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Exploiting a multi-omic data integration approach to assess the role of a gut related immune modulation in multiple sclerosis

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All the results presented here were obtained by myself, except for:

1) ***Whole Genome Sequencing for human microbiota characterization and statistical analysis on microbiota profiles***:

   Sequencing, pre-processing of shotgun metagenomic reads and taxonomic profiling of metagenomes (Results, Human Studies, subparagraph Impairments of BAM-producing bacteria in RRMS patients) were performed in collaboration with Prof. Nicola Segata, Computational Metagenomics Lab, Centre for Integrative Biology, University of Trento, Trento, Italy. Statistical analysis on microbiota profiles were performed in collaboration with Dr. Luca Massimino and Dr. Federica Ungaro, Experimental Gastroenterology Unit, IRCCS Ospedale San Raffaele, Milan, Italy.

2) ***Human bile acids metabolomic profiling via Ultra High Pressure Liquid Chromatography – Mass Spectrometry:***

   Samples preparation, chromatographic separation, and spectra acquisition (Results, Human Studies, subparagraph Defective BAMs production in RRMS patients) were performed by Dr. Annapaola Andolfo, Proteomics and Metabolomics Facility, Center of Omics Sciences, IRCCS Ospedale San Raffaele, Milan, Italy.

All sources of information are acknowledged by means of reference.

ABSTRACT

The gut microbiota and microbial metabolites play a pivotal role in modulating extra-intestinal autoimmune diseases like multiple sclerosis (MS). Changes in the gut microbiota composition and gut mucosal immunity, i.e., high frequency of intestinal Th17 cells, are detected both in preclinical models of MS and in relapsing remitting MS (RR)MS patients and correlate with disease activity. The microbiota-induced metabolic profile is crucial to modulate immunity at the intestinal and systemic level. Fox example, secondary bile acid metabolites (BAMs) released by gut microbial species are key regulators that modify Teff/Treg cell ratio by increasing $Foxp3^+$ Treg cells and limiting effector Th17 cells. Here we tested the beneficial effect of BAMs in modulating Central Nervous System (CNS) autoimmunity in the Experimental Autoimmune Encephalomyelitis (EAE) preclinical model and analysed the presence of secondary BAMs together with BAM-producing bacterial species in RRMS patients and healthy controls and correlate with the immunological profile. Oral administration of secondary deoxycholic acid (DCA) and lithocholic acid (LCA) significantly ameliorated clinical and neuropathological signs of EAE. Also, BAM-treated EAE mice showed decreased Th17 cell frequency and increased percentages of $Foxp3^+$ Treg cells and IL-10 secreting Tr1 cells in the gut but also in draining lymph nodes (DLN) and CNS. Notably, our data suggest that BAM-modulated Treg cells travel from the gut to DLN where they regulate the functional phenotype and dampen the differentiation of MOG-reactive Th17 cells. In support of the key role of BAMs in the suppression of CNS autoimmunity, our metabolomic analysis revealed a significantly decreased concentration of the highly immunoregulatory DCA in the intestine of RRMS patients, associated with a reduced abundance of BAM-producing bacteria. Those alterations were associated with increased percentages of circulating Th17 cells in RRMS patients. Our data suggest that secondary BAMs play a central role in the prevention of CNS autoimmunity both in humans and preclinical models.

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ACRONYMS AND ABBREVIATIONS

MS: Multiple Sclerosis

RR: Relapsing remitting

RRMS: Relapsing remitting multiple sclerosis

BAMs: Bile acid metabolite(s)

CNS: Central Nervous System

EAE: Experimental Autoimmune Encephalomyelitis

DCA: Deoxycholic acid

LCA: Lithocholic acid

DLN: Draining lymph nodes

IECs: Intestinal epithelial cell(s)

DCs: Dendritic cell(s)

APCs: Antigen-presenting cell(s)

PRRs: Pattern recognition receptor(s)

TLRs: Toll-like receptor(s)

GB: Gut barrier

GF: Germ-free

GALT: Gut-associated lymphoid tissues

PPs: Payer's patches

AMPs: Antimicrobial peptide(s)

SFB: Segmented filamentous bacteria

TGF-β: Transforming growth factor-β

PSA: polysaccharide A

Tr1: Type 1 regulatory T (cells)

SCFAs: Short Chain Fatty Acid(s)

HDACs: Histone deacetylase(s)

GPCRs: G protein-coupled receptor(s)

NF-kB: Nuclear factor KB

TNF: Tumor Necrosis Factor

TJs: Tight junction(s)

AHR: Aryl Hydrocarbon Receptor

PXR: Pregnane X Receptor

CA: Cholic Acid

CDCA: Chenodeoxycholic Acid

BSH: Bile salt hydrolase

FXR: Farnesoid X receptor

TGR5: Takeda G-protein receptor 5

VDR: Vitamin D receptor

ZO-1: Zonulin 1

IBD: Inflammatory Bowel Diseases

IEB: Intestinal epithelial barrier

GVB: Gut vascular barrier

AJs: Adherent junction(s)

RA: Rheumatoid Arthritis

T1D: Type 1 Diabetes

BBB: Blood brain barrier

GM-CSF: Granulocyte-macrophage colony stimulating factor

SPF: Specific pathogen free

MOG: Myelin oligodendrocyte glycoprotein

TUDCA: Tauroursodeoxycholic acid

JAM-A: Junctional adhesion molecule A

GGT: Gamma-glutamyl transferase

AST: Aspartate aminotransferase

ALT: Alanine transaminase

WGS: Whole Genome Sequencing

PBMCs: peripheral blood mononuclear cell(s)

MAIT: Mucosal-associated invariant T

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INTRODUCTION

1. THE GUT MICROBIOTA AND THE INTESTINAL IMMUNE SYSTEM

Mucosal surfaces, such as the one lining the human gastrointestinal tract, represent physical and functional barriers which isolate the internal host milieu from the external environment. Being continuously exposed to foreign stimuli, such as pathogens entering the body and immunomodulatory agents deriving from the diet and the commensal microbiota, the intestinal mucosa is equipped with structural and biological barriers in addition to a large and well-structured mucosal immune system. The surface area is composed of a single layer of columnar intestinal epithelial cells (IECs) together with antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages that are crucial to detect danger signals and to build appropriate adaptive immune responses as well as preventing unnecessary immunity against food and commensal bacteria. These sensing abilities are exemplified by their expression of a broad range of pattern recognition receptors (PRRs) which include Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Iwasaki & Medzhitov, 2004)(Williams *et al*., 2010)(Maloy & Powrie, 2011). The capacity of the gut mucosal immunity to finely discriminate the invasive organisms and the harmless antigens which enter in contact with, simultaneously determinates the host's defence as well as the maintenance of the immune tolerance which is crucial to guarantee the immune homeostasis at intestinal and systemic level. Thus, intestinal mucosal surfaces are constantly surveilled by T effector and T regulatory cells which respectively defend the host from pathogens and limit overwhelming effector T cell responses. In the intestine, a proper balance between immune response and tolerance is normally maintained by driving the differentiation of effector or regulatory T cells from naïve-T cells, but also by recruiting already differentiated Treg or T eff cells into the gut mucosa, or reprogramming differentiated Treg or Teff cells towards different lineages (Littman & Rudensky, 2010)(Brucklacher-Waldert *et al*., 2014). The terminal differentiation and/or modulation of the functional phenotype of T cells depend on key mediators, including immune differentiating factors and cytokines, which are in large part produced in response to

immunostimulatory signals provided by the gut microbiota. Hence, the gut microbial community exerts a pivotal role in regulating the development and function of the mucosal immune system by establishing a complex and dynamic interaction which is finely and reciprocally governed at the gut barrier (GB) level in this "three-dimensional interplay". Indeed, while the enteric commensals affect both the mucosal immune response and the GB integrity, the immune system prevents bacterial translocation across the GB which in turn, not only limits the interaction between the gut mucosa and luminal content, but actively contributes to determine the functional phenotype of T cells. The GB performs this action by providing immunostimulatory signals which are necessary to induce immune tolerance, avoiding inflammation and orchestrating an appropriate immune response against lumen pathogens and commensal bacteria (**Figure 1**).

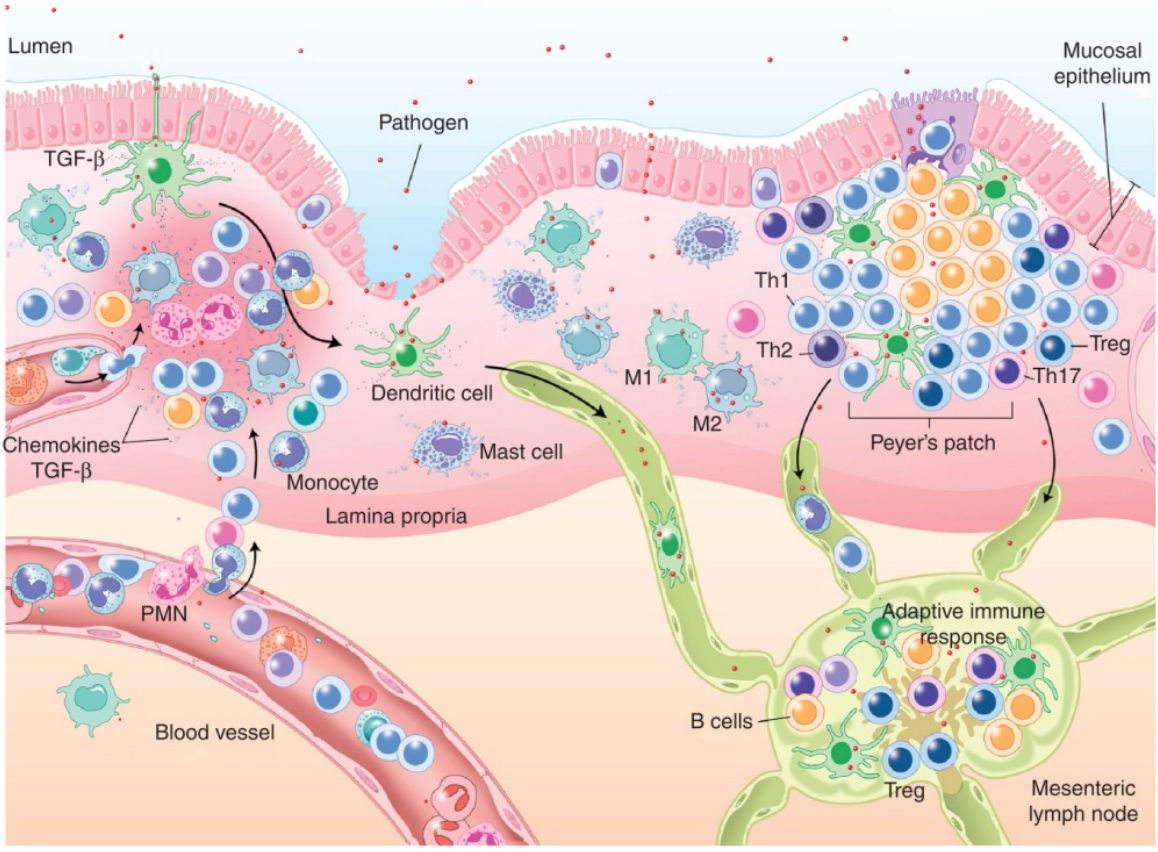


Figure 1. Mucosal exposure to pathogens and host response.
Schematic representation of the intestinal immune cell compartments under homeostatic and inflammatory conditions (P. D. Smith et al., 2011).

