by non-functional flagellin-mutants (Schlee et al., 2007; Troge et al., 2012). Interestingly, EcN is not a SCFA producer so the anti-inflammatory effects are not mediated through acetylation-mediated Treg induction. Recently, more evidence about outer-membrane vesicles (OMV) as the mediators of immunomodulation was documented. And indeed, in the case of EcN, OMVs induce distinct immunomodulatory activities. Purified EcN OMVs were functional in the treatment of Dextran sulfate sodium (DSS) colitis as proved by ameliorated clinical symptoms, reduced histological score and improved expression of selected cytokines and markers of barrier function (Fábrega et al., 2017). EcN-derived OMVs were shown to be the bearers of anti-inflammatory effects in simplified in vitro stimulations of RAW macrophages too with

triggering more anti-inflammatory cytokine production (IL-10) than pro-inflammatory (IL-6, TNF) (Hu et al., 2020). OMV may thus represent an alternative to using live organisms having non-replicative nature which could address the problematic of individual probiotic effects.

The Czech Institute for Drug Control recommends prescribing Mutaflor in following indications: prophylaxis of UC relapse, constipation, diarrhoea, allergies, eczema, infections of the urinary tract, ATB-associated dysbiosis and general enhancement of immunocompetence (State Institute for Drug Control, 2021b).

1.4.2. *Escherichia coli O83:K24:H31*

EcO is commercially produced and sold under the name of Colinfant New Born. As the name suggests, the probiotic is mainly targeted to treat specifically immune-challenged infants. EcO was found to be beneficial for infants from two "high-risk" groups: new-borns of parents with allergic background and new-borns in an infectious environment. Both of these groups take advantage of the immunomodulatory properties of the microbe which induce favourable immune tuning. The history of the microbe and its discovery is unclear, however, apparently, EcO might have been recognised at war for its protective effects against diarrhoea infections similarly as was EcN.

Although EcO may not be as extensively studied as EcN, being applicable to infants, EcO allows us to study long-term effects of microbes, tuning the IS, in early development. As documented in multiple studies, EcO decreases incidence of allergies of infants coming from genetically-predisposed background (J Hrdý, Kocourková, Lodinová-Žádníková, Kolářová, & Prokešová, 2016; Jiří Hrdý et al., 2018). These effects are probably partially mediated by following mechanisms. EcO treatment reduces IgE levels, a critically important trait in ameliorating allergic response (Liška et al., 2019). And, as it seems, EcO polarises the IS more towards Th1 response. Typical Th2 cytokines were reduced and pathological Th2 allergic traits like eosinophilia diminished in an EcO treatment allergy study (Zwicker et al., 2018). This is further supported by elevated IFN-y levels in serum of treated infants (Jiří Hrdý et al., 2018). Decreased allergy incidence was significant ten years after the post-natal EcO treatment (Rája Lodinová-Zádníková, Cukrowska, & Tlaskalova-Hogenova, 2003). The incidence decrease is, however, usually limited only to atopic diseases and thus recently also experimental intranasal administration was tested. In a systemically sensitised mouse model an airway challenge led to an inhibited allergy effector phase in EcO-treated group

demonstrated by reduced eosinophilia, Th2 cytokine production and mucus secretion (Zwicker et al., 2018).

Interestingly, EcO induces anti-inflammatory effects too, as demonstrated by increased Treg functionality. Although EcO does not induce an increase in Treg counts, it enhances their effector traits including Foxp3 and IL-10 intracellular expressions exhibited by increased mean fluorescence intensity values of these markers in peripheral blood samples from treated children (J Hrdý et al., 2016). This is supported by elevated IL-10 serum levels. Anti-inflammatory effects are however also accompanied by an increased resistance to infectious diseases, which was difficult to grasp (Rája Lodinová-Zádníková et al., 2003). These effects were later suggested to be caused by antibody level changes. EcO may induce a decrease in IgE levels, however, levels of IgG and IgA are elevated by the microbe (Liška et al., 2019; R Lodinová-Zádníková, Sonnenborn, & Tlaskalová, 1998). Increased levels of secretory IgA (S-IgA) in serum, saliva and the gut may be especially beneficial for formula-fed infants who are handicapped by the absent transport of maternal antibodies. EcO-treated infants were less susceptible to infections for years after treatment. They exhibit decreased presence of pathogenic strains in the intestine and other mucosal surfaces. Nosomical infection, mortality or the need for antibiotic treatment were also all reduced in EcO-treated groups (R Lodinová-Zádníková et al., 1998). For its anti-inflammatory and barrier-supporting (S-IgA elevation) effects, EcO has also been tested for IBD therapy capacity. In a mouse model of DSS colitis, EcO decreased symptom score and proved to be promising in the context of inflammatory diseases as well (Kokešová et al., 2006).

The Czech Institute for Drug Control recommends prescribing Colinfant New Born mainly to newborns and infants in following indications: prevention of gastrointestinal infections, preventive colonization, dysbiosis (State Institute for Drug Control, 2021).

Both EcN and EcO thus exhibit distinct activities making them promising candidates for treating various, both intestinal and extra-intestinal immune-related diseases. Therefore, they were chosen, for the purposes of our present study, to be tested for EAU therapy.

2. Aims

The aim of this thesis was to search for differences in capacities of immunomodulation provided by EcO and EcN, which could determine their potential to reduce the severity of EAU. In several mouse and mouse-derived models, we aimed to assess:

- Colonization abilities of EcO and EcN
- Response of antigen presenting cells to EcO and EcN
    - Activation traits
    - Antigen presentation traits

3. Materials and Methods

3.1.1. *Animals*

Initial experiment assessing immunomodulatory effects of *E. coli in vivo* was performed on 5 to 8 weeks old female C57BL/6J mice supplied to us by The Centre for Experimental Biomodels, First Faculty of Medicine, Charles University in Prague. Mice were housed at a conventional animal facility at the Department of Pharmacology, First Faculty of Medicine, Charles University in Prague. Mice with congenital defects were excluded from the study. Animals were distributed from a bulk randomly into cages.

Stimulation of bone marrow-derived macrophages, stimulation of bone marrow-derived dendritic cells and colonization experiment were performed on 10 weeks old female C57BL/6J mice obtained from the breeding colonies of the Institute of Microbiology of the Czech Academy of Sciences. All studies were carried out in accordance with the recommendations of the ethics standards defined by the EU legislation on the use of experimental animals (2010/63/EU) and the Czech animal welfare act. The protocols were approved by The Commission for AnimalWelfare of the First Faculty of Medicine of Charles University in Prague, The Ministry of Education, Youth and Sports (MSMT 9993/2017-2).

3.1.2. *Experimental Autoimmune Uveitis Induction*

EAU was induced by subcutaneous injection of interphotoreceptor retinoid-binding protein peptide 1—20 (IRBP; [Homo sapiens] H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, MA, USA) 500 µg per mouse emulsified in CFA containing 3.3 mg/mL of heat-killed Mycobacterium tuberculosis H37Ra (Difco, Franklin Lakes, NJ, USA) immediately followed by intraperitoneal application of pertussis toxin (PTx; List Biological Laboratories, Inc., Campbell, CA, USA) 1.2 µg, as previously reported (Klimova et al., 2016).

3.1.3. *Probiotics*

Escherichia coli Nissle 1917 (EcN; serotype O6:K5:H1; Ardeypharm GmbH, Herdecke, Germany) and *Escherichia coli* O83 (EcO; serotype O83:K24:H31; Dyntec spol. S.r.o., Terezin, Czech Republic) were cultured in Lennox´s version of Luria Bertani broth (Sigma-Aldrich, St. Louis, MO, USA) in a shaker incubator. Their optical densities (OD) were periodically measured by spectrophotometer at 600 nm (A_{600}) to monitor population density. OD and volume was matched to cell count using previously established growth

curves (growth curves were evaluated by bacterial growth on 0.05 % agar plates (from the same broth) in various concentrations followed by CFU counting matched to corresponding optical densities). Once bacteria reached target population density and underwent series of washing they were diluted in sterile seline (0.9% NaCl, Ardeapharma, a.s., Ševětín, Czech Republic) solution to target concentration 10^{10} mL^{-1}. Aliquotes were stored at 4–8°C as wet pellets for up to two weeks (bacteria were periodically checked on viability by culture on agar) and re-suspended in sterile seline immediately prior to administration. Oral treatment was a 100 µl dose of 10^9 bacteria administered by gavage. Mice in colonization experiments received a single dose. Mice in the experiment assessing the immunomodulatory effect of probiotics received the treatment 3 times a week (every other day) and were split in four different schedule groups. First group´s treatment started 14 days before EAU induction and continued to experiment termination 28 days after induction (Prevention and Treatment). Second group only received prevention and treatment was stopped on the day of induction (Prevention). Third group´s treatment started on the day of induction and continued to the experiment´s termination (Early and Late Treatment). Fourth group received its first dose 14 days after EAU induction and treatment went on to the experiment termination as well (Late) (**Figure 2**). Mice from different groups were house separately to avoid cross-contamination.

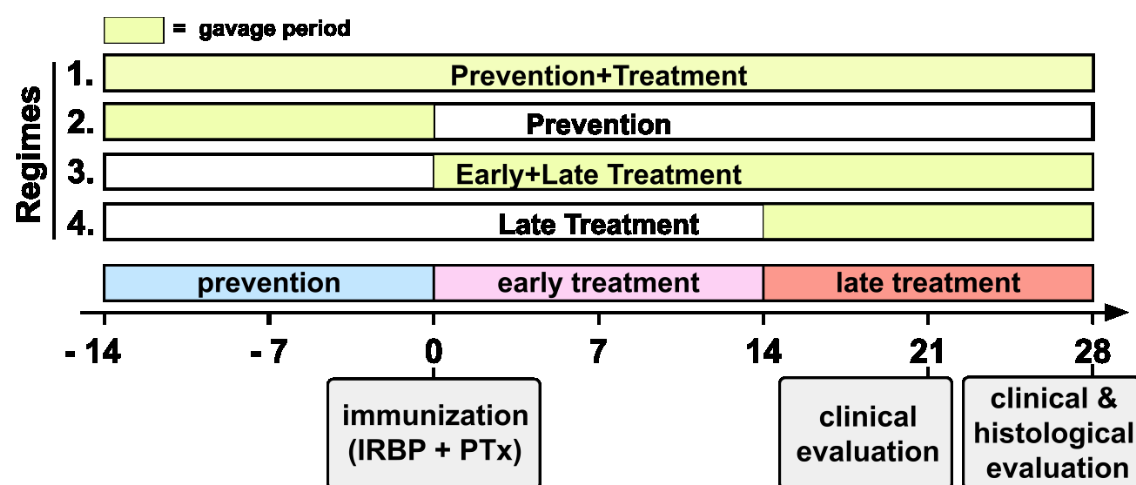

Figure 2. Experimental schedules.

3.1.4. *Clinical Evaluation*

In vivo clinical examination was carried through eye fundus biomicroscopy—imaging of the posterior central retina of each eye. Using topical endoscopy fundus imaging system with additional 4,0 diopter lens between the camera and the octoscope interior surface of the eye was imaged through dilated pupil (Tropicamide, Unitropic 1% oph. gtt., Unimed Pharma, Bratislava, Slovakia) and phenylephrine (Neosynephrin-POS 10% oph. gtt.,

Ursapharm, Prague, Czech Republic). The otoscope was applied to the cornea using carbomer eye gel (Vidisic gel, Bausch and Lomb, Prague, Czech Republic). Mice were under general anesthesia (ketamine 80 mg/kg and xylazine 5 mg/kg (both Bioveta, a.s., Ivanovice na Hane, Czech Republic)) administered intraperitoneally during the procedure. Retinal inflammatory changes were evaluated in: optical disc, retinal vessels and retinal tissue of the central fundus. The level of inflammation was evaluated independently by two experienced ophthalmologists and graded from 0 (no detectable changes) to 4 (most severe disease features) according to the scoring strategy described before (Heissigerova et al., 2016). Each mouse is shown as a mean of both eyes.

3.1.5. *Histological Evaluation*

Mice were sacrificed on day 28. Eyes were enucleated and immediately immersed in Tissue-Tek® O.C.T. COMPOUND™, (Sakura Finetek USA, Inc., Torrance, CA, USA) frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO, USA) in liquid nitrogen and stored at -70 °C until sectioning to 7-µm-thick slices (at -17 °C to -21 °C). Sections were taken at 3 levels: both eye peripheries and centrally through the optic nerve. Samples were cut by cryostat (Leica CM 1850) and then stained with hematoxilin and eosin. Two experienced ophthalmologists evaluated disease severity from 0 (no detectable changes) to 4 (most severe disease features) using previously established standardised grading system which considers following disease traits: non-granulomatous infiltrate, vasculitis, vitritis, retinal folds, granulomas and photoreceptor loss (Heissigerova et al., 2016). Final grade for each mouse was obtained averaging scores for each eye.

3.1.6. *DNA Isolation and RT-PCR*

Stool was collected directly into eppendorf tubes. Intestinal contents were isolated from dissected intestines at the end of the experiment. Samples were then homogenised using ceramic beads Lysing Matrix D on FastPrep-24 (both MP Biomedicals, Solon, OH, USA) DNA was isolated by MasterPure DNA purification kit (Epicentre, Madison, WI, USA) and diluted to 5 ng/µl. Concentration was measured using NanoDrop 2000 (ThermoFisher Scientific). A dilution series of a corresponding PCR product with known copy number or DNA isolated from a known amount of CFU were used as a standard. PCR contained primers specific for EcN and EcO (**Supplementary Table S1**), SG PCR Master Mix (Generi Biotech, Hradec Kralove, Czech Republic) sterile dH_2O and 4 µl of template. Cycling parameters were set to: 4 min at 95 °C, 40 cycles of 30 sec at 94 °C, 30 sec at 60 °C, 1 min at 72 °C followed

by 1 min at 54 °C, a melt curve of 10 sec from 54 °C to 95 °C and 10 min at 10 °C. Reaction was carried on LightCycler® 480 Real-Time PCR System. Final CN/CFU was normalized to sample weight.

3.1.7. *Preparation of Bacterial Lysates, Cultivation Filtrates and Controls*

Bacteria were cultivated as described above. After a washing series, to prepare lysates, each cell suspension was passed three times through French Press, lyophilised and re-suspended in a complete RPMI medium (Merck; Cat# R0883) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom GmbH), 1% Antibiotic-Antimycotic solution (Merck), 1% Glutamine (Merck; Cat# G6392) and 50 μM 2-mercaptoethanol (Merck) to target concentrations. Filtrates were prepared by passing the liquid bacterial culture through a 0.22 μm filter (Merck; Cat# SLGV004SL) to filter out all bacterial cells and only allow metabolites in. LPS-S (Enzo; Cat# ALX-581-009) diluted in complete RPMI to 1 μg/ml was used as a positive control. Complete RPMI served as a negative control.

3.1.8. *Generation and Culture of Bone Marrow-Derived Macrophages*

10-week-old C57BL/6J mice were euthanized be cervical dislocation. Lower limb bones were collected and cleared of all muscle tissue. Femur and tibia were isolated and epiphyses were cut off. Using a 0.45 mm needle (MEDOJECT), bones were washed through with sterile saline (0.9% NaCl, ardeapharma, a.s., Sevetin, Czech Republic) through a 70 μm filter (Falcon; Cat# 352350). After re-washing with sterile saline, bone marrow cells were resuspended in 10 ml of culture medium and placed into a 90 mm petri dish (GAMA GROUP; V400974) at a concentration of 1,6 x 10^6 cells/ml. Culture medium contained RPMI 1640 (Merck), 10% heat-inactivated fetal bovine serum (Biochrom GmbH), 1% Antibiotic-Antimycotic solution (Merck), 1% Glutamine (Merck; Cat# G6392), 50 μM 2-mercaptoethanol (Merck) and 20% conditioned L929 cell (ATCC CCL-1) medium. L929 medium contains macrophage colony stimulating factor (MCSF) produced by L929 cells in a 10-day cultivation in a complete RPMI medium. Cultivation was carried in a humidified incubator (37°C, 5% CO_2). Medium was changed after 3 days. On d6 of culture, cells were seeded into a 96F-well plate (ThermoFisher Scientific; Cat# 167008) at 200,000 cells per 200 μl per well and on d7 cells were stimulated. On d8 experiment was terminated and cells underwent analyses.

3.1.9. *Generation of Bone Marrow-Derived Dendritic Cells*

Bone marrow was harvested from lower limb bones of 10-week old C57BL/6J mice as described above (see chapter 3.1.8. Generation of Bone Marrow Derived Macrophages). Cells were then re-suspended in 10 ml of a dendritic cell differentiation medium to a 5×10^5/ml concentration and cultured in a petri dish in a humidified incubator (37°C, 5% CO_2). Differentiation medium contained: RPMI medium (Merck) with 10% heat-inactivated fetal bovine serum (Biochrom GmbH), 1% Antibiotic — Antimycotic solution (Merck), 1% Glutamine (Merck Cat# G6392), 50 µM 2-mercaptoethanol (Merck), 1% Sodium Pyruvate (Merck; Cat# S8636), 1% minimum essential medium (Merck; Cat# M7145), IL-4 (Merck; Cat# I1020) at 4,5 ng/ml and GM-CSF (Merck; Cat# SRP3201) at 20 ng/ml concentration. Medium was changed on d4 and d7. On d8 cells were harvested, concentration was adjusted to 2×10^6/ml, cells were seeded into a 96F-plate at 2×10^5 per 200 µl per well and stimulated for 24 hours. Cells and supernatants were then collected for analysis.

3.1.10. *Cell Preparation and Immunophenotyping by Flow Cytometry*

Cells from mesenteric, cervical and inguinal lymph nodes were harvested at the experiment termination. Cell suspensions were prepared by mechanical disruption and passed through a 70 µm cell strainer (Becton Dickinson; Cat# 352350) and washed (300 × g, 5 min, 4°C). To analyse macrophages resident to the lamina propria of the gut, 2 cm sections of ileum were collected, outside layer was scraped off and sample was placed into extraction buffer containing: 10 ml of PBS (Institute of Molecular Genetics of the CAS), 20 µl of 0.5M EDTA (Institute of Molecular Genetics of the CAS), 150 µl FBS (Institute of Molecular Genetics of the CAS) and 30 µl of 5% 1,4-Dithiothreitol (DTT) (Merck; Cat# D9779) solution per sample. Sample was then incubated for 15 minutes in a NB-205 shaking incubator (N-Biotek) at 37 °C and 320 RPM, washed in sterile saline (0.9% NaCl) and placed in digestion buffer containing: 1 ml of RPMI, 1,5 mg of type IV collagenase (Merck; Cat# C5138) and 12 µl of FBS per sample. In this buffer the sample was cut into approximately 1 mm^2 fragments and then incubated for 30 min at 37 °C and 500 RPM in Thermo-shaker (LAB MARK). Ultimately, samples were washed in sterile saline, filtered through a 70 µm cell sorter (Falcon; Cat# 352350), centrifuged, (300 x g, 5 min) re-suspended in 300 µl of PBS and transferred to a 96U-well plate by 150 µl per well.

The analysis of intestinal samples and lymph nodes was performed as follows: Cell suspension was washed with PBS, cells were labelled with Fixable Viability Dye

(ThermoFisher Scientific, Waltham, MA, USA), fixed and permeabilised with eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific), non-specific antibody binding was blocked by 10 % normal mouse serum and anti-CD16/CD32, and cells were stained for: T cell, monocyte and macrophage markers using fluorescence-labelled antibodies (**Supplementary Table S2**).

Analysis of IC cytokine production was evaluated on cells (2×10^5 cells in complete RPMI) derived from mesenteric, cervical and inguinal lymph nodes. Non-specific TCR stimulation response was tested by 18-hour cultivation with plate-bound 5 µg/mL anti-CD3" and 4 µg/mL of anti-CD28 (both from Biolegend). Specific response to IRBP was tested by 40-hour incubation with 20 µg/mL of IRBP and a mixture of 3 µg/mL of Brefeldin A and 2 µM Monensin (both ThermoFisher Scientific) added for the last 4 h of incubation. Next, (after both of the cultivation strategies) cells were washed, labeled with Fixable Viability Dye (ThermoFisher Scientific), fixed and permeabilized with eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (ThermoFisher Scientific), blocked with anti-CD16/CD32 antibody, and stained for CD3, CD4, CD8 (all Biolegend), IFN-γ, IL-17, and TNF-α (all ThermoFisher Scientific) (**Supplementary Table S2**).

BMDMs and BMDDCs were collected in PBS with cultivation plate placed on ice to release cells attached to the surface. Upon gentle scraping with pipette tips approximately 100,000 cells were transferred per well. Analysis was performed as follows: Cells were washed in PBS, unspecific staining was blocked by 10% normal mouse serum and anti-CD16/32 antibody (Fc receptors) and eBeads were added for single stain controls. BMDMs were than stained for viability, I-A/I-E, CD14, CD40, CD80, CD86 and CD38. BMDDCs were stained for viability, CD80, CD86, I-A/I-E, CD40, CD11b, CD103, F4/80 and CD11c (**Supplementary Table S2**).

All samples were measured in a 96U-well plate (Merck; Cat# Z707899) by LSRII (BD Biosciences, San Jose, Ca, USA) cytometer. Data were analysed by FlowJO software (Tree Star Inc., Asjland, OR, USA). Gating strategies and list of antibodies are attached in supplementary data (**Supplementary Figure S1, Supplementary Table S2**).

3.1.11. ***Analysis of Cytokine and NO Production***

To analyse cytokine production at the intestinal immune induction sites, Peyer´s patches were cultured in 500 µL of complete RPMI medium (Merck; Cat# R0883) containing 10% heat-inactivated fetal bovine serum (Biochrom GmbH, Berlin, Germany; Cat# S 0115) and 1% Antibiotic-Antimycotic solution (Merck) in a humidified incubator (37 °C, 5% CO^2)

for 48 h. The supernatants were collected and stored at -20 °C until analysis. Cytokines were then quantified by indirect ELISA according to the manufacturer´s instructions as described below. We assessed: TNF-α, IL-6, IL-1β and IL-33 (all Bio-Techne, Minneapolis, MN, USA; Cat# DY410, DY406, DY401, DY3626 and DY3059).

NO production in BMDM cell culture supernatants was measured on the day of experiment termination. Standard—$NaNO_2$ (Merck, Cat# 237213)—was diluted in RPMI medium to concentrations from 62.5 μM to 0.98 μM. Griess reagent (Merck; Cat# G4410) in 40 mg/mL concentration was added to samples in 1:1 ration and incubated for 15 min in dark at RT. Absorbance was measured in a 96F-well plate (ThermoFisher Scientific; Cat# 167008) at 540 nm by spectrophotometer (Multiskan Ascent Plate Reader 96/384, MTX Lab Systems).

Cytokines in BMDM and BMDDC culture supernatants were stored at -20 °C and subsequently quantified by indirect ELISA as follows: 96F-well plates (NUNC Maxisorp; Cat# 442404) were coated with primary antibodies, incubated for 12 hours, washed with 1x PBS–0.05% Tween 20 (Merck KGaA, Darmstadt, Germany), blocked with 1% Bovine Serum Albumine (BSA; Merck) for one hour and washed again. Samples were diluted with 1x PBS 1% BSA to target concentrations according to each cytokine, applied on plates in dublets and incubated for 1.5 hours. After washing, corresponding antibodies were added to each well and incubated for 1.5 hours, then, plates were washed again. Next, streptavidin-HRP (R&D Systems) was applied to each well and incubated for 20 minutes in the dark. After last washing substrate was added, incubated in the dark and periodically checked on reaction progression. After 5–30 minutes (according to the cytokine) reaction was stopped by adding 2M H_2SO_4. Absorbance was measured at 450 nm and 650 nm by spectrophotometer (Multiskan Ascent Plate Reader 96/384, MTX Lab Systems). Quantified cytokines were IL-1β, IL-10 and IL-12 (all Bio-Techne, Minneanapolis, MN, USA; Cat# DY401, DY417 and DC419).

3.1.12. *Primary Intestinal Epithelial Cell Derivation and Cultivation*

Primary intestinal epithelial cell culture was performed according to an established methodology as follows (Psichas, Tolhurst, Brighton, Gribble, & Reimann, 2017). 3 month-old C57BL/6J mice were euthanized by cervical dislocation. Colons were removed and placed in L-15 medium (Merck; Cat# L5520) on ice. Next, colons were washed with PBS, intestinal content was flashed out, colons were cleared of all mesentery and muscle layer on top was peeled off under a dissecting microscope. Ultimately, colons were minced with dissecting scissors into approximately 1 mm^2 fragments, further washed with PBS and placed in

Dulbecco´s Modified Eagle Medium (DMEM; Institute of Molecular Genetics of the CAS). Next, tissue fragments underwent a series of enzymatic disintegration by a digestion medium containing XI crude collagenase (Merck; Cat# C9407) diluted in DMEM to a 0.4 mg/ml concentration. The series consisted of 6 cycles of incubation in 37 °C water bath for 10 min interspaced with 10 sec shaking every 5 min. Every incubation was followed by digestion media change. Supernatants from "digests" 3 to 6 containing intestinal crypts were collected, centrifuged, re-suspended in pre-warmed culture medium and set aside. By digest 6 most of the tissue has been disintegrated. Supernatants were combined, centrifuged, re-suspended in culture medium supplemented with Y-27632 dihydrochloride (Merck; Cat# Y0503) (to prevent anoikis) into total volume of 7 ml per colon and filtered through a 100-µm cell strainer (Merck; CLS431752). Cell suspension was seeded in 24F-well plates (ThermoFisher Scientific; Cat# 142475) and 24 well-transwell plates (Corning; Cat# CLS3413) simultaneously. Plates were pre-coated with 2% basement membrane matrix (Corning; Cat# 356234) solution diluted in chilled DMEM and incubated for 1 hour in a humidified incubator (37°C, 5% CO_2). Culture medium consisted of: high-glucose DMEM (Institute of Molecular Genetics of the CAS) supplemented with 10% heat-inactivated fetal bovine serum serum (Biochrom GmbH, Berlin, Germany; Cat# S 0115), 1% Antibiotic-Antimycotic solution (Merck) and 1% Glutamine (Merck; Cat# G7513). Cells were then cultivated for 2 weeks and periodically measured on TEER values and checked under a microscope for growth progression.

3.1.13. *MODE-K Cultivation*

MODE-K cells used in experiments were a 20th and a 23rd passage (third and fifth after thawing an aliquot stored at -120 °C). Cells were primarily cultured in flasks. Upon reaching 3rd passage (at minimum) after defrosting they were transferred to 24F-well plates (ThermoFisher Scientific; Cat# 142475) and 24-well transwell plates (Corning; Cat# CLS3413). Every transfer/passage was mediated by adding a solution of 0.25% Trypsin (Biowest; Cat# P5957) and 0.01% EDTA (Merck; Cat# E5134) in PBS and incubating for 5 minutes to detach the cells form the surface. Reaction was then stopped by adding culture medium containing fetal bovine serum, cells were centrifuged, re-suspended in new culture medium and quartered in concentration for passage or adjusted to the target concentration 5×10^5 cells per transwell. Culture medium consisted of: RPMI medium (Merck) with 10% heat-inactivated fetal bovine serum (Biochrom GmbH), 1% Antibiotic-Antimycotic solution (Merck), 1% Glutamine (Merck; Cat# G7513), 50 µM 2-mercaptoethanol (Merck), 1

mM Sodium Pyruvate (Merck; Cat# S8636), 1% minimum essential medium (Merck; Cat# 51412C), 1mM HEPES (Institute of Molecular Genetics of the CAS; Prague, Czech Republic) and $NaHCO_3$ (Institute of Molecular Genetics of the CAS).