1.1. The impact of the commensal microbiota on the mucosal immune response

The human intestinal compartment hosts about 10^{12} microbial cells. (Sender et al., 2016). The enhancements of technical approaches in molecular biology which have been obtained in the last decades, such as rRNA 16S sequencing and shotgun metagenomics, were fundamental to allow the identification of the large biodiversity of the mammalian microbiota. This includes five predominant *phyla* (Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia). Bacteroidetes include *Bacteroides*, *Prevotella* and *Xylanibacter* genera; Firmicutes include *Clostridium*, *Lactobacillus*, *Ruminococcus*, as well as *Eubacterium*, *Fecalibacteirum* and *Roseburia*; proteobacteria include *Escherichia* and *Desulfovibrio*, while *Akkermansia* genus belong to Verrucomicrobia phylum (Schroeder & Bäckhed, 2016). Each commensal microbial community is extremely heterogeneous among the different individuals, being impacted by several environmental and genetic factors. Nevertheless, there is a balance in its organization which normally confers a broad range of benefits toward the host, while eventual alterations compromise the overall health status (Charbonneau *et al*., 2016). Studies conducted in germ-free (GF) or antibiotic-supplemented mice revealed that intestinal microorganisms are crucial to regulate different biological functions, including for example the maturation of the immune system as well as physiological processes like resistance to pathogenic infections, nutrient absorption, and maintenance of GB integrity (L. V. Hooper *et al*., 2001)(Hill & Artis, 2010). Such microbial-mediated regulatory mechanisms are essential in maintaining the homeostasis at intestinal and systemic level. In the recent years, increasing evidence of a central role of the microbiota in orchestrating immune responses in the intestinal tissues has been collected. It has been estimated that the gut mucosa hosts the largest immune compartment in the human body (Mestecky & Mcghee, 1987). In the mice intestine, most of immune cells house in the gut-associated lymphoid tissues (GALT) mainly including Payer's patches (PPs) and mesenteric lymph nodes (MLNs) and in the lamina propria, which represent the main locations for priming adaptive immune cell response in the gut. In line with the importance of commensal microbiota for gut immunity, GF mice display underdeveloped intestinal lymphoid tissues when compared to wild type, with lower frequency of intestinal lymphocytes and impaired levels of IgA and antimicrobial peptides (AMPs), which are essential to counter enteric infections (Kabat *et al*., 2014). Importantly, in the gut microbial commensals play

a key role in the maintenance of a proper T effector and T regulatory cell balance. For example, GF mice display lower frequency of Th17 and Th1 cells, together with a reduced production of IL-17 and IL-22 cytokines and overall, a decrease in the mucosa associated $CD4^+$ T cells (Mazmanian *et al*., 2005)(Chung *et al*., 2012)(Macpherson *et al*., 2002). In the murine microbiota, a reduction of Th17 cell population has been linked to the absence or reduction of segmented filamentous bacteria (SFB), thus suggesting the microbial capacity to trigger Th17 cell differentiation (Gaboriau-Routhiau *et al*., 2009)(Ivanov *et al*., 2009). In contrast, conclusive data describing human-specific SFB are still lacking, although recent advances suggest that this lineage may exist even in humans (Jonsson et al., 2020). Gut microbes can modulate Th17 cell expansion even via the production of bioactive molecules. Indeed, commensal organism-derived adenosine 5'-triphosphate triggers the activation of colonic CX_3CR1^+ mononuclear phagocytes to promote Th17 cells differentiation (Atarashi *et al*., 2008). Immune responses in the intestine need to be finely regulated along the gastrointestinal tract and even more in the colon where the necessity to counteract pathogens must be balanced with the need to maintain tolerance toward a larger variety of commensal microorganisms. Hence, the gut microbiota is also essential for the differentiation of regulatory T cells such as $FoxP3^+$ Treg cells. In support to this idea, the frequency of intestinal $FoxP3^+$ Treg cells is significantly compromised in the colonic lamina propria in the absence of commensals; this can be reversed by re-introducing the gut microbiota with certain microbial species (Kamada & Núñez, 2014)(Geuking *et al*., 2011)(Atarashi *et al*., 2011). For example, *Clostridium* strains have been shown to induce the production of transforming growth factor-β (TGF-β) in IECs, thereby promoting $FoxP3^+$ Treg cell differentiation in the colonic lamina propria (Atarashi *et al*., 2011). Similarly, the commensal *Bacteroides fragilis* by inducing the production of the tolerogenic cytokine IL-10, promotes the induction of immunosuppressive $FoxP3^+$ Treg cells through a polysaccharide-A (PSA)-mediated mechanism, a molecule expressed on its surface (Round & Mazmanian, 2010). Additionally, *Bifidobacterium breve* stimulated intestinal tolerogenic $CD103^+$ DCs to induce the development of IL-10-releasing type 1 regulatory T (Tr1) cells in the large intestine, thus preventing inflammation in a model of colitis (Jeon *et al*., 2012). Commensal microorganisms also affect the induction of intestinal B cells which reside in the lamina propria (Wesemann *et al*., 2013). Intestinal B lymphocytes are responsible for

the production of IgA, which in turn, are key regulators of the gut microbial composition. Thus, a proper induction of intestinal B cells is required not only to reach a large diversification of IgA in the gut mucosa, but also to establish and maintain immune tolerance toward enteric bacteria (Fransen *et al*., 2015). Indeed, in steady-state conditions, commensal coating with Immunoglobulin-IgA allows the induction of a more diversified amount of species-specific IgA (Fransen *et al*., 2015). All these findings reveal that our gut microbiota exerts a large variety of effects on both the maturation and continuous induction of innate and adaptive immune cell responses in the intestinal tract. Simultaneously, the gut mucosal immunity acts to discriminate between pathogenic microorganisms and the harmless commensals, thus adopting immune tolerance toward the latter.

1.2. The role of microbial metabolites in the regulation of the intestinal immune system

In the steady-state, the host relies on its commensals for a heterogeneous collection of metabolic enzymes (Lora V. Hooper *et al*., 2002). Indeed, a "healthy" microbiota releases a large variety of metabolites trough the fermentation of undigested dietary components as well as endogenous products which are synthetized by both intestinal bacteria and the host. IECs at mucosal interface between the host and enteric commensals allows the passage of those microbial metabolic products that access the mucosa and interact with the host immune cells. Particularly, those metabolites are essential for a correct functioning of the immune system and their interaction with the host mucosal immune cells is crucial to orchestrate the immune response of both effector and regulatory T cells (**Figure 2**).

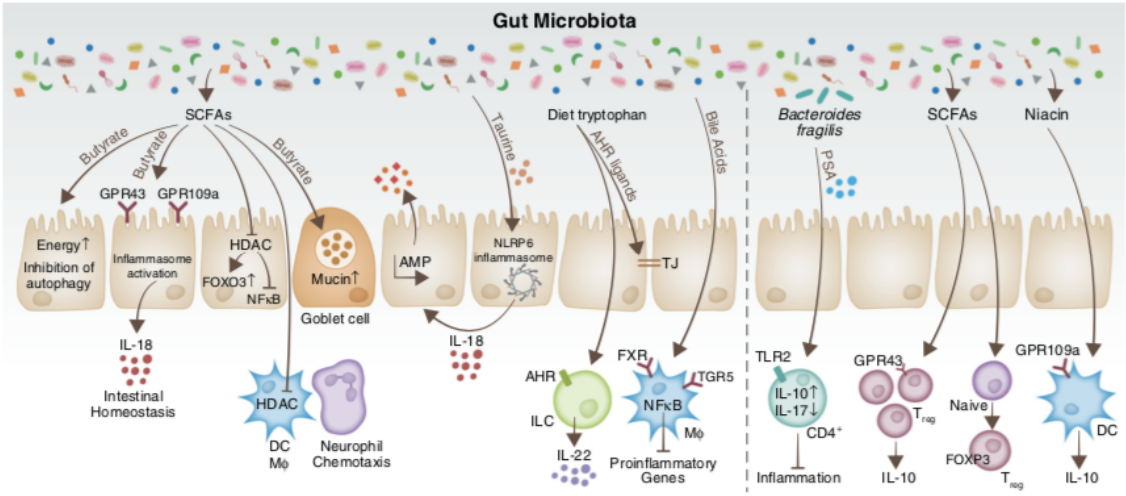


Figure 2. Modulation of the immune system by microbial metabolites.
Examples of microbial metabolites produced by enteric commensal affecting the host mucosal immune response. In the gut, the effects of microbial metabolites are direct on the immune cells or conveyed by the GB (Levy *et al*., 2017).

1.2.1. Short Chain Fatty Acids

Short Chain Fatty Acids (SCFAs), mainly including acetate, propionate and butyrate, are metabolites produced by the host microbiota resulting from dietary fiber fermentation. Beside their role as source for energy production, SCFAs possess different regulatory

functions that affect the host physiology and immunity. Indeed, acting as inhibitors of histone deacetylases (HDACs) and ligands for G protein-coupled receptors (GPCRs), they regulate differentiation and function of several cell lineages. By inhibiting HDACs, SCFAs drive anti-inflammatory and tolerogenic immune response, thus revealing the epigenetic effect that the gut microbiota has toward the host. The inhibition of HDAC by SCFAs, also results in the downregulation of nuclear factor -kB (NF-kB) and reduces the release of the pro-inflammatory tumor necrosis factor (TNF)(Usami *et al*., 2008). Notably, the inhibition of HDACs mediated by SCFAs increases and regulates the frequency of suppressive $FoxP3^+$ T reg cells in a model of colitis (Tao *et al*., 2007). Many regulatory properties related to SCFAs are exemplified by binding specific GPCRs such as GPR43, GPR41 and GPR109A, which are expressed by both immune cells and IECs. For example, SCFAs supplementation in colitis models ameliorates the disease course by increasing tolerogenic $FoxP3^+$ Treg cells via a GPR43-mediated mechanism (P. M. Smith *et al*., 2013)(Maslowski *et al*., 2009). Analogues tolerogenic effect have been observed for SCFAs-mediated activation of GPR109A, which by inducing the differentiation of IL-10 producing Tr1 cells, prevents colitis in pre-clinical models (Singh *et al*., 2014). SCFAs also play a beneficial role toward the GB integrity. A direct exposure to SCFAs or the supplementation of SCFAs-producing bacteria, such as *Bacteroides tethaiotamicron* or *Facalibacterium prausnitzii*, preserve the integrity of the GB by stimulating the production of immunoregulatory mucins (Willemsen *et al*., 2003)(Gaudier *et al*., 2004)(Wrzosek *et al*., 2013). Also, these metabolites preserve the GB properties by controlling the adhesion between tight junctions (TJs), thus avoiding the spreading of harmful molecules from the lumen to the systemic circulation (Fukuda *et al*., 2011). Those evidence highlight the key role of microbial SCFAs in regulating immune responses and in contributing to mucosal homeostasis.