3.1.14. *Transepithelial Electrical Resistance Evaluation*

To measure Transepithelial electrical resistance (TEER) cells were cultured on 24-well transwell plates (Corning; Cat# CLS3413). Each well was measured twice by Millicell ERS-2 Voltohmmeter and evaluated on electrical resistance values every other day. Before every evaluation electrodes were put in 70% sterile ethanol for 15 min and then in corresponding culture medium for 15 min. Control wells had no cells cultivating and thus represent the base values of corresponding medium in the system. Half of the medium was changed every day of TEER measuring. Resistance values were measured in Ohms.

4. Results

4.1. Initial in Vivo Experiments

4.1.1. *Theory and Experimental Design*

These experiments were performed in collaboration with my group in a research testing the immuno-modulatory properties of EcN and EcO in an EAU model (Dusek et al., 2020). My role was minor (**Supplementary Table S3**), however, presenting the data is essential to form a comprehensive overview of the studied mechanism based on which we have designed additional experiments to better characterise the process.

In the initial *in vivo* experiments, C57BL/6J mice with induced EAU were orally treated with one of the two probiotics—EcO or EcN—in various experiment schedules. Disease severity was evaluated by ophthalmoscopy and histology, immune phenotypes in lymph nodes were analysed by flow cytometry and immune environment of the gut was evaluated by gut tissue culture.

4.1.2. *Results*

EcN reduces severity of EAU, whereas EcO does not. Mice treated with EcN exhibited lower clinical as well as histological disease scores (**Figure 3**). While in some cases, probiotics accomplish beneficial effects being in a form of autoclaved fragments, in our study only live probiotics reduced disease severity significantly. Timing of treatment had a pivotal impact as well. EcN has to be given prophylactically. Schedules with late onset were found to be ineffective. Treatment had to start prior to or at the time of EAU induction to effectively reduce EAU severity (**Figure 4**). This suggests that EcN mediates its effects during the antigen (IRBP) presentation phase of EAU induction.

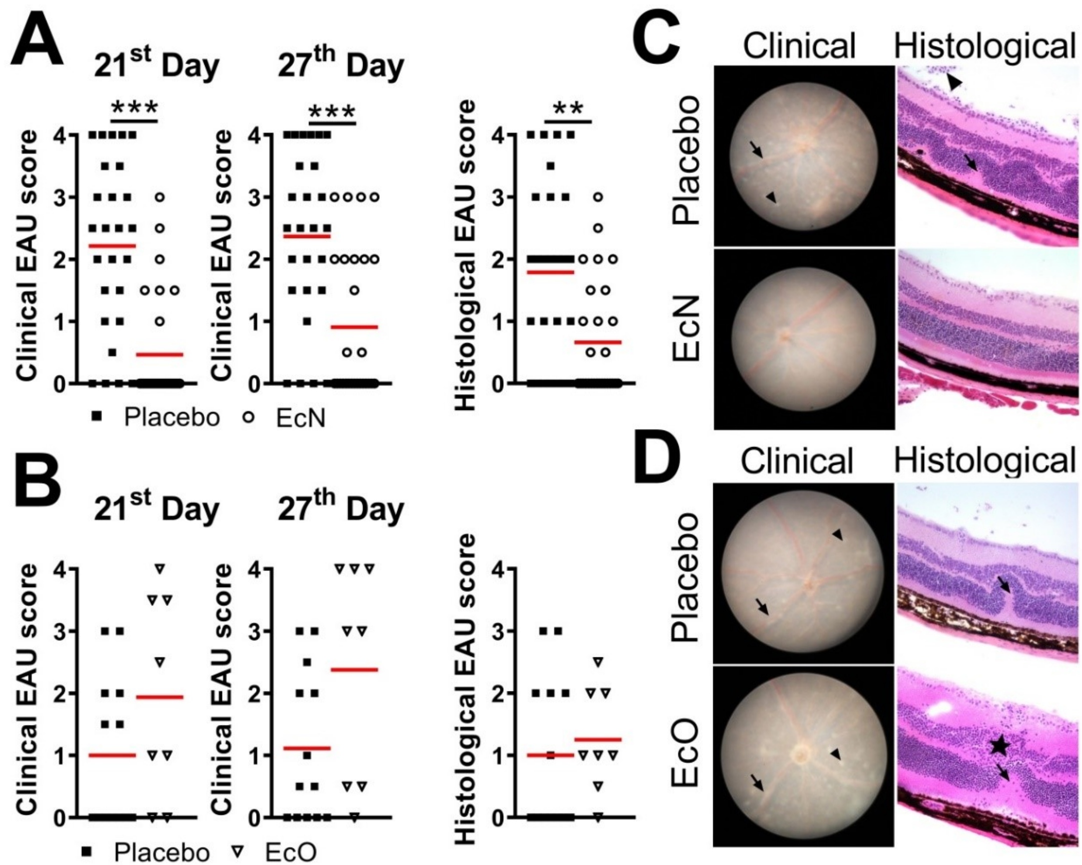

Figure 3. Treatment with live EcN reduces EAU severity while EcO treatment has no effect. Clinical score was assessed via in vivo fundus biomicroscopy—imaging of the posterior retina on the 21st and 27th day post-induction. Histology was performed on sections cut by cryostat and stained by hemotoxilin and eosin on the 28th day post-induction. Scores were evaluated on scale from 0 to 4 (4 being the most severe). Differences were quantified by unpaired Mann–Whitney test; graphs show individual values and red lines represent mean, ** $p < 0.01$ *** $p < 0.001$. Data are pools from 5 (EcN) or 2 (EcO) independent experiments. Figure shows representative pictures from prevention-and-treatment experiment schedule group. **(A)** and **(C)** Clinical and histological scores of EcN-treated mice showing reduced EAU scores [n = 26 (placebo), 28 (EcN) in total]. **(B)** and **(D)** Clinical and histological scores of EcO-treated mice showing no significant effect on EAU score [n = 13 (placebo), 8 (EcO) in total]. Grading evaluated EAU-associated pathologies. These include chorioretinal lesions (arrowhead), vascular sheathing (arrow) and optic nerve inflammation during clinical examination and infiltration of cells in the inner retina (star), cell infiltration of the vitreous body (vitritis, arrowhead) and retinal folds (arrow) during histological examination (optical magnification 200 ×).

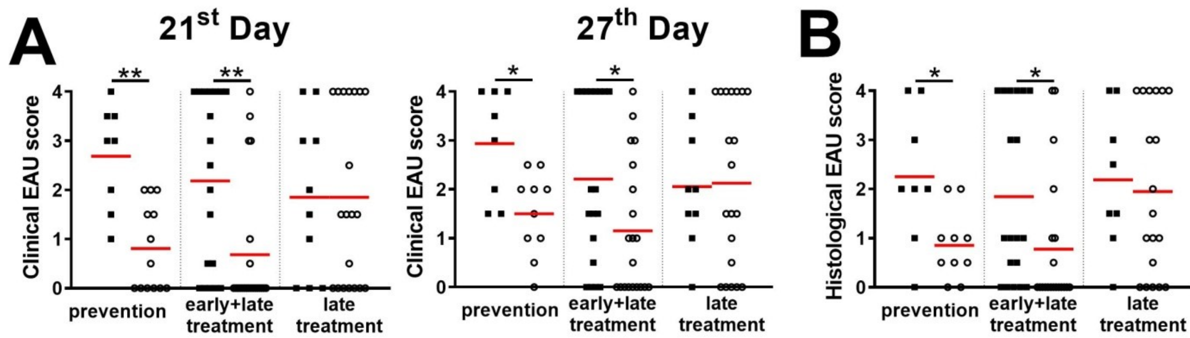

Figure 4. EcN treatment is only effective when given before or at the time of EAU induction. **(A)** EAU severity was analysed by *in vivo* fundus biomicroscopy on the 21st and 27th day post-induction and **(B)** by histological analysis at day 28 postinduction. Differences were quantified by unpaired Mann–Whitney test; * $p < 0.05$ ** $p < 0.01$; n = 8 (placebo prevention), 13 (EcN prevention), 19 (placebo early + late treatment), 22 (EcN early + late treatment), 10 (placebo late treatment) and 20 (EcN late treatment) in total, graphs show individual values and red lines represent mean. Black squares represent individual inflammation severity scores from the placebo group and open circles from the EcN group.

Next, we focused on describing the immunological background of the process. Non-specific TCR stimulation response (anti-CD3/anti-CD28) of T cells derived from EcN-treaded mice, evaluated by production of pro-inflammatory cytokines, was unchanged (**Supplementary Figure S2**) and the proportions of Treg, Th17 and innate lymphoid cells 3 (ILC3) derived from mLN and cLN showed no differences among the groups either (**Supplementary Figure S3**). However, *ex vivo* stimulation of LNs with IRBP induced a lower expression of inflammatory cytokines by CD4^{+} T cells in the EcN group compared to placebo (**Figure 5**). This reduced IRBP-specific T cells response occurred first in iLNs—at the site of immunisation on d7 (data not shown) after immunisation (before EAU manifestation) and propagated to other mLN and cLN later (d28). This suggests that at the time IRBP was presented, immunity was modulated and Th cells were primed to this specific antigen differently. This is in agreement with the timing of effective treatment which indicates that EcN affects antigen presentation of IRBP.

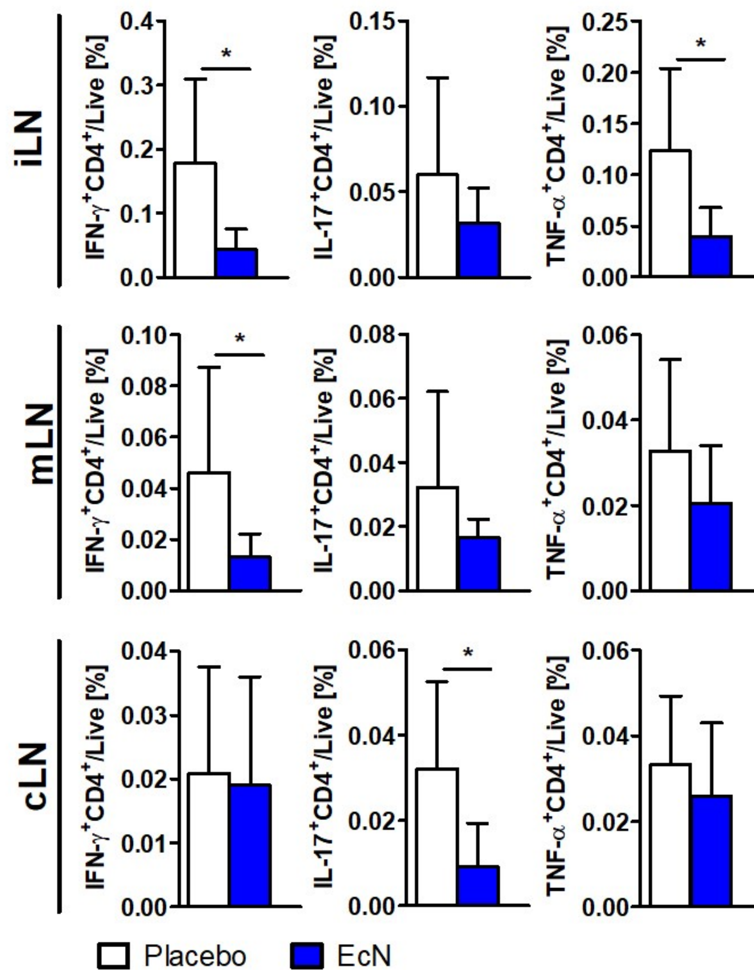

Figure 5. LN cells derived from EcN-treated mice are less responsive to IRBP stimulation. Specific response of inguinal, mesenteric and cervical lymph node-derived cells to IRBP was tested by a 40-hour incubation with 20 μg/mL of IRBP. Graphs show data from day 28 post-induction. Percentage of cytokine-producing populations was evaluated by immunophenotyping via flow cytometry. Differences were quantified by unpaired Mann Whitney test; * $p < 0.05$ ($n = 5–8$ per group).

To determine local gut mucosal immunity tuning we cultured PPs and assessed their cytokine production by ELISA. PPs derived from EcN-treated mice produced significantly lower amounts of inflammatory cytokines (TNF-α, IL-1β and IL-33) compared to placebo (**Figure 6**). Furthermore, EcN-treated mice harboured lower numbers of pro-inflammatory M1 macrophages ($CD38^+$, $Egr2^-$) in ileum (**Figure 7**) and a lower proportion of activated $iNOS^+$ macrophages in gut-draining mLNs compared to the placebo group (**Figure 8**). These data indicate that EcN modulates induction sites of the intestinal mucosal immunity to an anti-inflammatory state and that macrophages could be involved in the EcN-induced immune tuning.

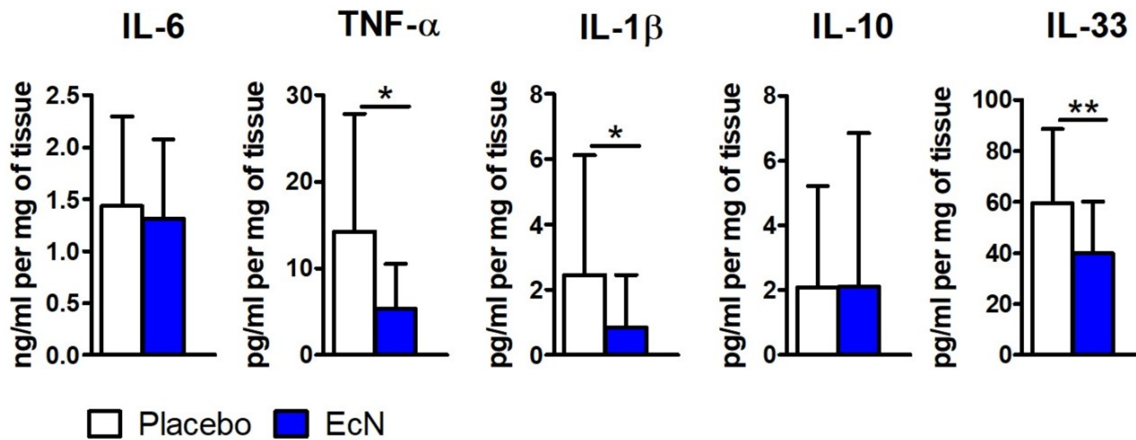

Figure 6. PPs derived from EcN-treated mice produce lower levels of inflammatory cytokines. PPs were collected at day 28 post-induction and cultured for 48h in a complete RPMI medium. Production of cytokines was evaluated by indirect ELISA. Data are pooled from 5 independent experiments (n = 25 per group in total) and differences were quantified by unpaired Mann–Whitney test; * $p < 0.05$ ** $p < 0.01$.