1.2.2. Tryptophan derivatives

Tryptophan derivatives microbial metabolites shape the intestinal immune system mainly by binding the aryl hydrocarbon receptor (AHR) and the Pregnane X Receptor (PXR). While these receptors were initially recognized for their role in xenobiotic metabolism, AHR and PXR are now intensively investigated for their involvement in

regulating mucosal immune response (Rooks & Garrett, 2016). The activation of AHR, which is expressed by immune cells, IECs as well as cancer cells, is affected both by dietary nutrients and gut microbiota composition. Indeed, only some bacterial species, particularly belonging to Lactobacilli strains, are able to metabolize dietary tryptophan and generate AHR ligands (i.e., microbial indoles). Of interest, tryptophan metabolism and synthesis of indole-3-aldehyde by commensal *L. reuteri* revealed to be crucial in counteracting the overgrowth of opportunistic *C. albicans* by binding AHR and stimulating the production of IL-22 by group 3 innate lymphoid cells (ILC3 cells) (Zelante *et al*., 2013). Indole-3-pyruvic acid suppresses gut inflammation by enhancing release of IL-10 , thus limiting the differentiation of Teff cells in the colonic lamina propria (Aoki *et al*., 2018). Similarly, oral supplementation of mice with *Lactobacillus murinus* and *Lactobacillus reuteri* increases the concentration of the tryptophan-derived indole-3-lactic acid, that induces immune regulation and reduces the Th17 cell polarization (Wilck *et al*., 2017). Indoleacrylic acid revealed similar protective features. Of note, indoleacrylic acid stimulates goblet cell function to enhance intestinal barrier properties and induces the secretion of tolerogenic IL-10 together with suppression of TNF-α and IL-6 (Wlodarska *et al*., 2017). In addition, indole-3-propionic acid and indole-3-acetic acid are two well-known tryptophan derived metabolites and PXR ligands which have been associated with epithelial protection in the intestine (Venkatesh *et al*., 2014)(Lamas *et al*., 2016). All together those findings reveal that along with conferring resistance to pathogens colonization, tryptophan derived metabolites play an immunomodulatory role which sustains the gut homeostasis and prevents inflammation at intestinal and systemic level.

1.2.3. Bile Acid metabolites

Largely studied for their emulsifying properties in enabling the absorption and the excretion of dietary fats in the intestine, more recently bile acids have been identified as key signalling molecules that regulate gut homeostasis and immunity by directly interacting with cellular receptors and enteric commensals. Indeed, while they shape microbial composition due to intrinsic bacteriostatic properties, they also interact with a broad range of cell subsets, such as hepatocytes, IECs and immune cells to regulate

mucosal immune response (Swann *et al*., 2011)(Inagaki *et al*., 2006a)(M. L. Chen *et al*., 2019). In mammals, the bile acid pool involves primary and secondary bile acids. Primary bile acids are synthetized in the liver via the cholesterol catabolism while secondary BAMs are produced through the microbial metabolic activity from primary bile acids (Ridlon *et al*., 2006) (Russell, 2003). The most abundant primary bile acids in humans are represented by cholic acid (CA) and chenodeoxycholic acid (CDCA). The hepatic biosynthesis of those primary bile acids involves two overlapping pathways known as "classical" and "alternative" pathways, both under regulation of cytochrome P450 (CYP) enzymes, such as CYP7A1, CYP27A1 and CYP7B1 (Chiang, 2009). Of interest, the microbial community regulates the expression of the aforementioned enzymes (Sayin *et al*., 2013), thus indirectly having an effect on the primary bile acids secretion from hepatocytes. The final step of bile acids synthesis includes the conjugation to the amino acids taurine or glycine, a reaction which is critical for their lipids emulsifying properties. The conjugation of primary bile acids with taurine and glycine groups is catalysed by the bile acid-coA:amino acid N-acyltransferase enzyme and requires active transport by the host to enable their transfer into bile ducts and storage in the gall bladder (Dawson *et al*., 2009). The majority of conjugated bile acids are post-prandially released into the duodenum, while in the terminal ileum they undergo an active re-uptake to enter the enterohepatic circulation and circulate back to the liver trough an active transporter system. The enterohepatic circulation is finely regulated to both encounter the necessity of the host to absorb lipids and emulsify fats as well as to avoid excessive concentrations of bile acids which can be toxic for the gastrointestinal tract (Russell, 2003). In fact, due to their detergent-like activities, bile acid can be pro-inflammatory and cytotoxic when dysregulated. Bile acids that are not reabsorbed at ileal level (about 5-10%) are mostly unconjugated and enter the large intestine becoming substrate for further bacterial-mediated transformation. Different intestinal bacterial taxa such as *Firmicutes*, *Bacteroides*, *Eubacterium* and *Clostridium* release bile salt hydrolase (BSH) enzymes that allow the deconjugation of taurine- and glycine-conjugated bile acids (Ridlon *et al*., 2006). The bacterial-mediated deconjugation of primary bile acids first takes place in the terminal ileum and proceeds in the large intestine. Since only conjugated bile acids are actively reabsorbed in the ileum, the bacterial deconjugation represents an example of how microbial metabolism directly affects ileal bile acids re-

uptake and, most important, bile acid concentration in the large intestine. At colonic level, unconjugated bile acids are further transformed by many enteric commensals in secondary BAMs through a reaction of hydroxysteroid dehydrogenation. Although among secondary BAMs the most common are represented by DCA and LCA, advances in bile acid-targeted metabolomics allowed the identification of other secondary bile acids in both mice and humans, which are produced trough reactions of epimerization, isomerization and oxidation of -OH groups (Wahlström *et al*., 2016). Recently, a key immune regulatory role of secondary BAMs produced by the gut microbiota and, specifically DCA and LCA, has been identified. BAMs exert these immune regulatory properties trough activation of specific receptors that mainly include the nuclear Farnesoid X Receptor (FXR), the membrane-type Takeda G-protein receptor 5 (TGR5), together with the nuclear vitamin D receptor (VDR) and PxR (Makishima *et al*., 1999)(Duboc *et al*., 2014)(Makishima *et al*., 2002)(Staudinger *et al*., 2001). These receptors are largely expressed by several cell lineages, particularly within the gastrointestinal tract, and show different affinities according to the different BAMs (Guo *et al*., 2003)(T. Li & Chiang, 2013). Indeed, while both CA and CDCA are potent FXR agonists, TGR5 displays highest affinity for secondary BAMs DCA and LCA, while VDR and PxR preferentially bind LCA (M. L. Chen *et al*., 2019). Recently, it has been demonstrated that the activation of such receptors in both innate and adaptive immune cell induces tolerogenic signals. A comprehensive overview of the role of bile acid metabolites in regulating gut homeostasis and mucosal immunity is represented in **Figure 3**. For example, increased levels of DCA and LCA in the large intestine shape the profile of colonic macrophages from a pro-inflammatory M1 toward an anti-inflammatory M2 phenotype. Such polarization is mediated by the activation of TGR5 receptor and promote the production of high level of tolerogenic IL-10 together with higher percentage of $Foxp3^+$ Treg cells, that results in attenuated colitis in mice (Biagioli *et al*., 2017). Similarly, FXR activation by a natural isomer of DCA induces DCs to diminish their immunostimulatory properties and drive peripheral $FoxP3^+$ Treg cell differentiation (Campbell *et al*., 2020). BAMs were also shown to directly shape adaptive immunity. For example, LCA inhibits the activation of Th1 cell by binding VDR (Pols *et al*., 2017). Of note, Hang *et al*., found that two distinct derivatives of LCA, 3-oxo-LCA and isoalloLCA, act as T cell regulators in mice. While 3-oxo-LCA inhibited the differentiation of Th17

cells by directly binding to the transcription factor RORγt, isoalloLCA increased the differentiation of FoxP3$^+$ Treg cells through the production of mitochondrial reactive oxygen species. Also, the administration of such metabolites in mice increases T reg cells in the intestinal lamina propria (Hang *et al*., 2019). An elegant study showed that secondary BAMs directly induce a population of FoxP3$^+$ T reg cells expressing the transcription factor RORγ (FOXP3$^+$ RORγ$^+$ Treg cells), which are fundamental in maintaining mucosal immune homeostasis in the colonic lamina propria. Experiments in Treg specific VDR knock-out mice revealed the key role of VDR in triggering colonic FOXP3$^+$ RORγ$^+$ Treg cell expansion and preventing gut inflammation upon BAMs supplementation in a model of colitis (Song *et al*., 2020). Similarly, administration of DCA and LCA dampen inflammation occurring during colitis by binding TGR5 and blocking the release of pro-inflammatory cytokines and chemokines (Sinha *et al*., 2020a). While enteric bacteria can control the BAMs concentration in the gut through their metabolic activity, BAMs reciprocally regulate microbial colonization due to direct bacteriostatic properties as well as via BAMs-dependent signalling on IECs that promote production of AMPs (Inagaki *et al*., 2006b). BAMs also play important effects on GB integrity. The activation of FXR induces transcriptional responses in IECs which reinforce GB function and stimulate the release of AMPs, thus limiting the bacterial translocation across the GB during colitis (Gadaleta *et al*., 2011). More recently, supplementation of mice with the BAM-producing bacteria *Parabacteroides distasonis*, as well as by directly treating them with secondary Ursodeoxycholic Acid and LCA, showed to repair the GB integrity in obese mice by significantly increasing the expression of the TJs zonulin 1 (ZO-1) and Claudin-1 (Wang *et al*., 2019). Secondary BAMs have been found to be decreased in fecal and serum samples of individuals affected by Inflammatory Bowel Diseases (IBD), while primary conjugated bile acids were significantly higher in active disease, thus suggesting that an impaired bacterial metabolism associated to BAMs production can result in immune dysregulation and intestinal inflammation (Duboc *et al*., 2013).