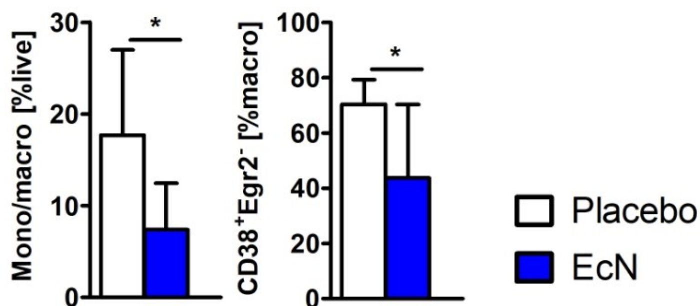

Figure 7. EcN treatment induces a decrease in macrophage populations in ileum. Tissue was collected at day 7 post-induction. Proportions of populations were assessed by FACS. Differences were quantified by unpaired Mann-Whitney test (n = 9 (placebo) or 7 (EcN)); *$p<0.05$, **$p<0.01$. Macrophages were defined as live $CD45^{+}F4/80^{+}CD11b^{+}$ cells and M1 ($CD38^{+}Egr2^{+}$). Markers were selected according to previously published data (Jablonski et al., 2015).

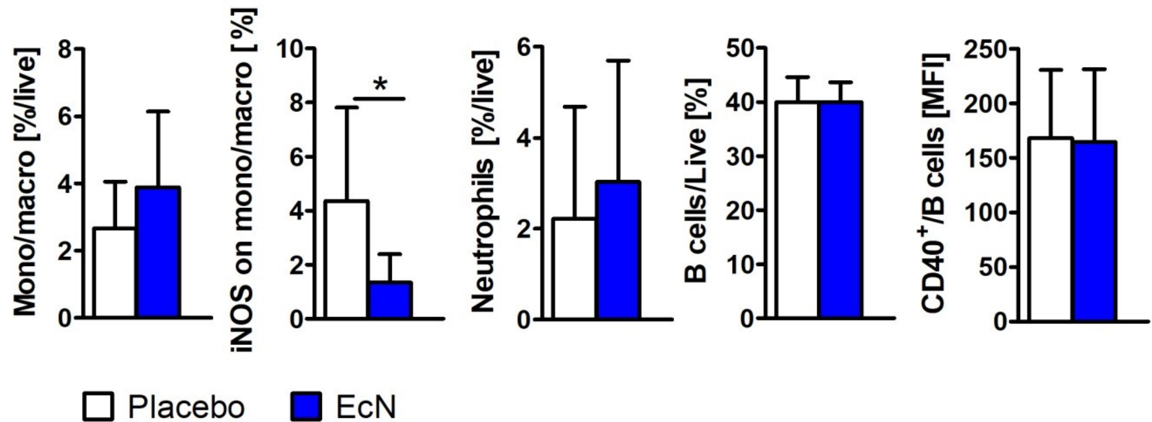

Figure 8. EcN treatment lowers the proportion of activated iNOS+ macrophages in mLN. The proportion**s** of populations in mesenteric lymph nodes were assessed by FACS. Graphs show data from day 28 post-induction. Data are pooled from 2 independent experiments (n = 11 per group in total) and differences were quantified by unpaired Mann–Whitney test; * $p < 0.05$. The monocytes/macrophages are defined as live cells CD45+CD11c-B220-CD3-CD49b-Ly-6G-SSClo, neutrophils are defined as live cells CD45+CD11c-B220-CD3-CD49b-Ly-6G+ and B cells are described as live cells CD45+CD11c-B220+.

Collectively, the data from the initial in vivo experiments reveal several hallmarks suggesting the immune mechanism involved in treatment of EAU with *E coli*. First, only specific probiotics with immunomodulatory properties (EcN) are capable of reducing EAU severity. Second, EcN realizes its effects during the phase of Ag presentation and T cell priming. Third, the effects are not propagated through modulated proportions of Tregs, Th17 or ILC3 and fourth, immunomodulation is accompanied by an anti-inflammatory tuning at the induction sites of intestinal mucosal immunity with a shift in macrophage populations.

Based on the mentioned results we thus asked: What are the differences in immunomodulatory capacities responsible for the distinct effects of the microbes and how could the microbes affect the process of antigen presentation?

4.2. Colonization Experiments

4.2.1. *Theory and Experimental Design*

A simple explanation of the difference in immunomodulatory properties of the two microbes would be a distinct capacity to colonise the host´s gut. A long term colonization could provide a constant feed of immunomodulatory molecules to the host which may be crucial to effectively tune the immune system and decrease the inflammation.

We thus tested whether EcO and EcN differ in the ability to colonise C57BL/6J mice. Mice were orally treated with a single dose of EcO or EcN probiotic solution. For the next 48 hours stool was periodically collected, then mice were euthanized and their intestinal contents were collected too. Samples were then analysed by RT-PCR with specific primers for each microbe and matched to copy number (CN) or colony forming unit (CFU) per gram of stool.

4.2.2. *Results*

In the first (trial) experiment mice from the breeding colonies of the Institute of Microbiology of the Czech Academy of Sciences (CAS) were used and only EcN treatment was examined. EcN DNA appeared in stool as soon as 2 hours after the dose and maintained its presence for the whole 48 hours suggesting a temporary colonization (**Figure 9A**). However, when the experiment was repeated with mice from a different animal facility—Department of Pharmacology, First Faculty of Medicine, Charles University in Prague (FM1) i.e. the animal facility of the initial in vivo EAU experiment—both EcN´s and EcO´s DNA in the stool was not detectable 48 hours after the oral administration (**Figure 9B**). This indicates that ability to colonize depends on the microbes already present in the individual mice, which are determined by the animal facility. Interestingly, when we inspected the concentration of the target DNA in intestinal content samples collected from FM1 mice 48 hours after the probiotic dose we found both strains at similar detectable levels (**Figure 9C**). Additionally, 5 days after the dose, no target DNA was detected (data not shown). With the analysis of intestinal contents we were thus able to detect the microbial DNA for longer than in stool samples.

These data indicate that the two probiotics do not differ in their capacity to colonise mice from FM1 animal facility and colonization advantage does not seem to be the cause EcN´s effectiveness in the treatment. Both microbes colonise for at least 48 hours. Since in the initial in vivo experiment a dose was given 3 times a week both bacteria provided a continuous feed of an immunomodulatory input.

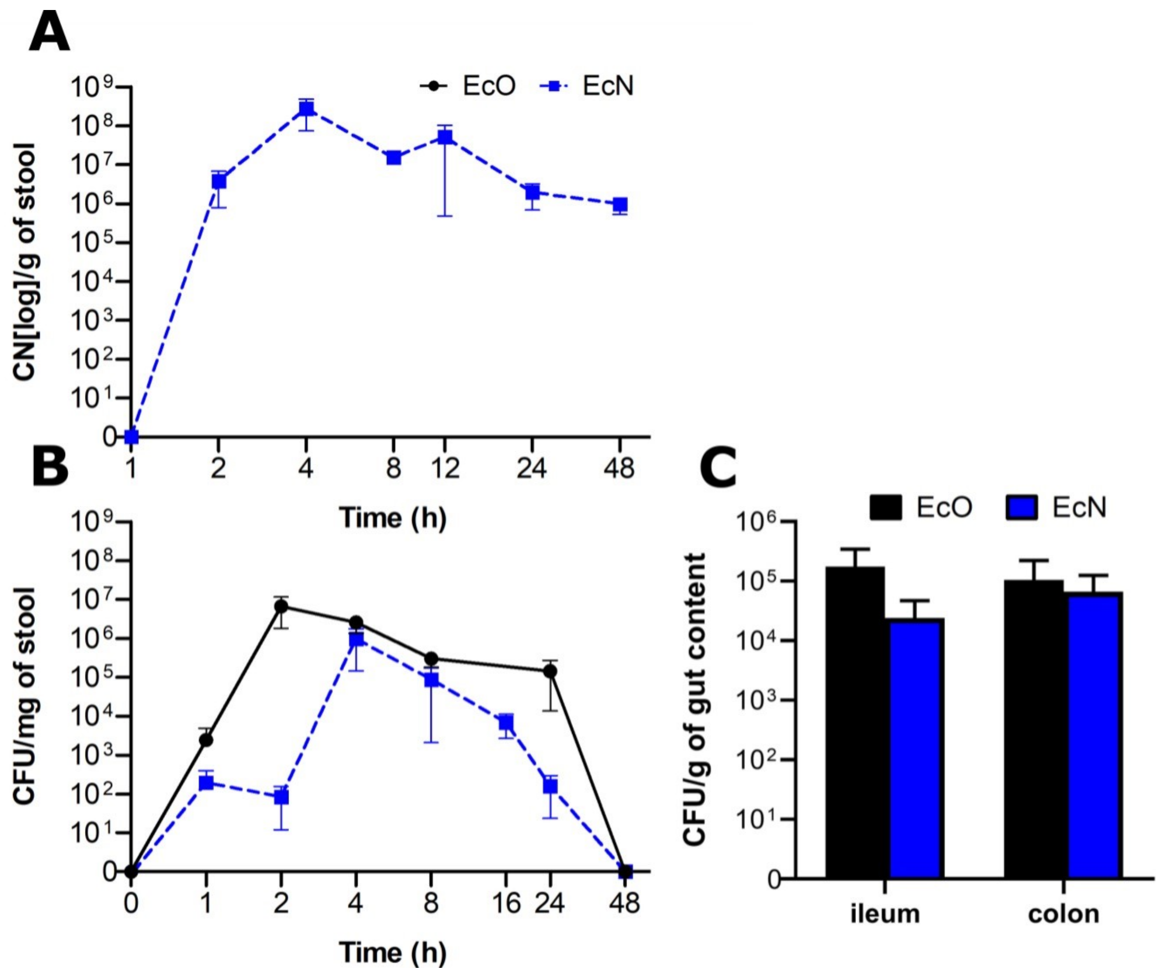

Figure 9. **EcN and EcO do not differ in the capacity to colonise C57BL/6J mice**. Colonization ability was measured by RT-PCR quantifying specific bacterial DNA in stool samples and intestinal contents collected from mice treated with one corresponding dose of 10^9 bacteria. (**A**) C57BL/6J mice obtained from the breeding colonies of the Institute of Microbiology of the Czech Academy of Sciences got transiently colonised upon a single dose of 10^9 probiotics. (**B**) In contrast, neither of the two microbes was detectable in stool of C57BL/6J mice housed at the animal facility at the Department of Pharmacology, First Faculty of Medicine, Charles University in Prague for longer than 48 hours. (**C**) The intestinal contents derived from EcN-treated mice housed at FM1 collected 48 hours after the probiotic dose, however, still contained detectable levels of the probiotic DNA. Each graph shows data from a single experiment (n = 4 (EcN) and 4 (EcO)).

4.3. Stimulation of Bone Marrow-Derived Macrophages

4.3.1. *Theory and Experimental Design*

Next, we wanted to assess how the microbes affect antigen-presenting cells. As mentioned above, data from the initial in vivo experiment indicate that the EcN-induced immune modulation affects antigen (IRBP) presentation.

Macrophages are candidates for an important cell type in this immune-regulatory process. They are antigen-presenting cells. They lay underneath the gut epithelium and in Peyer´s patches so they could be affected by probiotic IMMMs and they are one of the effector cells that mediate uveal inflammation in EAU. Changes in their characteristics are also suggested by results from the initial *in vivo* experiment. Anti-inflammatory response included decreased production of typical M1 macrophage cytokines in PPs like TNF and IL-1β. And a decreased proportion of proinflammatory M1 ($CD38^{+}$ $Egr2^{-}$) macrophages and activated $iNOS^{+}$ macrophages was detected in tissues isolated from EcN-treated mice compared to the placebo group. We thus focused our interest on macrophages in a series of experiments exploring the effects of each microbe in a simplified in vitro environment.

We produced equivalent stimuli of each probiotic. Microbes were cultured separately, lysed, lyophilised, weighted and diluted in a culture medium. Furthermore, to search for the nature of immunomodulatory molecule which mediates this mechanism a second stimulus was produced. This time a cultivation filtrate from each bacterium. Cultivation filtrate represents free microbial products released in the intestinal lumen whereas lysates represent more structural molecules that microbes consist of. A wide range of 4 different concentrations was used since it is unknown what concentration of specific antigens or IMMMs could reach macrophages in physiologic conditions. Filtrates were diluted to mirror the concentration of lysates. Thus if the lysate stimulus contained 100 lysed cells in 100 ul, filtrates would match this ration with 100 µl of media that originally contained 100 cells too.

We stimulated bone marrow derived macrophages (BMDMs) with lysates and filtrates derived from EcO and EcN. After 24 hours of stimulation, supernatants were collected and analysed by Griess Reaction and later by ELISA. Cells were collected too and underwent flow cytometry.

4.3.2. *Results*

During a reaction of a macrophage to a microbe, mediated via PRRs and MAMPs, macrophages are activated to effectively phagocyte and destroy the pathogen. These changes include production of reactive oxygen species, hydrolytic enzymes, inflammatory cytokines and nitric oxide (NO). BMDM, however, responded in these activation traits differently to EcO compared to EcN stimuli.

EcN lysate stimulated a lower production of NO compared to EcO lysate (**Figure 10A**). That is in agreement with data from our initial in vivo experiment in which EcN treatment significantly decreased proportion of NO synthase-expressing macrophages in mLN. Furthermore IL-1β production was also lower in response to EcN lysate stimulation (**Figure 10B**). Again, following the same traits seen in PPs derived from EcN-treated mice. Production of IL-10 remained with no differences among the bacteria (data not shown).

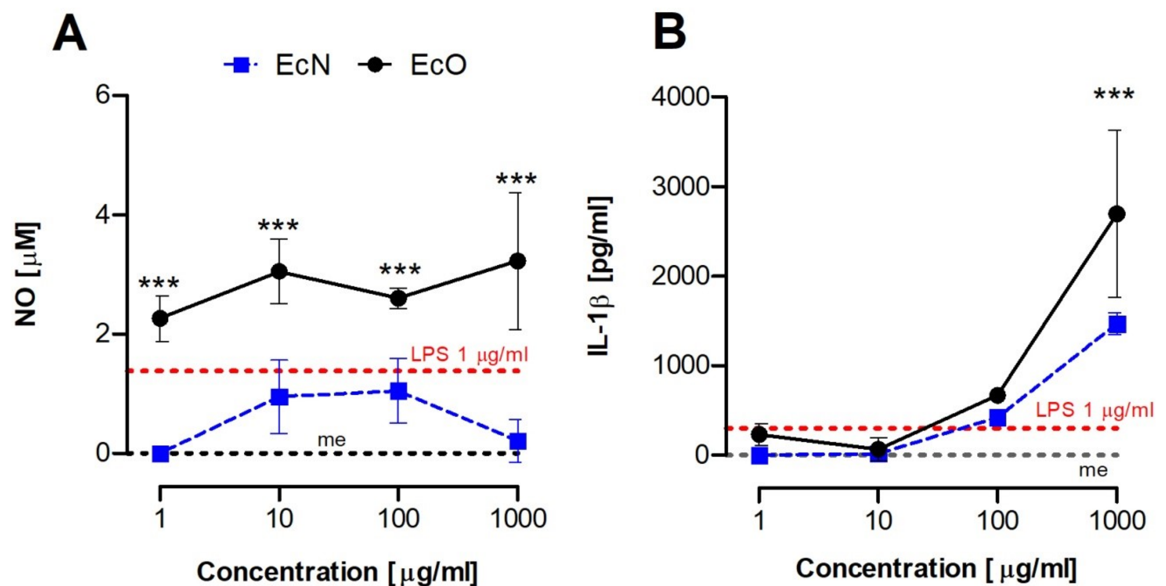

Figure 10. EcN lysates induce lower inflammatory response of BMDM compared to EcO lysates. (**A**) Molar production of NO in 100 μl of the culture supernatant was measured by Griess reaction. (**B**) Production of IL-1β was quantified by indirect ELISA. X axis shows concentration of the stimulus—the bacterial lysate. Red dotted line represents positive control — 1 μg/ml of LPS, black dotted line represents negative control—culture medium only. Figure shows representative graphs each from 1 out of 3 independent experiments. Differences were quantified by Two-way ANOVA with Bonferroni posttest; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

Next, we assessed the expression of several activation markers in reaction to the lysates by flow cytometry, mainly focusing on antigen presentation and co-stimulatory traits of BMDMs. While the expression of CD86 increased with increasing concentration of lysate stimuli, expression of CD40 decreased (**Figure 11**). CD38, CD80 and MHC II expression did not reveal any consistent concentration-dependent trends of response (**Figure 11, Supplementary Figure S4**). Compared to each other, the two stimuli, however, did not significantly differ in the expression they triggered.

Response to filtrates was inconsistent in all analyses (data not shown). Suggesting a lack of a robust metabolite, that could induce a concentration dependent response of BMDMs.

Collectively, the data indicate, that EcN induces a lower inflammatory response in macrophages compared to EcO. Two important activation traits are down-regulated after EcN lysate stimulation compared to EcO lysate. Presentation markers and co-stimulation membrane markers are, nonetheless, expressed no differently in reaction to the two microbial lysates.

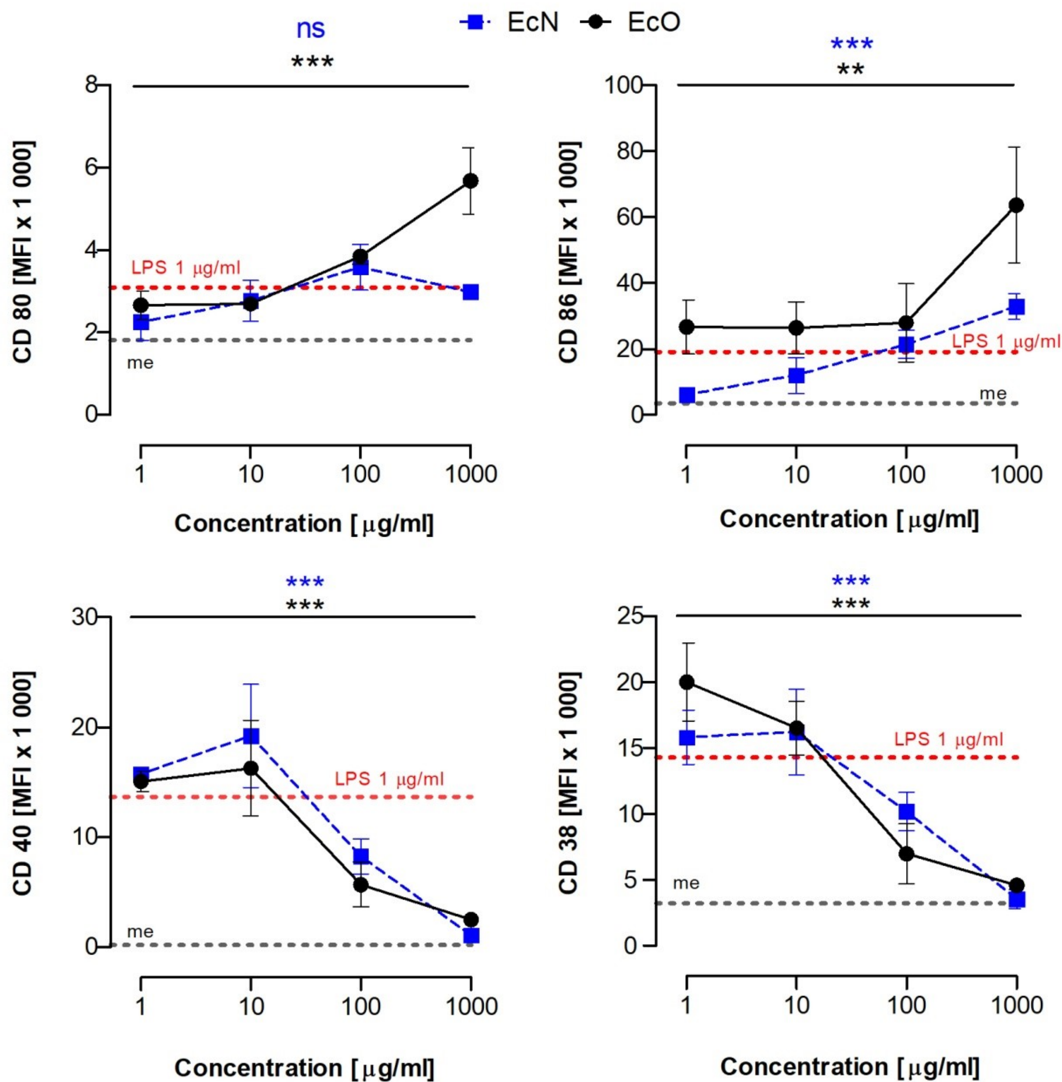

Figure 11. EcN and EcO lysates do not differ in the expression of activation markers they trigger in BMDMs. The expression does, however, follow concentration dependent trends in some cases. Data were obtained by FACS, gated to singlet, live, F4/80 positive cells and adjusted to mean fluorescence intensity (MFI) of each sample. X axis shows concentration of the stimulus—the bacterial lysate. Red dotted line represents positive control—1 μg/ml of LPS, black dotted line

represents negative control—culture medium only. Figure shows representative graphs each from 1 out of 3 independent experiments. Differences were quantified by One-way ANOVA with Tukey´s multiple comparison test; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

4.4. Stimulation of Bone Marrow-Derived Dendritic Cells

4.4.1. *Theory and Experimental Design*

To explore the immunological background of antigen presentation under the influence of EcO and EcN further, we, next, wanted to assess the response of dendritic cells (DCs). To better reflect physiological situation we first focused on dendritic cells in PPs and in LP, laying underneath the epithelium. We tried to develop a model of primary intestinal epithelial cells cultivated on a Transwell system. Epithelial cells could then be stimulated and pass on a signal to the studied dendritic cells in a co-culture experiment.

Primary cells were derived from 10-week old C57BL/6J mice intestines and cultivated for 14 days. To assess their maturation and growth we periodically measured their transepithelial resistance. After three attempts, cultivating both primary IECs and an epithelial cell line (MODE-K) we were, however, unable to detect values at a level of a confluent barrier (**Figure 12**).

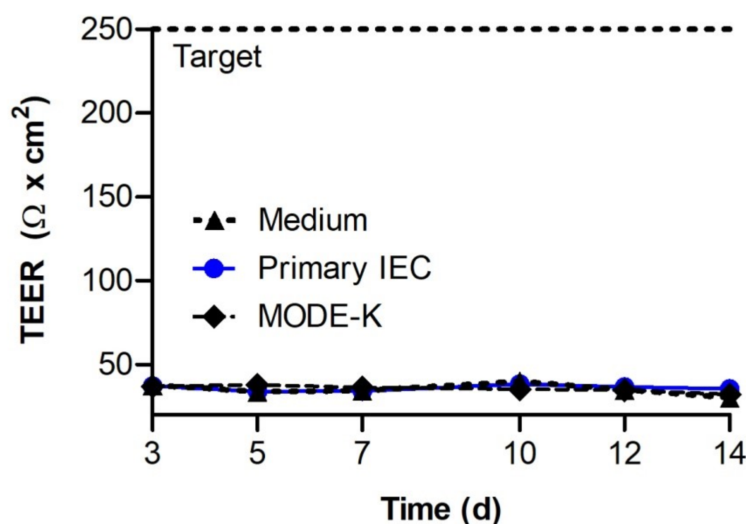

Figure 12. Primary IECs and MODE-K cells failed to form a confluent barrier. Cells were cultured for 14 days on transwell plates and periodically measured on transepithelial electrical resistance. Target was set to 250 $\Omega.cm^2$ representing a confluent intestinal epithelial barrier (Béduneau et al., 2014). Medium represents negative control. Data are pools from 3 (primary IEC) or 2 (MODE-K) independent experiments.

We thus instead focused on direct interaction of DCs with bacterial lysates, simulating the situation of snorkelling intestinal dendritic cells. Bone marrow derived dendritic cells (BMDDCs) were stimulated as BMDM were—with 4 concentrations of both EcO and EcN lysates. This time, however, no cultivation filtrates were applied (considering their inconsistent results in BMDM experiments). After 24-hour stimulation, cells were harvested for flow cytometry, supernatants were subsequently analysed for cytokine production quantification by ELISA.

4.4.2. *Results*

In flow cytometry we again focused on co-stimulatory and antigen-presentation markers. We generally encountered similar reaction as we did from BMDMs. While co-stimulatory CD80 and CD86 as well as MHCII increased their expression with increasing lysate concentration, CD40 significantly decreased (**Figure 13**). There were no major repetitive differences among the bacteria in these traits. However, CD103, a marker connected with migration to LN and Treg induction, was repeatedly expressed more in response to EcN lysate in comparison to EcO lysate in two out of four stimulus concentrations (**Figure 13**).

Next, we assessed production of two key cytokines which accompany T cell priming and determine its polarisation. IL-12 did not prove to be secreted differently in response to the two stimuli (data not shown). On the other hand, IL-10 was repeatedly produced significantly more upon EcN lysate stimulation compared to EcO in one or more stimuli concentrations (**Figure 14**).

These data collectively suggest, that there is a difference in the cytokines DCs produce and accompany their Ag presentation with in response to the two microbial lysates, but not in the expression of molecules which mediate the process of T cell priming (CD80, CD86, MHC II). Furthermore, increased CD103 expression could also take a part in the EcN-mediated immune modulation by enhancing migration to LNs and favouring anti-inflammatory priming.