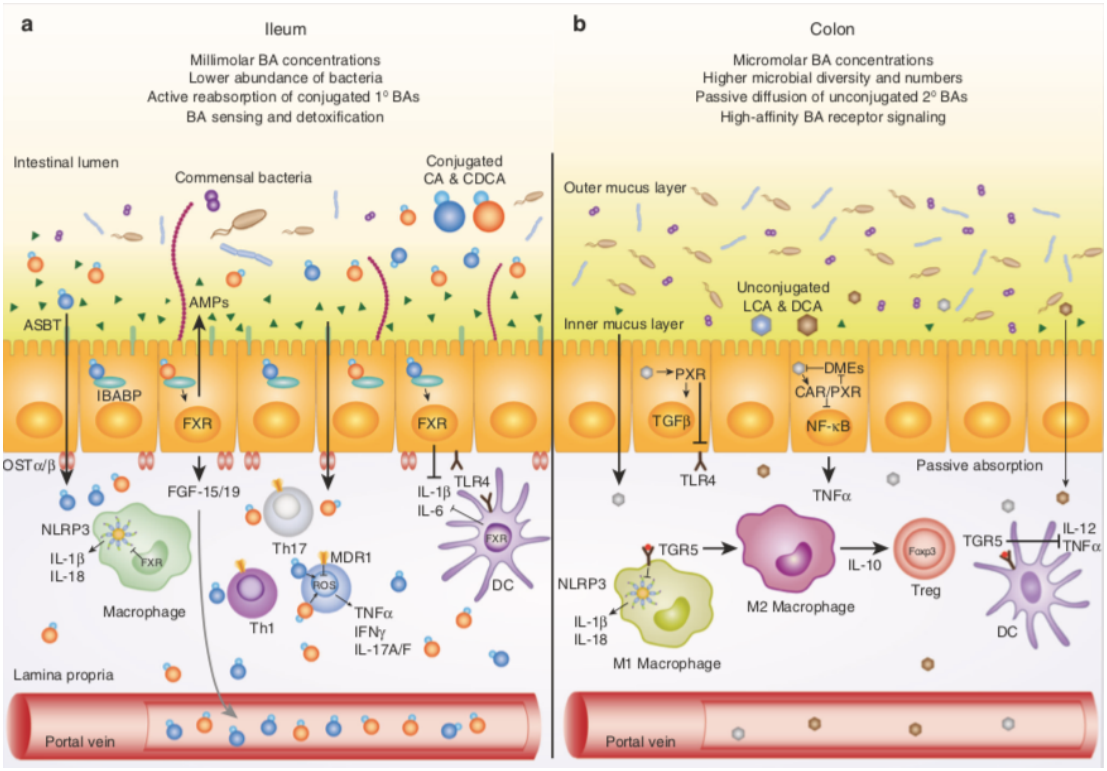


Figure 3. Bile acid microbial metabolites regulate gut homeostasis and mucosal immunity. *Schematic representation of the BAMs-mediated immunomodulatory microenvironments in the small and large intestine. a) conjugated primary bile acids are actively reabsorbed by the apical sodium-dependent bile acid transporter (ASBT), the cytoplasmic bile acid chaperone ileal bile acid-binding protein (IBABP) and the basolateral organic solute transporter (OST)-α/β. In the ileal mucosa they interact with FxR to promote the secretion of fibroblast growth factor 15/19 that suppress hepatic synthesis of bile acid and production of AMPs to protect the epithelial barrier. Also, bile acids activate FxR expressed by DCs and macrophages to suppress NLRP3 inflammasome activation as well as TLR-4-dependent pro-inflammatory cytokine production. Furthermore, bile acids induce the activation of the multidrug resistance protein 1 in mucosal Th17 and Th1 cells, thus suppressing the oxidative stress; b) unconjugated BAMs passively diffuse across the epithelial barrier and reach the mucosa where they activate PxR in IECs to induce the expression of TGF-β and suppress TLR-4-and NF-kB-dependent pro-inflammatory cytokine production. Both DCA and LCA bind with high affinity TGR5 expressed on colonic DCs and macrophages thereby limiting the release of TNF and IL-12 by DCs, inhibiting NLRP3 inflammasome activation in M1-type macrophages and inducing the polarization of IL-10 producing M2 macrophages which in turn increase the frequency of colonic Foxp3^{+} Treg cells* (M. L. Chen *et al*., 2019).

1.3. The role of the gut barrier in regulating the crosstalk between the gut microbiota and the intestinal immune system

Although microbial commensals are involved in immune system development and function, an abnormal response to microbial antigens may lead to significant changes in the activation status of mucosal immune cells. To avoid this, enteric microbes are confined within the intestinal lumen and separated from the lamina propria compartment, where immune cells are located, by intestinal barriers. These specialized structures consist of the mucus layer, the intestinal epithelium and the gut vascular barrier (GVB), which work in a coordinated manner to preserve the intestinal homeostasis (Hill & Artis, 2010). The mucus barrier is mainly composed by mucins representing complex agglomerates of glycoproteins released by goblet cells (Sicard *et al*., 2017). On the one hand, the mucus layer avoids harmful molecules present in the lumen to come in direct contact with the epithelial mucosa. On the other hand, the mucus layer is crucial in shaping gut microbiota composition. Indeed, through the mucus barrier the host is able to tolerate beneficial microbial strains, while it releases factors able to reduce adhesions and colonization of harmful microorganisms (McLoughlin *et al*., 2016). Mucin proteins also provide a nutrient source for many mucins-degrading commensals, such as *Bacteroides thetaiotamicron*, *Akkermansia muciniphila* and *Bacteroides fragilis* (Xu *et al*., 2003)(Derrien *et al*., 2004)(Macfarlane & Gibson, 1991). Of note, by regulating bacterial mucin degradation, mucin glycans can control microbial translocation. Beside acting as a diffusion barrier, the mucus layer actively regulates the interaction between the gut microbiota and the immune system. For instance, structural MUC2 is fundamental for the interaction between secretory goblet cells and tolerogenic $CD103^+$ DCs, which drives Treg cell differentiation and immune tolerance (Velcich *et al*., 2002). MUC 1 also plays anti-inflammatory functions by limiting adherence of bacteria, their translocation and consequent inflammation (Linden at al 2009). Beyond the mucus barrier, the intestinal epithelial barrier (IEB) maintains the segregation between the host and gut microorganisms thank to a single layer of IECs which are closely interconnected through TJs, adherent junctions (AJs) and desmosomes. While desmosomes and AJs serve for the stabilization of TJs, the latter include transmembrane proteins, such as occludins and claudins, membrane proteins like zonula occludens, and regulatory proteins (Turner, 2009). This anatomical structure maintains the integrity of the epithelium and regulates

the passage of fluids and solutes (Farhadi *et al*., 2003). A compromission of IEB has been associated with the onset of several inflammatory and T cell-mediated disorders, probably due to an increased bacterial translocation (Berg, 2000). Indeed, as a result of impairments of intestinal barrier permeability, including downregulation of ZO-1 and occludin, there is an uncontrolled passage of microbiota-derived factors, including bacterial products or toxins across the epithelium, which can lead to the onset of inflammatory disorders both at intestinal and distal level (Nagpal & Yadav, 2017)(Rakoff-Nahoum *et al*., 2004). By detecting antigens and secreting AMPs in response to the microbiota, the IEB shapes the commensal community and, at the same time, provides immunostimulatory signals to intraepithelial lymphocytes and lamina propria immune cells, thus regulating the mucosal immune response. For example, the thymic stromal lymphopoietin released by IECs in homeostatic conditions inhibits the DCs-mediated production of IL-12 and the induction of a Th1 response toward intestinal bacteria, thus maintaining an anti-inflammatory milieu (Ewaschuk *et al*., 2008). Enteric bacteria also promote IECs-mediated production of immunoglobulin IgA, which participate in preventing the microbe-IECs interaction. It was shown that inflammasomes associated to IEB play a pivotal role in tissue homeostasis and host defence to infection (Zaki *et al*., 2011). These protective mechanisms prevent intestinal dysbiosis (i.e., alteration of the commensal community) and avoid an aberrant microbiota profile which may enhance a mucosal inflammatory response that has been supposed to be linked to the pathogenesis of IBD (Zaki *et al*., 2011)(Elinav *et al*., 2011). The GVB represents the inner layer of defence in the multi-layered intestinal barrier system which finely regulates the translocation of elements from the lumen to the systemic circulation. The GVB is mainly composed by TJs and AJs together with different cell type including endothelial cells, pericytes and fibroblasts (Spadoni *et al*., 2015)(Ciccia *et al*., 2017). The correct maintenance of the integrity of the GVB avoids the invasion of microbial components into the gut mucosa and their peripheral dissemination, which may abnormally activate immune cells including self-reactive T cells. Hence, intestinal barriers at mucosal surface level are central actors in the maintenance of a healthy intestinal environment, which requires a permanent but finely regulated biological interaction between the gut microbiota and the mucosal immunity.

2. BEYOND THE GUT: FROM THE INTESTINE TO ORGAN-SPECIFIC AUTOIMMUNE DISEASES

Autoimmune disorders, such as rheumatoid arthritis (RA), type 1 diabetes (T1D), and MS, are driven by uncontrolled immune responses to self-antigens, thus causing chronic inflammation in the target organs. The peripheral immune system contains a considerable fraction of immune cells with high affinity for self-autoantigens (Danke *et al*., 2004) (Wekerle *et al*., 2013). However, while these cells remain dormant throughout the life in healthy individuals, under pathologic conditions those cells can be activated and expanded at peripheral level, thus leading to autoimmune responses against target organs (Danke *et al*., 2004)(Naparstek *et al*., 1983)(Wekerle *et al*., 2013). The triggering events remain to be elucidated but it has been supposed that the activation and/or the modulation of autoimmune T cells may take place in the intestine (Wekerle, 2017). Indeed, as previously discussed, in steady state conditions T cells continuously circulate in the gut mucosa, where they are modulated by the gut microbiota, which through the production of immunoregulatory metabolites such as SCFAs, tryptophan derivatives or BAMs, regulates the immune system by maintaining a proper balance in the differentiation of T effector cells and T regulatory cells. This regulation is finely coordinated by the intestinal barrier, which keeps separated highly immunogenic agents in the intestinal lumen from a highly immunoreactive T cells in the submucosa, physically and actively participate in maintaining the intestinal immune tolerance. Therefore, impairment of GB functions together with the presence of a dysbiotic (pro-inflammatory) microbiota, may switch T cell differentiation in the gut mucosa toward an effector phenotype. An imbalanced immune response with higher differentiation of T effector cells and impaired induction of Treg cells ($FoxP3^+$ Tregs and IL-10-producing Tr1 cells) may enhance aggressiveness of T cells circulating within the intestinal mucosa, possibly including self-reactive T cells that have escaped thymic selection (Berer *et al*., 2011). Simultaneously, a GB damage due to a condition of inflammation, could favour the uncontrolled translocation of bacterial components from the gut mucosa into the systemic circulation, thus increasingly activating autoreactive T cells either in the peripheral lymphoid tissue or in the target organs. Hence, in the contest of autoimmune disorders, the current hypothesis holds that in genetically predisposed individuals, autoreactive T cells specific for self-antigens normally circulate through the gut mucosa where they are activated and rendered more

pathogenic by a pro-inflammatory intestinal environment. From the intestine those self-reactive T cells migrate into the secondary lymphoid organs and infiltrate target tissues therefore inducing the autoimmune response (**Figure 4**).