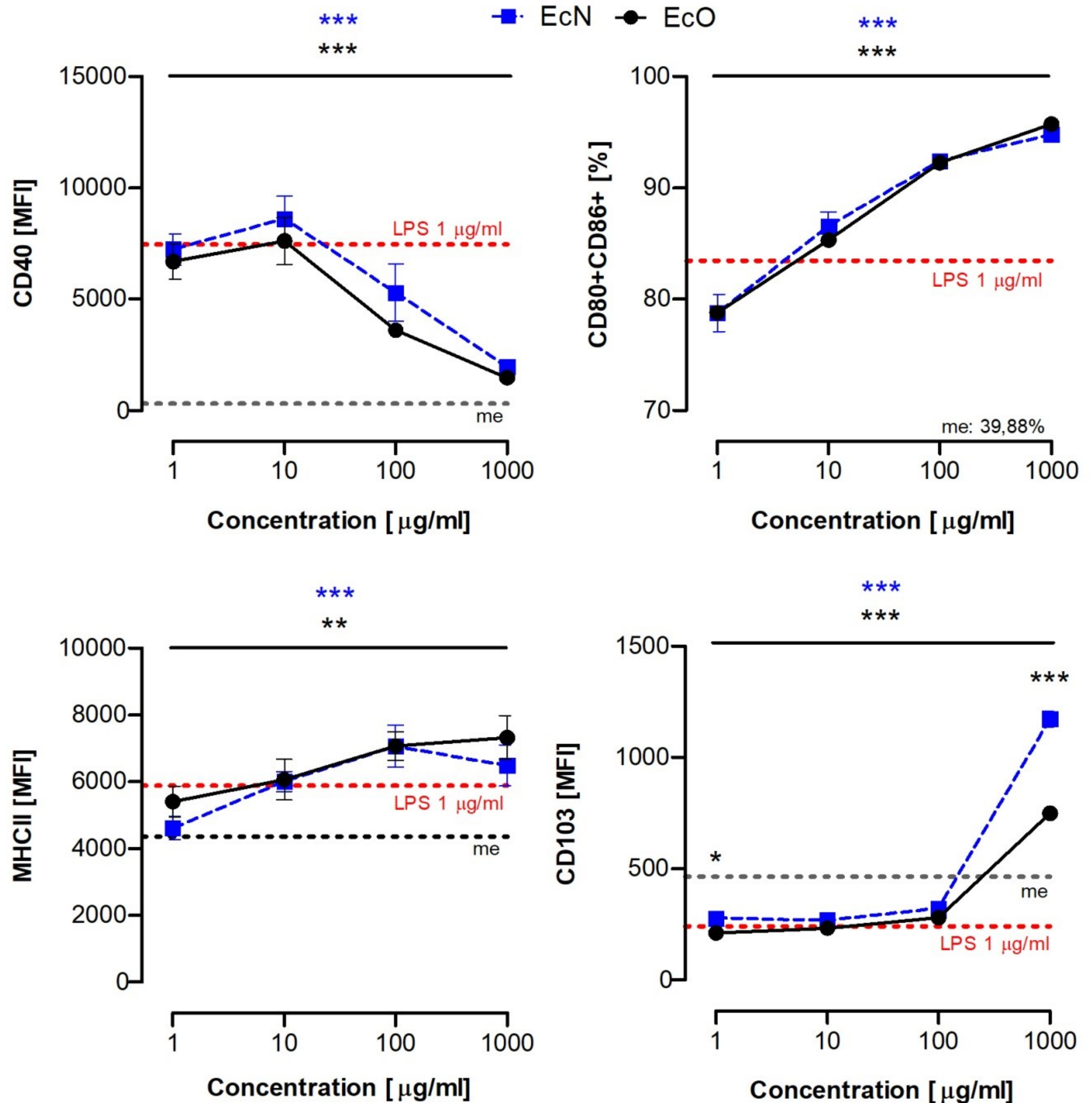

Figure 13. BMDDCs express more CD103 in response to EcN lysate compared to EcO lysate. Other assessed markers are expressed no differently among the two stimuli groups. Data were obtained by FACS, gated to singlet, live, CD11c positive cells and adjusted to mean fluorescence intensity (MFI) or positivity percentage of each sample. X axis shows concentration of the stimulus—the bacterial lysate. Red dotted line represents positive control—1 μg/ml of LPS, black dotted line or abbreviation "me" represents negative control—culture medium only. Figure shows representative graphs each from 1 out of 3 independent experiments. Differences in concentration were quantified by One-way ANOVA with Tukey´s multiple comparison test. Differences among bacteria were quantified by Two-way ANOVA with Bonferroni post-test; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

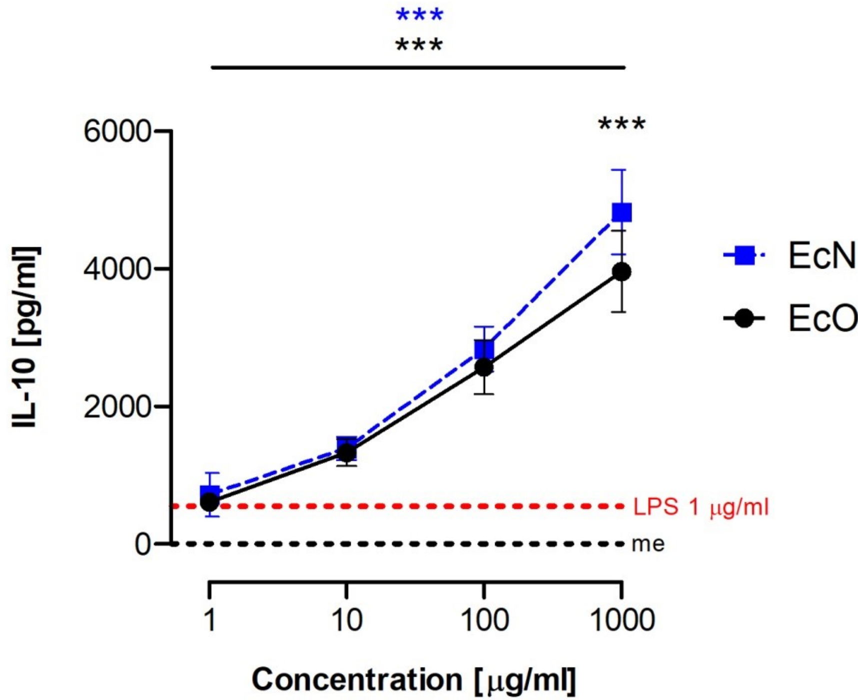

Figure 14. EcN lysate induces a higher production of an anti-inflammatory cytokine compared to an EcO lysate. Production of IL-1β was quantified by indirect ELISA. X axis shows concentration of the stimulus—the bacterial lysate. Red dotted line represents positive control—1 μg/ml of LPS, black dotted line —"me" abbreviation represents negative control—culture medium only. Differences in concentration were quantified by One-way ANOVA with Tukey´s multiple comparison test. Figure shows a representative graph from 1 out of 3 independent experiments. Differences among bacteria were quantified by Two-way ANOVA with Bonferroni post-test; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

5. Discussion

Initial experiment testing the immunomodulatory effect of *E. coli* in vivo revealed that oral treatment with EcN reduces the severity of EAU but treatment with EcO does not. Additionally, the results suggested that EcN realizes its immunomodulatory effects during antigen presentation as only prophylactic or early treatment was effective, antigen-independent anti-inflammatory effects were detectable mainly at the induction sites of the intestinal mucosal immune system, the abundance pro-inflammatory of antigen-presenting cell populations in GALT decreased and reactivity of $CD4^{+}$ T cells was altered specifically to the immunised retinal antigen outside the gut—i.e. at the site of immunization as early as day 7 post immunization. In a series of in vivo and in vitro experiments modelled on mice and mouse-derived cells, we aimed to describe the differences in the immunomodulatory effects of the two microbes. We found out that EcO and EcN do not differ in colonization capability but do differ in their capacities to provoke an immune response. In comparison to EcO, EcN induced lower activation of BMDMs demonstrated by decreased NO and IL-1β production. Furthermore, EcN-stimulated BMDDCs, compared to EcO-stimulated, exhibited significantly higher levels of traits inducing a tolerogenic response demonstrated by elevated IL-10 production and CD103 expression. This data suggest that macrophages and dendritic cells may be involved in the documented immunoregulatory process induced by EcN.

Both EcO and EcN are being successfully used in models of inflammatory diseases. However, their effectiveness is disease-dependent. EcO and EcN were previously shown to reduce the severity of DSS colitis (Kokešová et al., 2006; Rodríguez-Nogales et al., 2018). On the other hand, in EAU, EcO proved to be ineffective and only EcN reduced the clinical and histological scores of EAU significantly. This suggests that even closely related microbes induce very specific immune effects and their efficiency in treatment depends on the nature of the pathophysiology involved which cannot be broadened to inflammatory diseases in general.

The effectivity of the treatment was also dependent on the timing of probiotic intervention. Only prophylactic and early treatment by EcN were effective. Similar results have been reported in an EAE study where two oral doses of EcN before the induction of the disease decreased the disease severity later on (Libbey et al., 2020). This timing of treatment is particularly of note as it suggests the affected phase of the immune response—antigen presentation and T cell priming. Through this approach we can identify candidate cells and sites for mediation of the immunomodulatory effects—antigen presenting cells, immune

induction sites. This is supported by results from stimulation of $CD4^+$ T cells derived from LNs of EAU-diseased mice. While non-specific TCR stimulation (anti-CD3/anti-CD28) responsiveness or proportions of $CD4^+$ T cell populations were unaffected, specific stimulation with IRBP induced significantly lower inflammatory (IFN-γ and IL-17) response in the EcN-treated group compared to placebo, suggesting that the response to this antigen was primed differently. Interestingly, a different immune tuning was associated with the treatment of EAU with a mixture of *Lactobacilli*, *Bifidobacterium bifidum* and *Streptococcus thermophilus* which led to altered proportions of T cell populations in draining lymph nodes (J. Kim et al., 2017). This indicates that the same pathological mechanism can be approached from two different stand-points—two different probiotic-induced immunomodulatory mechanisms—and both can achieve amelioration of the disease. To assess the immune environment at the intestinal mucosal immune induction sites, we analysed the production of cytokines in PPs. The inflammatory tuning was significantly lowered in the EcN-treated group as demonstrated by reduced levels of IL-1β, TNF-α and IL-33. Furthermore, proportions of proinflammatory M1 macrophages in ileum and activated $iNOS^+$ macrophages in mLN were significantly decreased.

Collectively, the data from the initial in vivo experiment thus suggest that: first, EcO and EcN differ in their capacity to affect the immune system which determines their effectiveness in EAU treatment and second, that modulation of IRBP antigen-presentation may be an important aspect of the induced immune mechanism.

Therefore, we have assessed the abilities of each microbe to colonise their host. This would provide a simple explanation to EcO ineffectiveness—EcO would fail to provide a continuous feed of an immunomodulatory effect. However, the microbes have shown no differences in their colonization capacities as both sustained their presence in stool for roughly 48 hours. This suggests that the ineffectiveness of EcO treatment was not caused by inferior colonization abilities and animals from the initial in vivo experiment were under a continuous feed of signals from both microbes given that the treatment was administered every other day. 48 hours is a relatively standard time-period after one dose as was reported for common probiotics (Grimm, Radulovic, & Riedel, 2015). The stool shedding of supplemented microbes usually peaks at about 4 hours upon gavage and then gradually decreases dependent on the specific microbe and microbiota context (Zmora et al., 2018). Interestingly, supplemented microbes can colonise for the long-term as well. Human studies have documented persistence of *Bifidobacterium longum* in stool for over 6 months after an

oral supplementation. Thus, there are species which are able to prevail, if the context is favourable (Maldonado-Gómez et al., 2016).

In this study, the origin of the animal—the animal facility was indicated to impact the colonization. In the first trial experiment, mice housed at CAS showed persistent presence of EcN´s DNA in stool after 48 hours. In comparison, no presence of the two probiotics was detected in the stool of mice housed at FM1 after 48 hours. This indicates that colonization by these species may be microbiota-context dependent. The dependence of colonization on individual host factors is a well-known phenomenon. The success-rate of probiotic colonization is determined by hosts microbiota composition and factors like innate and adaptive immunity activation and differentiation, digestion and metabolism gene expression signature (Zmora et al., 2018). The microbiota dependence is in fact so pronounced that faecal transplantation from colonization-permissive or colonization-resistant individuals to GF mice determines the probiotic colonization rate. Furthermore, some evidence indicates that there are bacterial species which are more dependent on host´s unique characteristics. EcN´s flagellin is important for the colonization of the intestinal mucosa as it binds to human mucin. However, this interaction is missing in murine intestinal mucosa so the colonization might be more challenging (Troge et al., 2012). There are also other factors affecting this process. Colonization by EcO was significantly more successful in the group of breast milk-fed infants compared to formula-fed, so nutrition during treatment might be important as well (Slavíková et al., 1990). Some of these factors could cause the differed colonization-permissibility seen among the two animal facilities in our experiments.

The probiotic DNA was detectable for longer in the intestinal contents than in the stool samples. The limited reliability of stool for an accurate description of intestinal microbial colonization cannot be overlooked as almost 30% of the detected bacteria in stool occupies the gut significantly different. The target DNA concentration in stool samples might be under the detection limit while the microbes still remain present at low numbers in the intestine as was detected by the analysis of intestinal contents. This phenomenon–under-representation of bacterial species in stool compared to the intestine presence–was previously described and might be common (Zmora et al., 2018). There are more advanced techniques like red fluorescence protein Dsred2 labelling of target microbes which are more precise and reflect the path of the microbe through the gastrointestinal tract better which would be useful to definitely assess the colonization progress (Li et al., 2019). Interestingly, for the microbe to effectively induce immunomodulation of the host, colonization is not always necessary. Treatment with probiotic lysates—fragments of microbes incapable of colonization—can

induce immunomodulatory effects detectable weeks after treatment. For example, a treatment once a week for 1 month by *Lactobacillus casei* lysate protected mice from DSS colitis induced a week later. Also, the effects of a changed gut microenvironment and improved gut barrier function were detected two weeks later (Zakostelska et al., 2011).

Nonetheless, considering data from animals housed at CAS can only be viewed as preliminary, missing the EcO counterpart and having a different standard, more research needs to be made on this topic and the results may not be relevant to this study.

Next, we aimed to evaluate the effects of the two microbes on antigen-presenting cells. The first tested cell type were macrophages. Macrophages represent a promising candidate in the observed immune mechanism. They lie in the intestinal epithelium and in PPs providing them access to transported luminal antigens and IMMMs, they present antigens, while they are also the main effector cell type mediating the inflammation during EAU and the effects of EcN treatment in the initial in vivo experiment pointed to their alteration (reduced cytokines produced by macrophages and reduced counts of M1 and activated iNOS^{+} macrophages in GALT tissues). We have thus stimulated BMDMs directly with lysates and cultivation filtrates derived from each microbe and analysed their response by evaluating activation and antigen-presentation traits. The response differed significantly among the microbes. Lysates derived from EcN induced lower NO and IL-1β production compared to EcO lysates. This is in agreement with data from the initial experiment where the levels of IL-1β in PPs were significantly reduced upon EcN treatment compared to EcO. Decreased NO production also interestingly mirrored the lowered proportion iNOS^{+} macrophages detected in mLNs of EcN treated mice. There were thus some interesting traits of reaction translated from the in vivo experiments suggesting that macrophages could be among the cells responsible for the altered microenvironment at the induction sites observed in the initial in vivo experiment. Similar results of (lowered IL-1β production) were observed also in colonic tissue of mice in a study of DSS colitis treated with EcN, suggesting that lowered production of IL-1β could be among the general anti-inflammatory mechanisms EcN mediates its protection against inflammatory diseases with. Antigen presentation and co-stimulatory markers (MHCII, CD80, CD80, CD40) evaluated by FACS did not differ between the two microbial stimuli. However, since IL-1β was decreased in the EcN-stimulated cells and has a critical impact on antigen priming, the antigen presentation may be affected nonetheless.