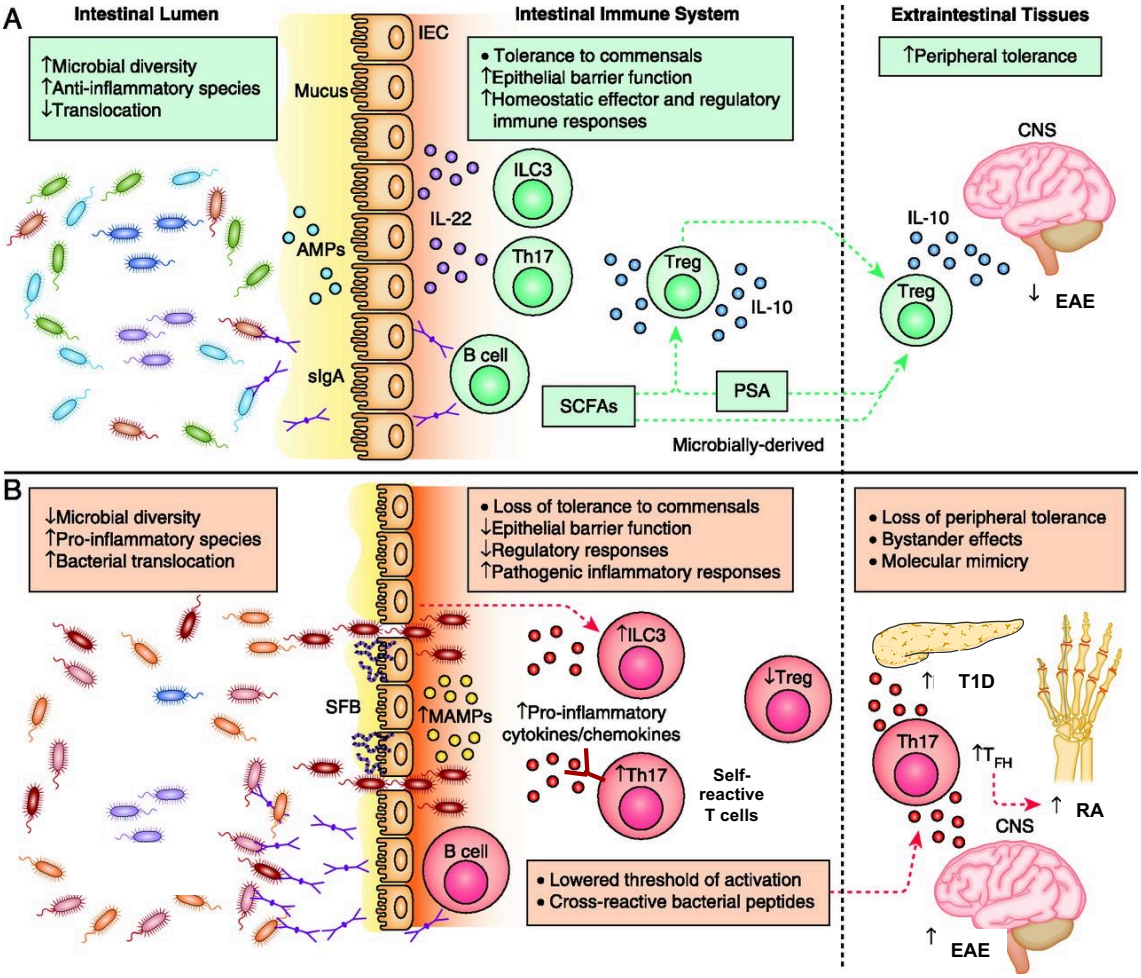


Figure 4. Host-microbiota interactions regulate homeostasis and inflammation at intestinal and extra-intestinal level. *(**A**) In homeostatic conditions, the gut microbiota is compartmentalized in the lumen and the interaction with the mucosal immune system is finely regulated by the mucus and the epithelial barrier which contribute to maintain immune tolerance toward commensals. In such conditions, microbial derived SCFAs and PSA induce the expansion of IL-10 producing T regulatory cells which protect against neuroinflammation in EAE mouse model of MS. (**B**) During intestinal inflammation and microbial dysbiosis, the loss of GB integrity allows an uncontrolled interaction between pathogens and/or enteric bacteria and the intestinal immune system, thus contributing to the breakdown of immune tolerance while incrementing the production of pro-inflammatory cytokines and the differentiation of effector cell population, including also self-reactive T cells. Activation of pro-inflammatory Th17 cells via bystander effects and/or molecular mimicry can initiate autoimmunity and exacerbate inflammation in extraintestinal tissues due to the migration of autoreactive T cells to the peripheral target organs (Grigg & Sonnenberg, 2017).*

2.1. Organ-specific autoimmune diseases

In the past decades it has been recorded a dramatic peak in the incidence of autoimmune disease in the western countries. Both genetic and environmental factors are relevant in the pathogenesis of organ-specific autoimmune diseases such as MS, T1D and RA. Indeed, studies conducted in asymptomatic twin carriers of risk genetic variants (i.e., genetic polymorphisms involving the human leukocyte antigen and other genes in immune pathways), reveal that additional factors are required for disease establishment (Marson *et al*., 2015). Nowadays, modifications in lifestyle such as changes in dietary habits, higher hygiene conditions or an extensive use of antibiotics, are examples of environmental factors that significantly changed in the past decades and may be held responsible for promoting autoimmune disease in genetically predisposed individuals. While being extremely heterogeneous, those environmental factors have a common target which is the human intestinal microbiota. In support of such hypothesis, recent studies exploiting multi-omics approaches revealed that when compared to healthy controls, patients suffering from autoimmune disorders show alterations of the intestinal microbiota and microbial metabolic profile and, importantly, such alterations are often associated with the clinical outcome of the diseases. Those findings strongly reinforce that the pathogenesis of extra-intestinal autoimmune diseases is regulated, at least in part, at the intestinal level. For example, individuals affected by RA, an autoimmune disease characterized by inflammatory processes and tissue damage at the synovial joints level, have high frequency of fecal *Clostridium perfrigens* and an increased abundance of *Prevotella* spp., (Olhagen & Månsson, 1968)(Scher *et al*., 2013). Of note, antibodies against *Prevotella copri* has been detected in new-onset RA, where *P.copri*-specific IgA correlated with Th1 and Th17 related cytokines in serum (Pianta *et al*., 2017). Also, changes related to the absence of certain gut bacteria correlate with clinical indices in patients with RA. Indeed, a decrease in the relative abundance of genus *Haemophilus* is associated with lower levels of RA-specific autoantibodies and rheumatoid factors (Zhang *et al*., 2015). Additionally, different studies revealed a decreased richness of bacteria biodiversity in children affected by T1D, with an overall decrease in *Prevotella* spp. and an increase in *Bacteroidetes* spp (Alkanani *et al*., 2015)(Kostic *et al*., 2015). T1D is driven by self-reactive T cells and autoantibodies directed against insulin-producing pancreatic β-cells and, of note, children with genetic predisposition show an

overgrowth of *Bacteoroides dorei* which precedes seroconversion (Vatanen *et al*., 2018). This evidence strongly suggests a pathogenic role of *B. dorei* in the onset of the disease. T1D patients revealed an imbalance in the production of SCFAs that together with a reduction of genera *Akkermansia*, both implicated in the correct maintenance of the GB, may lead to the "leaky gut" condition which has been detected in T1D children (Stewart *et al*., 2018)(Chelakkot *et al*., 2018)(Harbison *et al*., 2019). Importantly, the loss of GB integrity revealed to be sufficient to trigger the activation of islet-reactive T cells in the intestine and initiate autoimmune T1D in mice (Sorini *et al*., 2019). At mucosal level, the activation of self-reactive T cells can occur mainly via bystander activation or via antigens which closely resemble self-antigens, a mechanism known as molecular mimicry (Grigg & Sonnenberg, 2017). Bystander effects involve mechanisms by which pathogens can activate autoreactive cells in an antigen-nonspecific manner during infection by promoting an inflammatory environment instead of tolerogenic signals (Ruff *et al*., 2020). Several microbial associated molecular patterns, such as bacterial lipopolysaccharide and peptidoglycan, have been shown to activate TLRs and NOD-like receptors thus contributing to the onset of autoimmune responses in pre-clinical models (Tsuji *et al*., 2012)(Costa *et al*., 2016)(Huang *et al*., 2019). Simultaneously, similarities between microbial agents and host proteins can activate immune cells through a mechanism of cross-reaction, which in autoimmunity is commonly recognized as molecular mimicry (Ebringer, 1979). Indeed, in steady state, a broad variety of antigens from enteric microorganisms are constantly presented to adaptive immune cells and recently, specific bacteria with the capacity of inducing the activation of cross-reactive T cells have been identified. Mucosal T cells activated upon stimulation with bacterial antigens could mediate the development of extra-intestinal autoimmune disease by travelling into target organs. For example, a microbial mimic peptide derived from the Mgt protein of intestinal *L. goodfellowii*, can activate islet-specific-glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific NY8.3 $CD8^+$ T cells and facilitate T1D onset in non-obese diabetic (NOD) mice (Tai *et al*., 2016). Furthermore, Pc-P27 peptide derived from *P.copri* can trigger the activation of T and B cells in patients suffering from RA (Pianta *et al*., 2017). Overall, these findings support the concept that altered interactions between the gut microbiota and the immune system participate to the onset of autoimmune disorders in the gut but also at sites distal from the intestine.