Stimulation with cultivation filtrates was unsuccessful as it failed to induce a robust response of macrophages and results were inconclusive. This indicates that neither EcO nor EcN produce a soluble IMMM which would be actively produced into the environment and

could induce a concentration-dependent response of macrophages. Some of the immunomodulatory effects of EcN have, however, been documented to be induced by supernatant (cultivation filtrate) only. The induction of human beta defensine-2 production by Caco-2 cells was more effective through stimulation by EcN cultivation filtrate than by the cell pellet. This indicates that there may be more IMMMs produced by EcN as there are by B. fragilis for instance. Deferent IMMMs could be released into the environment variably and could target various cell types and immune processes.

Next, we examined the reaction of the main antigen-presenting cell type—dendritic cells. Using the same methodology, we stimulated BMDDCs with EcN and EcO-derived lysates. Indeed we observed differences in the response. While co-stimulatory markers and antigen presentation markers (CD40, CD80, CD86, MHCII) matched the reaction of BMDMs with no differences among the microbial stimuli, CD103 was significantly more expressed in response to EcN lysate. Furthermore, BMDDCs also reacted to EcN lysate with significantly higher production of IL-10 in comparison to EcO lysate. CD103 is an integrin typical for migrating mucosal DCs. $CD103^+$ DCs are associated with regulatory effects as they were reported to preferentially induce regulatory T cells and conversely induce significantly less IFN-γ-producing Th1 cells than their $CD103^-$ counterparts (Annacker et al., 2005; Coombes et al., 2007). This mechanism might be also supported by the elevated IL-10 production, DCs could accompany the antigen-presentation and T cell priming with. A decrease in Th1 cytokine IFN-γ response mediated by $CD103^+$ DCs priming is of interest as it affects the main population mediating EAU inflammation. A decreased IFN-γ-producing $CD4^+$ T cell proportion was also one of the few effects found on T cells derived from EcN-treated mice. $CD103^+$ DCs were, interestingly, also documented to be the main cell type that receives luminal antigens from goblet cells—a newly proposed antigen-transporting intestinal cell type (McDole et al., 2012). Such DCs then migrate to LNs and induce Treg polarisation. Notably, $CD103^+$ DC were isolated even from cLNs of *B. fragilis*-treated mice. These DCs were shown to be the most potent Treg inducers when compared to the $CD103^-$ and *B. fragilis* untreated counterparts (Ochoa-Repáraz et al., 2010). This suggests that probiotic treatment may propagate its effects through $CD103^+$ DCs even on the peripheries, outside the intestine. Another mechanism which could contribute to the effects is the decreased expression of CD40 in response to increasing concentrations of the lysates. It was recently documented that CD40 signalling abrogates the induction of Tregs by $CD103^+$ DCs. Constitutive CD40 signalling reduced $CD103^+$ DC numbers and induced colitis (Barthels et al., 2017). Thus a reduced signalling mediated by the downregulation of CD40 expression could be an important

mechanism involved. Collectively the data from the in vitro stimulation of bone-marrow derived cells suggest that EcN, compared to EcO, induces a reduction of inflammatory tuning in macrophages and an enhancement of anti-inflammatory tuning in dendritic cells. Macrophages represent a tissue resident cell type with low migratory abilities, so we can speculate they might be more responsible for the reduced inflammatory tuning at the induction sites, while dendritic cells with high migratory activities could be more responsible for the propagation of the anti-inflammatory effects.

The EcN-mediated reduced severity of EAU is indisputably a complex immune process. While the immunisation and priming to the ocular antigen happens in iLNs, the treatment is received through the intestinal mucosal immune system. Furthermore, the inflammation is located outside these tissues in an originally immune-privileged organ—the eye. To speculate about the possible underlying mechanism, EcN might reduce the activation of intestinal mucosal macrophages and tolerogenically imprint DCs. That would lead to a decreased IL-1β production in PPs and either a decreased or a tolerogenic antigen presentation. These effects could spread systematically, affect IRBP presentation, decrease IRBP-specific response and ultimately reduce EAU severity. Although most DCs migrate from the intestinal mucosa to mLNs, as already indicated, there are documented tolerogenic DCs in peripheral LNs induced by oral probiotic treatment too (Ochoa-Repáraz et al., 2010). Thus, it could be that when the immunisation to IRBP occurs, there are EcN-induced tolerogenic DCs in the iLNs creating a microenvironment which primes the response to IRBP to be lower and more tolerogenic through a bystander effect. This notion is supported by the observed localisation and timing of reduced IRBP-specific $CD4^+$ T cell response. These cells occur first in iLNs—at the site of immunisation on d7 post-immunisation (before EAU manifestation) and propagate to other mLN and cLN later (d28). Thus, tolerogenic imprinting of effector T cells happens at the site of immunisation. Considering that the initial effects on d7 also include established anti-inflammatory environment at the induction sites, it may be the innate immune cells, to propagate the tolerogenic effect, which is followed by altered priming and later also systemic migration of less responsive IRBP-specific T cells.

Alternatively, EcN could also mediate the effects through strengthening of barrier integrity—a well-described phenomenon induced on the epithelial intestinal cells (Guo et al., 2019). A reduced translocation of microbes in the intestine could generally set the IS to be more tolerogenic. However, we could also speculate that EcN might propagate its barrier supportive effects indirectly to other barriers such as the hemato-ocular barrier. Since the integrity of this barrier is a crucial aspect, protecting the uvea from inflammation, its

fortification might be a robust defence. A supporting mechanism involved could also be mediated by antagonistic abilities of EcN. Through a reduction of deleterious stimuli from pathogenic microbes in the intestinal tract, EcN might aid to induce a preventive setting of intestinal microbiota. Our research is not good enough to definitely confirm what effects are responsible for the reduction of EAU severity by EcN. It has, however, pointed to promising candidates.

6. Conclusions

Intestinal microbiota represents a promising target for the therapy of autoimmune and inflammatory diseases. While dysbiotic microbiota affects host deleteriously, healthy microbiota and specific microbes can provide beneficial effects and ameliorate the severity of inflammatory diseases. Microbiota´s plasticity allows us to manipulate the composition through nutrition, prebiotics, probiotics, antibiotics or faecal microbiota transplantation and shape the microbial signals to be favourable. EcN has been clinically used for over a hundred years for its beneficial effects blocking intestinal infections, supporting intestinal barrier function or providing anti-inflammatory tuning of the IS. Similarly, EcO has been used for its immunomodulatory effects preventing infections and providing a balancing IS tuning, preventing allergies and inflammatory diseases.

In our present study, EcN reduced the severity of an extra-intestinal inflammatory disease—EAU—but EcO did not. The EcN treatment was characterised by a reduced inflammatory tuning in PPs—involving mainly typical macrophage cytokines, a decreased proportion of M1 macrophages and activated iNOS^{+} macrophages in ileum and mLN respectively, a reduced responsiveness of T helper cells to IRBP antigen (first occurring at the site of immunisation) and no changes in non-specific TCR stimulation responsiveness or in proportions of Treg, Th17 or ILC3 populations in cLN or mLN. The two probiotics did not differ in their capacity to colonise mice, thus the ineffectiveness of EcO was not caused by a reduced immunomodulatory feed provided by EcO. However, the microbes differed in the response of antigen-presenting cells they triggered. In reaction to EcN lysate, compared to EcO, BMDMs have decreased their inflammatory tuning as demonstrated by a reduced production of IL-1β and NO, while BMDDCs, on the other hand, have increased their anti-inflammatory traits by elevated IL-10 production and increased CD103 expression.

Our study provides the first data documenting a reduction of EAU severity by EcN treatment. This thesis has identified several differences in the immune effects of EcO and EcN which could impact their effectiveness in treatment of EAU. More research needs to be made on this topic to ultimately define the immune mediators responsible for the beneficial effects of EcN in treatment of EAU. Nonetheless, EcN continues to be among the most promising probiotics with an increasingly wide array of clinical use potential.

7. References

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8. Supplementary Materials

Target	Forward primer (5’->3’)	Reverse primer (5’->3’)
EcN	AACTGTGAAGCGATGAACCC	GGACTGTTCAGAGAGCTATC
EcO	CTAATGCGGGCAGAGAAATAAAGT	ATAAAGACGGCAGGGTAACACAC

Table S1. List of used primers.

References**:** (Blum-Oehler et al., 2003; Hejnova et al., 2005)**.**

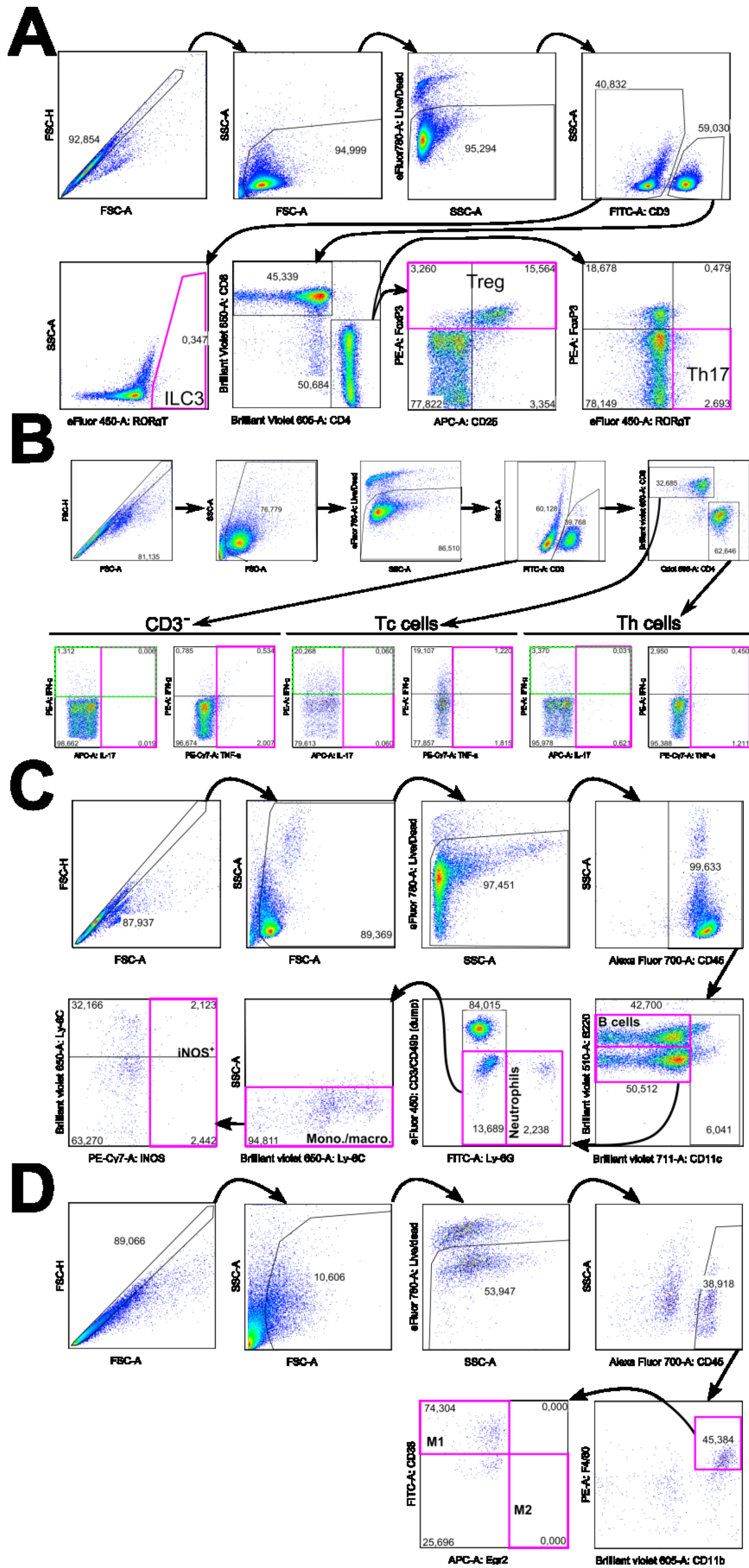
Epitope — Fluorochrome	Clone	Manufacturer	Cat#
CD3ε (purified)	145-2C11	BioLegend	100359
CD28 (purified)	37.51	BioLegend	102121
CD16/32 (purified)	93	BioLegend	101302
Fixable Viability Dye eFluor 780[1, 2, 3, 4,6]	-	Thermo Fisher Scientific	65-0865-14
Fixable Viability Dye eFluor 450[5]	-	Thermo Fisher Scientific	65-0863
CD3 — eFluor 450[3]	17A2	Thermo Fisher Scientific	48-0032-82
CD3ε — FITC[1, 2]	145-2C11	BioLegend	100306
CD4 — Brilliant violet 605[1, 2]	GK1.5	BioLegend	100451
CD8 — Brilliant violet 650[1, 2]	53-6.7	BioLegend	100741
CD11b — Brilliant violet 605[4,6]	M1/70	BioLegend	1106285
CD11c — Brilliant violet 711[3,6]	N418	BioLegend	117349
CD14 - APC[5]	Sa2-8	Thermo Fisher Scientific	17-0141-81
CD25 — APC[1]	PC61.5	Thermo Fisher Scientific	17-0251-82
CD38 — FITC[4,5]	90	BioLegend	102705
CD40 — PE[3,5,6]	1C10	Thermo Fisher Scientific	12-0401-82
CD45 — Alexa Fluor 700[3, 4]	30-F11	BioLegend	103128
CD80 - PerCP/Cyanine5.5[6]	16-10A1	BioLegend	104722
CD80 - Brilliant violet 711[5]	16-10A1	BD Biosciences	740698
CD86 - Brilliant violet 605[5]	GL1	BioLegend	105037
CD86 - FITC[6]	GL1	Thermo Fisher Scientific	11-0862-81
B220 (CD45R) — Brilliant violet 510[3]	RA3-6B2	BioLegend	103247
CD49b — eFluor 450[3]	DX5	Thermo Fisher Scientific	48-5971-82
I-A/I-E - Alexa Fluor 700[5,6]	M5/114.15.2	Thermo Fisher Scientific	56-5321-82
CD103 - eFluor 450[6]	2E7	Thermo Fisher Scientific	48-1031-82
Egr2 — APC[4]	erongr2	Thermo Fisher Scientific	17-6691-82
F4/80 — PE[4]	BM8	BioLegend	123110
F4/80 - APC[6]	BM8	Thermo Fisher Scientific	17-4801-82
Foxp3 — PE[1]	FJK-16s	Thermo Fisher Scientific	12-5773-82
IFN-γ — PE[1, 2]	XMG1.2	Thermo Fisher Scientific	12-7311-81
IL-17A — APC[1, 2]	eBio17B7	Thermo Fisher Scientific	17-7177-81

iNOS — PE-Cyanine7[3]	CXNFT	Thermo Fisher Scientific	25-5920-80
Ly-6C — Brilliant violet 605[3]	HK1.4	BioLegend	128036
Ly-6G — FITC[3]	1A8	BioLegend	127605
Rorγt — Brilliant violet 421[1]	Q31-378	BD Biosciences	562894
TNF-α— PE-Cyanine7[1, 2]	TN3-19.12	Thermo Fisher Scientific	25-7423-82

Table S2. List of used antibodies. Used to analyze: [1] regulatory T cells, Th17 and ILC3, [2] cytokine production by T cells, [3] monocytes and macrophages, [4] M1/M2 monocytes and macrophages, [5] bone marrow derived macrophages or [6] bone marrow derived dendritic cells.

Task
Assistance with experiment termination (tissue processing, cell counting etc.)
Derivation of leukocytes from colon samples
FACS preparation
Colonization assessment (sample collection, DNA isolation, RT-PCR, analysis)
Stimulation of BMDMs (stimuli preparation, cell derivation, cultivation, ELISA)

Table S3. Contributions to the research: (Dusek et al., 2020).


A
B
C
D
ILC3
Treg
Th17
CD3⁻
Tc cells
Th cells
iNOS⁺
Mono./macro.
Neutrophils
B cells
M1
M2

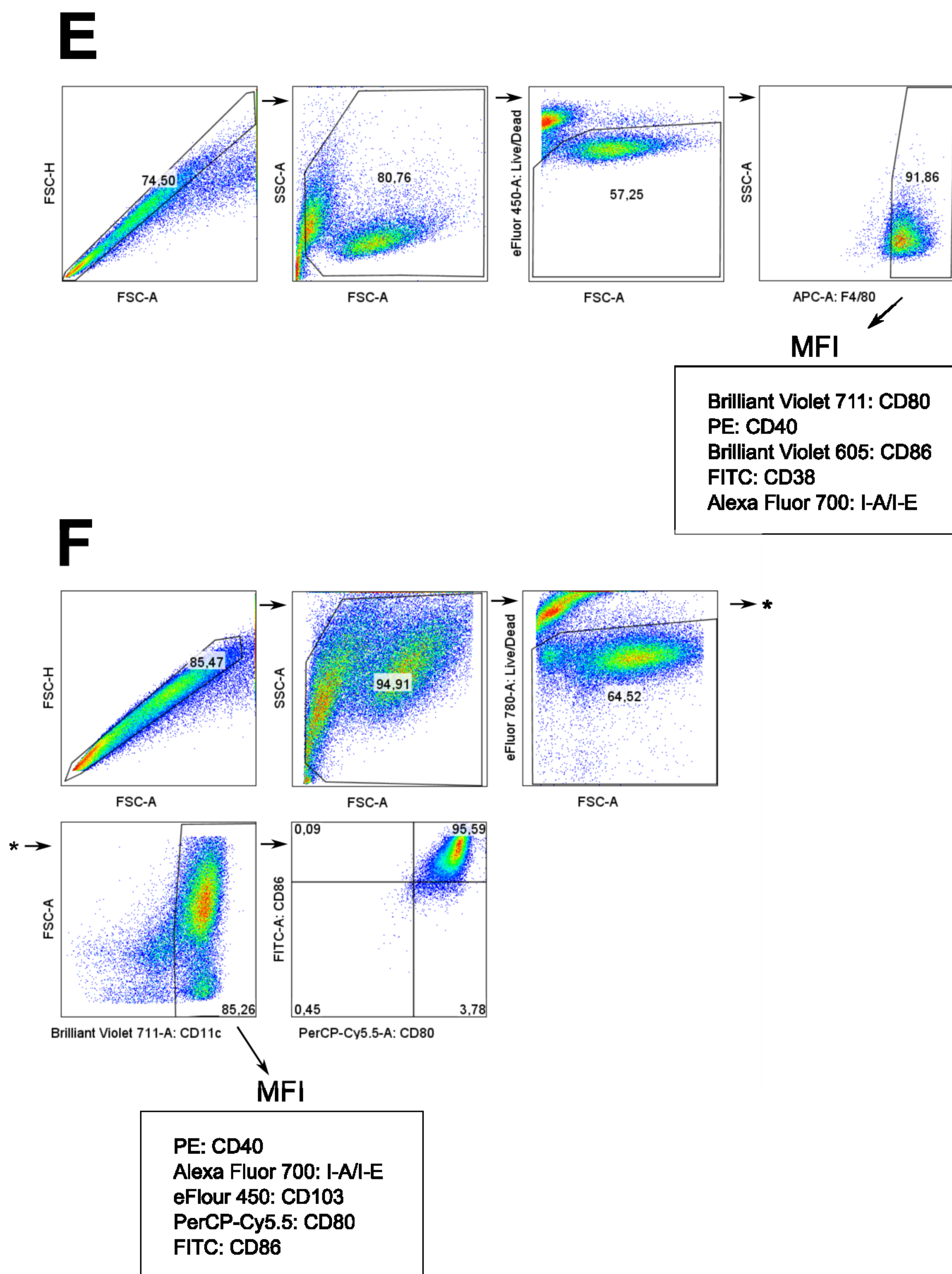


Figure S1. Gating strategies for regulatory T cells, Th17 and ILC3 **(A)**, cytokine production by T cells **(B)** and monocytes, macrophages and neutrophils **(C)** using mesenteric lymph nodes (mLN) of placebo-treated C57BL/6J mouse suffering from EAU. Gating strategy for M1/M2 macrophages using cells isolated from ileum of healthy placebo-treated C57BL/6J mouse **(D)**. Gating strategies for BMDMs **(E)** and BMDDCs **(F)**.

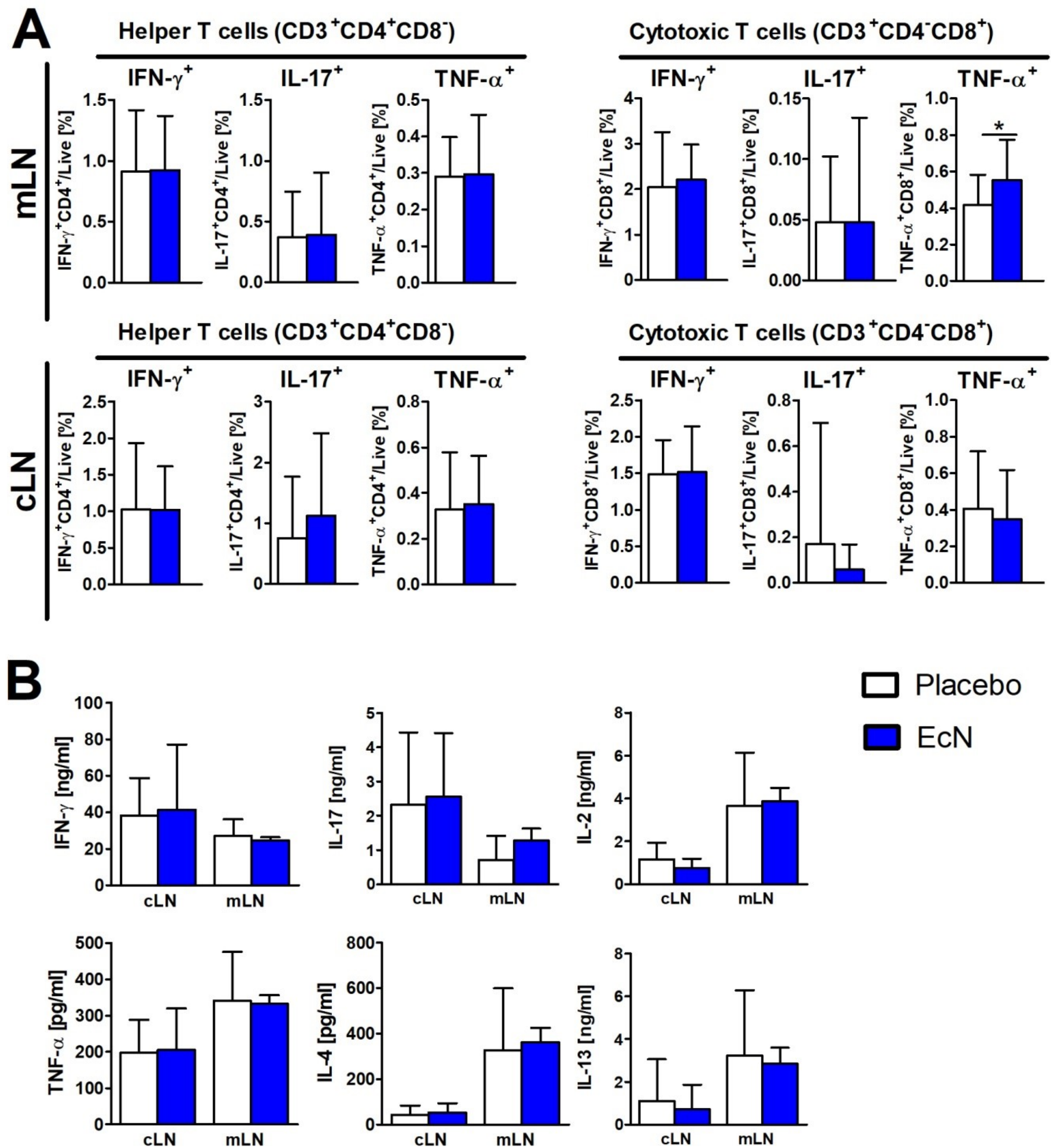


Figure S2. Responsiveness of T cells to anti-CD3/anti-CD28 stimulus remains unchanged by EcN treatment. Response was evaluated by intracellular expression of proinflammatory cytokines of T cells derived from cLNs and mLNs assessed by FACS (**A**) and production of cytokines by activated T cells derived from cLN and mLN assesed by ELISA (**B**). Data are pools from 5 independent experiments (n = 24 per group in total). Differences were quantified by unpaired Mann–Whitney test; *$p<0.05$. Data are from day 28 post-induction.

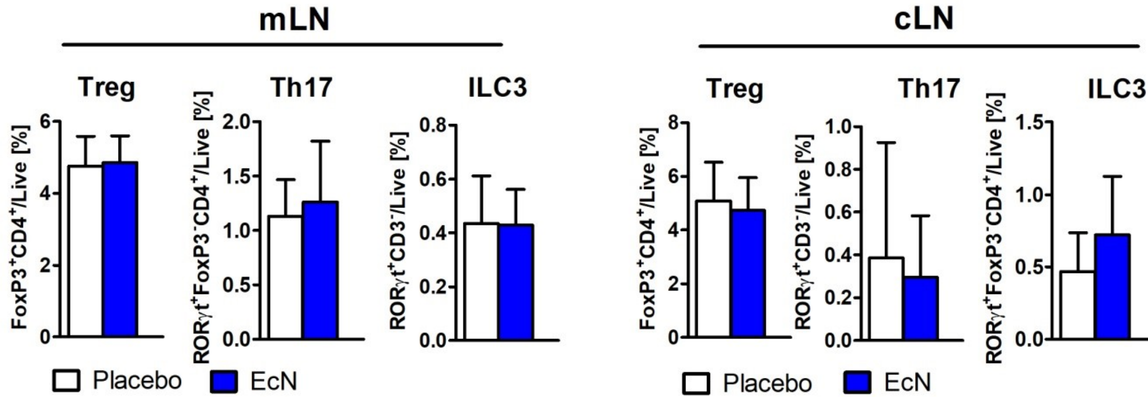


Figure S3. Treatment of EAU with EcN does not affect proportions of Treg, Th17 and ILC3 populations in mLNs or cLNs. Proportions of cells in mesenteric and cervical lymph nodes were evaluated by FACS at day 28 post-induction (n = 22 (placebo), 23 (EcN) in total).

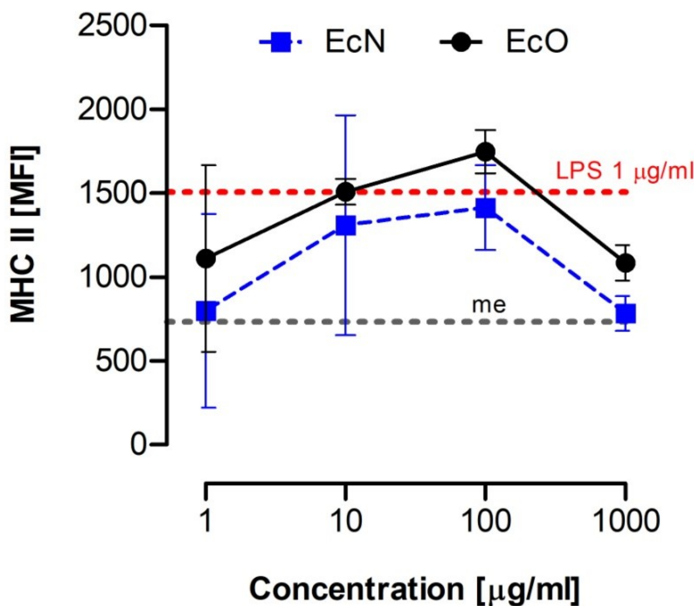


Figure S4. There are no significant differences in the expression of MHC II by BMDM in reaction to the two lysates. Data were obtained by FACS, gated to singlet, live, F4/80 positive cells and adjusted to mean fluorescence intensity (MFI) of each sample. X axis shows concentration of the stimulus—the bacterial lysate. Red dotted line represents positive control — 1 µg/ml of LPS, black dotted line represents negative control—culture medium only. Figure shows representative graph from 1 out of 3 independent experiments. Differences were quantified by One-way ANOVA with Tukey´s multiple comparison test; * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.