3. MULTIPLE SCLEROSIS

MS is a chronic inflammatory demyelinating and neurodegenerative disease induced by myelin-specific self-reactive lymphocytes. Clinical symptoms vary according to the site of neurological lesions which are often associated with infiltrates of inflammatory cells across the blood brain barrier (BBB) (Nylander & Hafler, 2012). Classification of the different types of MS has been largely based on clinical phenomenology and its course can be relapsing-remitting or progressive. RRMS is the most common form manifesting in relapse of symptoms followed by periods of remission. At the beginning of the disease course, RRMS patients generally return to normal neurologic functions after a relapse episode, however with time individuals experience irreversible progressive disability (secondary progressive MS) and in some cases the disease progresses between relapses (progressive relapsing MS). Disease relapses and shift into progressive MS can be now delayed through a large variety of therapeutic interventions (Gunnarsson *et al*., 2011). In a small percentage of MS cases (5-10%) , the disease course appears progressive from the diagnosis (primary progressive MS) with neurological deficits progressively worsening from disease onset (Miller & Leary, 2007) (Tullman *et al*., 2004).

3.1. Epidemiology

MS represents the most common neurological disorder affecting young individuals in western countries, with an almost double prevalence in women than men and disease onset which usually reveals in the third or fourth decade of life (Compston & Coles, 2002). According to the Atlas of MS, it is currently estimated that 2.8 millions of people suffer of MS around the world (Walton *et al*., 2020). This autoimmune neurological disorder is spread worldwide but not equally distributed. Indeed, over the past decades, prevalence has been rising across Central and North America and Europe while a lower incidence is recorded in Africa, Central Asia and Caribbean regions (Walton *et al*., 2020).

3.2. Etiology

Although it is largely accepted the multifactorial nature of the disease, the etiology of MS is currently unknown. Several genetic and environmental factors have been implicated to the pathogenesis of MS which can be involved in the breakdown of immune tolerance at the central and peripheral level. Indeed, genome-wide association studies highlighted polymorphisms in several genes encoding for different immunoregulatory molecules, cytokines and their receptors (Becker *et al*., 1998). Human Leukocyte Antigen (HLA)-DR15 located on chromosome 6 likely represents the main known gene involved in the susceptibility of MS (Hollenbach & Oksenberg, 2015). Recently, additional susceptibility loci have been recognized in IL2RA and IL7A normally expressed by Treg cells, whose role in the regulation of immune response in MS has been largely studied (Viglietta *et al*., 2004)(Verma, 2008). Although an increasing number of polymorphisms are associated with the disease, high rates of discordance in monozygotic twins, indicate that environmental factors play a key role in the development of MS (Willer *et al*., 2003). To date, the main putative environmental factors implicated in the onset of MS include vitamin D deficiency, smoking and Epstein-Barr virus infection, which remain the best-confirmed environmental contributors (Belbasis *et al*., 2015). Thus, environmental triggers such as diet, hygiene conditions and antibiotic treatment, which directly affect the intestinal microbiota, have begun to be investigated. In support, studies conducted in pre-clinical model of MS allow to observe that qualitative and functional changes of the gut microbiota can impact the incidence and severity of CNS inflammation (Berer *et al*., 2011).

3.3 Immunopathogenesis

MS is primary characterized by an inflammatory process determined by breakdown of the BBB and consistent infiltrations of myelin-specific self-reactive T lymphocytes into the CNS (Nylander & Hafler, 2012). The activation of these autoreactive T cells occurs at peripheral level, from where they migrate to the CNS together with activated B cells and monocytes (Wekerle *et al*., 2013)(Dendrou *et al*., 2015). This evidence supports the current method used to induce EAE in pre-clinical models: an emulsion of CNS antigens and immune adjuvants is administered to stimulate the generation of pathogenic Th1 and Th17 cells in the DLN; these cells then enter the circulation and once crossed the BBB as

well as the cerebrospinal fluid at the choroid plexus, reach the CNS tissue where they exert their inflammatory properties (Reboldi *et al*., 2009). The activation of pathogenic T cells at peripheral sites can potentially occurs via a mechanism of molecular mimicry as well as through bystander activation (**Figure 5**). Remarkably, it has been recently observed that a synergistic effect mediated by the commensal microorganisms, i.e., a strain of the *Erysipelotrichaceae* family (OTU002) and *L. reuteri*, worsen EAE by enhancing Th17 cell response and molecular mimicry to myelin oligodendrocyte glycoprotein (MOG), respectively. The effect of *L. reuteri* was mediated by the UvrABC system A, a protein which cross-reacts with MOG as it induces proliferation of MOG-specific CD4 T cells. However, UvrA-derived peptide alone does not elicit EAE, thus suggesting that intestinal microbes could act synergistically to promote the disease. (Miyauchi *et al*., 2020). Of note, while Th1 cells were previously thought to drive MS inflammation, it is now largely established that pro-inflammatory effector Th17 cells also play a critical role in the initiation of the disease pathogenesis (Steinman, 2007). Furthermore, IL-23 production together with transcription factor RORγt induce Th17 cells to produce the granulocyte-macrophage colony-stimulating factor (GM-CSF) which is required for encephalogenicity and strongly contributes to axoglial damage (Codarri *et al*., 2011)(El-Behi *et al*., 2011). In the CNS, autoreactive Th17 cells increase BBB permeability by releasing pro-inflammatory cytokines such as IL-22 and IL-17 which initiate a cascade of immune cell penetration including IFN-γ-producing Th1 cells, cytotoxic $CD8^+$ cells, B cells and immunoglobulin-secreting plasma cells, thus forming the perivascular infiltrates in the CNS tissue (**Figure 5**) (Nylander & Hafler, 2012).

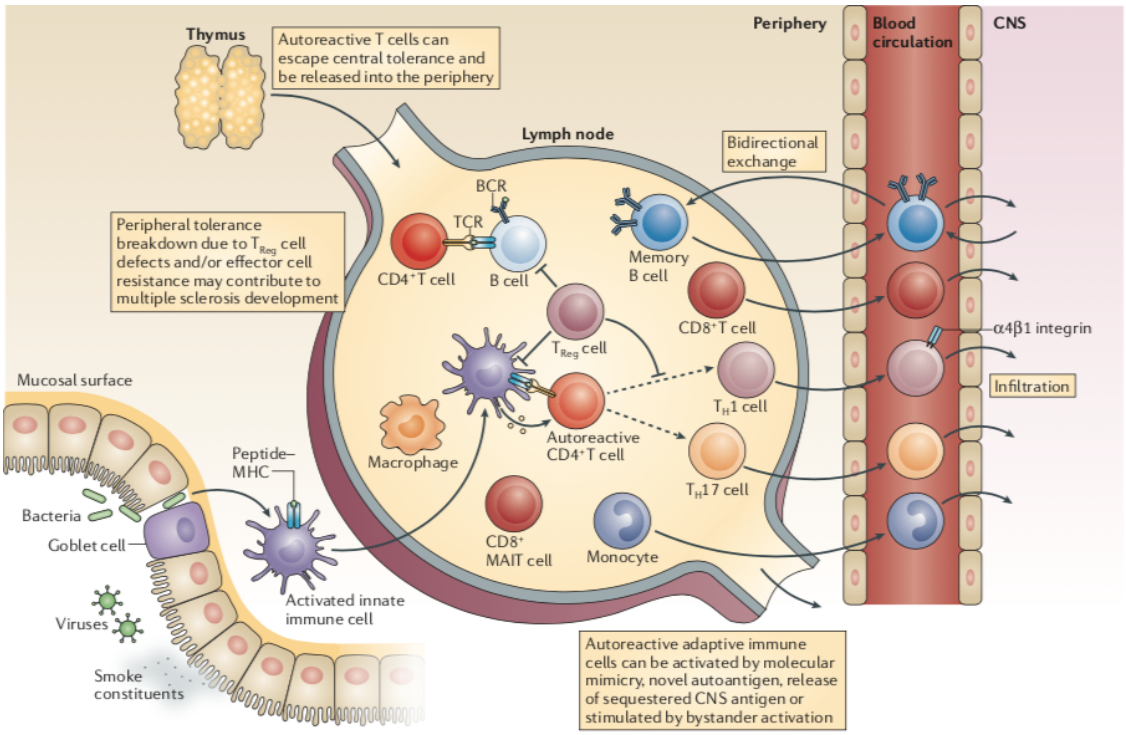


Figure 5. Immune dysfunction at peripheral level underlies the pathogenesis of MS.
The majority of autoreactive cells are deleted during the establishment of the central tolerance in the thymus while some self-reactive T cells can be released into the periphery. In normal conditions, the peripheral tolerance avoid the activation of those cells. However, the breakdown of immune tolerance involving a defect in the frequency and function of T reg cells, can allow the aggressiveness of silent CNS-antigen specific B and T cells activated via molecular mimicry and bystander activation. Environmental factors such as infections and smoke can facilitate this process. Once activated at lymph node level, CNS-self-reactive T and B cells travel toward the CNS thus initiating the autoimmune response (Dendrou et al., 2015).

4. HOW THE GUT MICROBIOTA AFFECTS THE AUTOIMMUNE RESPONSE IN MULTIPLE SCLEROSIS

First evidence for a microbial role in contributing to autoimmune phenomena at brain level came from the first transgenic mouse model of spontaneous EAE, which express a myelin-specific antigen T cell receptor. By exploiting this model, the researchers observed that when maintained in sterile conditions, mice failed to develop EAE, while under conventional not-sterile conditions, spontaneously developed the disease (Goverman *et al*., 1993). However, at that time, pathogens were suspected to be among the main triggers of the disease. The potential role of intestinal commensal microbes was considered later, when the administration of antibiotics to treat the clinical course of EAE revealed beneficial effects. Indeed, the treatment of EAE mice with broad spectrum antibiotics has been shown to counteract or delay the onset of the disease (Yokote *et al*., 2008)(Javier Ochoa-Repáraz *et al*., 2009)(Javier Ochoa-Repáraz *et al*., 2010). Although sometimes the protection was associated with a reduced response of circulating effector Th1 and Th17 cells and pro-inflammatory cytokines, in other cases it was accompanied by increased pro-tolerogenic $CD11c^{+}CD103^{+}$ DCs and higher level of anti-inflammatory interleukin 10 producing B cells (Javier Ochoa-Repáraz *et al*., 2010). The subsequent observation that GF mice are protected from spontaneous brain autoimmunity, demonstrated the instrumental gut microbial role in modulating this CNS disorder. To test whether the intestinal microbiota actively participates in triggering an autoimmune disease in the CNS, Berer *et al*., studied the onset of spontaneous EAE under specific pathogen free (SPF) and GF conditions. Authors exploited a relapsing-remitting mouse model expressing a transgenic T-cell antigen receptor recognizing the MOG peptide 92-106, which leads those mice to spontaneously develop EAE within 3-8 months of age. (Berer *et al*., 2011). When observing SPF RR mice, almost all transgenic mice developed RR-EAE, with clinical signs reflecting autoimmune infiltration in the CNS, such as classical paresis due to lesions of the spinal cord and an increase of anti-MOG autoantibody concentration. In contrast, GF RR mice remained fully protected, with no clinical symptoms or neuropathological lesions. Nevertheless, fecal microbiota transplantation from SPF RR mice promptly triggered the production of anti-MOG antibodies and the onset of RR-EAE (Berer *et al*., 2011). This observation suggests that the murine intestine harbours commensal microbial species that trigger activation and

pathogenicity of myelin-specific T cells. In the past decade, several commensal bacterial species that modulate CNS autoimmunity have been characterized. For example, commensal microbes SFB residing in the murine intestine, induce brain autoimmunity by selectively inducing the differentiation of effector Th17 cell (Lee *et al*., 2011). Conversely, *Bacteroides fragilis* prevents intestinal inflammation and brain autoimmunity through a protective mechanism dependent on the PSA contained in its capsule (Mazmanian *et al*., 2008)(J. Ochoa-Repáraz *et al*., 2010). Polysaccharide A signals directly act on TLR2 expressed on $CD4^+$ T cells to induce differentiation of $FoxP3^+$ Treg cells and prevent Th17 expansion in the gut through an IL-10-mediated mechanisms (Round & Mazmanian, 2010). The evidence that some intestinal microorganisms can enhance the disease, while others counteract it, strongly enforces the idea that an aberrant microbial stimulation of the immune response may represent a driving environmental factor of MS. Results in preclinical models stimulated a large body of research in humans aimed at finding possible links between clinical MS and changes in the composition of the intestinal microbiota. A large number of studies revealed that a condition of intestinal dysbiosis is present in individuals suffering from RRMS when compared to healthy controls and, interestingly, those alterations significantly differ between patients with active disease or under remission (J. Chen *et al*., 2016)(Cosorich *et al*., 2017). Despite differences related to geographic area and other confounding factors affecting the gut microbiota, most of the human studies revealed decreased microbial diversity in RRMS patients compared to healthy controls, with an overall altered levels in both Bacteroidetes and Firmicutes *phyla* (Cantarel *et al*., 2015)(Miyake *et al*., 2015). 16S ribosomal RNA sequencing of microbial DNA isolated from small intestinal tissues of RRMS patients revealed an increase in phylum Firmicutes and a decrease in phylum Bacteroidetes. Of interest, this study showed that a reduction in the relative abundance of *Prevotella* is present in RRMS patients with active disease, suggesting that those modification could be linked with activation of brain autoimmunity and onset of MS relapse (Cosorich *et al*., 2017). In line, oral supplementation of EAE mice with human *P.histicola,* but not *P. melaninogenica*, reduces inflammation and demyelination in the CNS through induction of $FoxP3^+$ Treg cells, tolerogenic DCs and suppressive macrophages (Mangalam et al., 2017). Others observed a reduction of the genus *Clostridium* and specifically of Clostridia cluster XIV and IV in patients with RRMS

(Miyake *et al*., 2015). Jangi *et al*., observed that MS patients have increased *Methanobrevibacter* and of *Akkermansia* and decreased levels of *Butyrycimonas* (Jangi *et al*., 2016). Recently, a decrease in the abundance of *Parabacteroides distasonis* in RRMS patients has been detected (Cekanaviciute *et al*., 2017). Furthermore, some studies report a greater abundance of Proteobacteria in line with reports in other autoimmune diseases (J. Chen *et al*., 2016). Although those findings have been crucial to demonstrate that a condition of intestinal dysbiosis occurs in patients suffering from MS, this kind of experimental approach is correlative and failed to establish causal relationship. Indeed, the results reported so far are highly variable, with few similarities, i.e., decrease in *Prevotella* genus, and do not allow to identify a specific pro-inflammatory microbiota profile which is distinctive and referable to MS patients. Furthermore, the descriptive nature of those evidence is unable to causally link the alterations in the microbial profile of those patients to the pathogenesis of this human CNS disease. A direct proof identifying the pathogenic role of the microbiota in modulating MS pathogenesis came from an elegant study conducted in a microbiota-humanized transgenic mouse model. With the aim of further exploring the link between MS and changes of intestinal microbiota, Berer *et al*., demonstrated that the human microbiota components from RRMS patients directly trigger CNS-specific autoimmune disease. They studied the gut microbiota from monozygotic human twins' pairs discordant for MS and observed that when transferred in RR mice expressing myelin-specific T cell receptor, the microbiota from MS-suffering twin triggered the onset of the disease, but not the microbiota transplanted from healthy twin. These results directly provide evidence that human MS-derived microbiota has a pathogenetic role and contains factors that initiate brain autoimmune disease in a humanized RREAE mice (Berer *et al*., 2017). Although it is clear that the gut microbiota plays a pathogenic role in RRMS, the mechanism underlying this effect are largely unknown. The pathogenetic and pro-inflammatory nature of MS-related microbiota could be due to an impairment of the microbial-associated metabolic pathways induced at intestinal level, which normally shape the gut mucosal immunity by maintaining an anti-inflammatory milieu. In particular, gut microbiota-derived metabolites such as SCFAs, tryptophan derivatives or BAMs that are instrumental to maintain intestinal and extra-intestinal immune homeostasis and tolerance can be fundamental in the microbiota-mediated regulation of RRMS. A defective release of

those tolerogenic bacterial products due to imbalances in gut microbial community could be involved in dysregulated immunity, gut inflammation and triggering of autoimmune responses in RRMS.

4.1. Alterations of gut barrier integrity affect brain autoimmunity

Several lines of evidence allowed to consider the intestine inhabited by commensals as a checkpoint for the modulation of adaptive immune cells and studies in pre-clinical models of MS showed that the gut microbiota have a fundamental role in triggering brain autoimmunity. However, the mechanisms underlying the activation of pathogenic extra-intestinal tissue-specific T cells are still object of scientific speculation. In steady state conditions, the interaction between intestinal microbes and immune cells circulating in the gut mucosa is tightly controlled by physical and biological intestinal barriers that maintain immune homeostasis and regulate translocation of bacterial components across the gut mucosa and peripheral tissue. Alterations of the intestinal permeability and microbial dysbiosis appear to be co-occurring events, which trigger a vicious circle responsible for pathogenic cascade in both intestinal and extra-intestinal tissue. Indeed, an intestinal dysbiosis could promote intestinal inflammation, thereby inducing a compromission of the intestinal wall. In turn, the loss of GB integrity may participate to the onset of MS by favouring the pathological interaction between unbeneficial commensals and circulating myelin-reactive T cells. In support to this hypothesis, there are some lines of evidence indicating a breakage of GB continuity in both pre-clinical models and patients suffering from MS. Of note, the loss of GB integrity during EAE is associated with an increase expansion of intestinal Th17 cells and positively correlates with the severity of the disease (Nouri *et al*., 2014)(Secher *et al*., 2017). Pre-clinical models of MS showed increased intestinal permeability both before the onset of clinical EAE and after the establishment of the disease (Nouri *et al*., 2014). Additionally, those alterations of intestinal barrier properties were accompanied by changes in the intestinal wall morphology, i.e., villus length, submucosal layer width and thickness of muscularis mucosa, which increased as the disease occurred. Of interest, along with increased intestinal barrier permeability and alteration of gut tissue morphology, a disturbed intestinal homeostasis was observed, with increased levels of circulating Th17 cells in the lamina propria, PPs and the spleen during the development of EAE. Specifically, the

frequency of Th17 cells was higher already before the onset of the EAE and enhanced after the establishment of the disease. The expansion of those pro-inflammatory effector cells was inversely correlated with the one of FoxP3^{+} Treg cells whose frequency revealed to be lower already before the onset of clinical signs of EAE (Nouri *et al*., 2014).

4.2. Perturbation of mucosal immunity in Multiple Sclerosis

In the perspective of clarifying the mechanisms through which the gut commensal bacteria can trigger the autoimmune response in the CNS, several lines of evidence showed that the intestinal environment enhances the pathogenicity of myelin-reactive T cells by favouring their acquisition of a Th17 cell phenotype. Indeed, although different B and T cell subsets take part in the pathogenesis of EAE, effector Th17 cells are supposed to be the first cell population to infiltrate the CNS (Kebir *et al*., 2007)(Korn *et al*., 2007). Studies in pre-clinical models first allowed to observe a gut microbial modulation of mucosal immunity occurring during autoimmune response in the brain. Indeed, SPF RR mice which spontaneously develop EAE have increased levels of Th17 cells in the intestinal lamina propria (Berer *et al*., 2011). Interestingly, monocolonization of GF mice with SFB, which are well-known inducer of intestinal Th17 cells, drove the development of EAE upon immunization with MOG peptide. In those mice, the Th17 cell response increased in both intestine and spinal cord, thus supporting the hypothesis that autoreactive T cells modulated in the gut mucosa reach the CNS where they govern the autoimmune pathogenesis of the disease (Lee *et al*., 2011). However, mono-colonization with SFB was of low efficiency in restoring CNS autoimmunity in RR GF mice, thus suggesting that in a spontaneous model the pathogenic activation of Th17 cells requires a synergistic effect mediated by gut microbes (Berer et al., 2011). Indeed, although SFB can induce the polarization of T cells toward a Th17 phenotype, spontaneous RR GF mice harbour autoreactive T cells with a TCR that specifically recognize one epitope of MOG peptide, which might not cross-react with SFB antigens, thus avoiding the induction of pathogenic T cell responses. Along this line, it was recently shown that blocking gut homing of myelin-specific Th17 cells confers protection from an adoptive transfer EAE model. Specifically, authors observed an altered intestinal immune response associated with an increase of MOG-specific Th17 cells in the colonic lamina propria of EAE mice. By preventing the migration of those auto-reactive Th17 cells in the intestinal

compartment before reaching the CNS and supplementing mice with antibiotics, Duc *et al.*, showed a reduced severity of EAE, thereby demonstrating that myelin-specific self-reactive Th17 cells are regulated at intestinal level by gut commensal bacteria during the disease (Duc *et al*., 2019). Th17 cells expansion in the gut mucosa could also be an important pathogenic mechanism in clinical MS. In fact, an increased frequency of Th17 cells is present in RRMS patients with active disease and, most importantly, this alteration of gut mucosal immunity correlates with precise modifications of the intestinal microbiota (Cosorich *et al*., 2017). By performing 16s ribosomal RNA sequencing analysis at genus level, authors revealed that RRMS patients with evidence of disease activity had statistically significant higher representation of *Streptococcus* strains, in particular *S. mitis* and *S. oralis*, which are capable to induce Th17 cell differentiation in humans (Engen *et al*., 2014). In addition, the same patients with active disease showed a significant decrease in the relative abundance of *Prevotella*, bacterial genus able to produce the SCFAs propionate, a pro-tolerogenic metabolite that limits gut mucosal Th17 differentiation in mice (J. Li *et al*., 2016). Imbalances in the gut commensal community can lead to impairments in the production of immunoregulatory microbial metabolites. In line, MS patients with dysbiotic gut microbiota also show significantly reduced levels of the SCFAs propionate in serum and feces, particularly after the first relapse (Duscha *et al*., 2020). In this report, authors supplemented propionate to MS patients on top on MS immunotherapy. After two weeks of intake, researchers detected a significant increase of activated Treg cells in the peripheral blood, together with a substantial decrease in Th17 cell differentiation. The modulation at immunological level reflected also in beneficial effects at neurological level with improved clinical parameters such as a reduction in relapse rate and disability progression (Duscha *et al*., 2020). Also, microbial metabolites can have beneficial effect on neuroinflammation. For example, tryptophan metabolites such as indoxyl-3-sulfate, indole-3-propionic acid and indole-3-aldehyde can modulate astrocyte activity and CNS inflammation by inducing AHR signalling and reducing the severity of EAE (Rothhammer *et al*., 2016). Similarly, the endogenous secondary bile acid tauroursodeoxycholic acid (TUDCA) prevents *in vitro* neurotoxic activity of astrocytes and pro-inflammatory polarization of microglia and *in vivo* ameliorates neuropathology (Bhargava *et al*., 2020). TUDCA as well as other secondary BAMs such as DCA have been found decreased in the plasma of RRMS patients (Bhargava *et al*.,

2020). Importantly, plasmatic levels of DCA as well as secondary taurodeoxycholic acid and alpha-muricholic acid are downregulated in concomitance with CNS lymphocytes infiltration and demyelination in a spontaneous model of RREAE, thus suggesting that microbial bile acid metabolism plays a beneficial role in CNS autoimmunity (Bhargava *et al*., 2020) (AK, 2013).

AIM OF THE WORK

Recent studies demonstrated that the maintenance of a balanced mucosal immunity induced by the gut microbiota is instrumental in order to regulate the autoimmune response at sites distal from the intestine, such as the CNS. Remarkably, the microbiota-related metabolic profile is crucial to modulate the immune response at intestinal and systemic level. For example, microbiota-produced secondary BAMs such as DCA and LCA play important immune modulatory functions being capable to shape the differentiation of $Foxp3^+$ Treg cells at expense of effector Th17 and Th1 cells. Although different studies have indicated the strong association between impairments of the intestinal microenvironment (i.e., alterations of the gut commensal community and mucosal immunity) and MS, the mechanisms by which the gut microbiota and the intestinal immune system can modulate the CNS autoimmunity has still to be characterized. Thus, the aim of this work is to assess how the gut microbiota modulates the autoimmune response in MS. Specifically, we want to assess whether microbiota-produced secondary BAMs modulate CNS autoimmunity by altering Teff/Treg cell ratio at intestinal level and systemically. Also, we want to test whether specific alterations of immunoregulatory BAMs and BAM-producing bacteria are present in patients suffering from RRMS and correlate with a pro-inflammatory immunological profile. By integrating clinical observations, multi-omics screening and immunological analysis, we aim at providing novel interpretations of the mechanisms underlying the pathogenesis of MS.

RESULTS

1. PRE-CLINICAL STUDIES

1.1. A pro-inflammatory intestinal environment underlies CNS autoimmunity in EAE

Previous studies revealed that EAE-immunized mice show alterations of the small intestinal environment including damage of GB integrity and changes in mucosal immunity which are detectable already before the onset of the disease and correlate with the clinical progression of EAE (Nouri *et al*., 2014). Furthermore, it was recently observed that encephalitogenic Th17 cells infiltrate the colonic lamina propria before the development of neurological symptoms of EAE (Duc *et al*., 2019). Hence, we asked whether there is a pro-inflammatory environment in the large intestine of EAE-immunized mice, particularly in the early phase of the disease. To this aim, we measured the mRNA level of key cytokines taking part in the regulation of inflammatory response such as IL-6, IL-18, 1L-1β, TNF-α and IL-10 as well as analyzed the immune profile in the large intestine of EAE mice after the induction of the disease and before the clinical onset (i.e., 7 days post immunization). Also, we evaluated the integrity of the GB by measuring gene expression of TJs proteins ZO-1 and Junctional Adhesion Molecule A (JAM-A). We detected a pro-inflammatory intestinal environment at large intestinal mucosal level of EAE mice, with an increased mRNA expression of inflammatory IL-6, IL-18, 1L-1β and TNF-α and decreased levels of tolerogenic IL-10 (**Figure 6**).

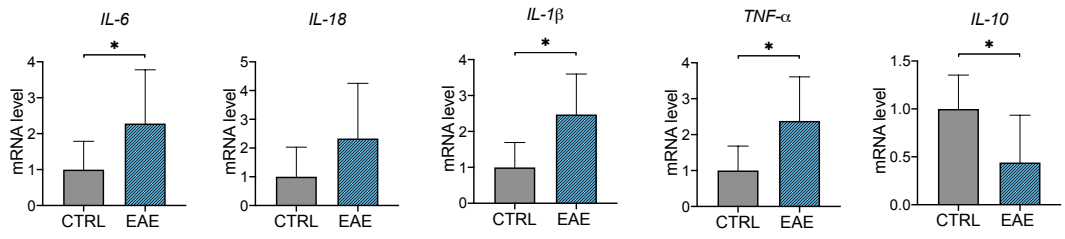


Figure 6. Relative gene expression of IL-6, IL-18, IL-1β, TNF-α and IL-10 in the large intestine of non-immunized C57BL/6 (CTRL) and EAE immunized mice. *Samples were collected at day 7 post-immunization. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=5 per group). Bars represent the mean ± S.D. of fold-change induction over the control group. *p<0.05.*

Immunological analysis of gut mucosal immunity of EAE immunized mice confirmed the pro-inflammatory profile with expansion of effector Th17 cells at the expense of FoxP3^{+} Treg cells (**Figure 7**). The pro-inflammatory gut environment in EAE-immunized mice was associated with GB damage as demonstrated by the decreased expression of TJs ZO-1 and JAM-A (**Figure 8**). Collectively, those data indicate that EAE mice are subjected to alterations of mucosal homeostasis in the large intestine which leads to impairment of the GB integrity and altered Teff/Treg balance towards the expansion of effector pro-inflammatory T cell populations.

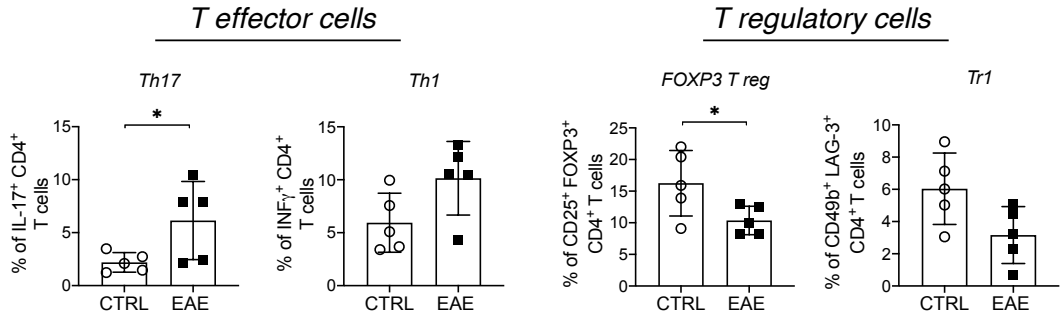


***Figure 7. T helper cell subsets in the large intestine of non-immunized C57BL/6 (CTRL) and EAE immunized mice**. Percentages of CD4^{+}T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the colonic lamina propria of non-immunized C57BL/6 mice (CTRL) and EAE immunized mice (n=5 mice per group). Samples were collected at day 7 post-immunization. Bars represent the mean ± S.D. *p<0.05.*

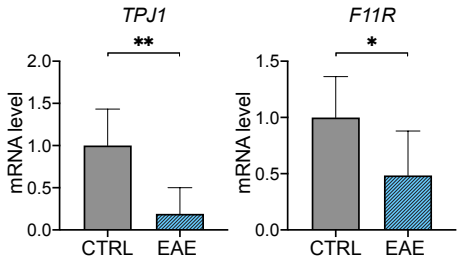


***Figure 8. Relative gene expression of TPJ1 (ZO-1) and F11R (JAM-A) in the large intestine of non-immunized C57BL/6 (CTRL) and EAE immunized mice.** Samples were collected at day 7 post-immunization. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=5 per group). Bars represent the mean ± S.D. of fold-change induction over the control group. *p<0.05, **p<0.01.*

1.2. Secondary DCA and LCA induce a tolerogenic gut environment in C57BL/6 mice

Previous studies showed that increasing concentration of DCA and LCA in the intestine ameliorates gut inflammation in a model of colitis by promoting an anti-inflammatory intestinal environment as well as the differentiation of $Foxp3^+$ Treg cells through the engagement of the TGR5 receptor (Sinha *et al*., 2020b)(Biagioli *et al*., 2017). Simultaneously, DCA and LCA have been shown to be able to limit the effector Th17 and Th1 cell expansion (Hu *et al*., 2021). To test whether increasing intestinal concentrations of immune regulatory BAMs could have a beneficial effect on CNS autoimmunity, we first aimed at creating a tolerogenic BAM-enriched intestinal environment by orally supplementing C57BL/6 mice with an emulsion of DCA and LCA or the vehicle (corn oil) for four weeks (**Figure 9**). After 28 days of treatment, mice were sacrificed and intestinal tissues recovered in order to analyze any changes in the expression of BAM-target receptors, genes encoding inflammatory and tolerogenic cytokines as well as modifications in the mucosal immune cell phenotype.

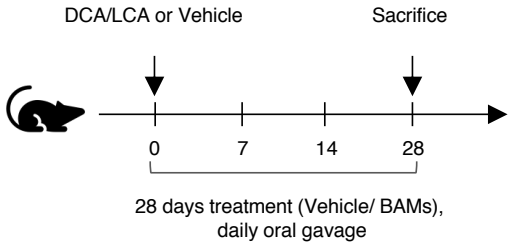


Figure 9. Scheme of BAMs treatment in C57BL/6 mice.
Seven weeks of age C57BL/6 female mice were orally supplemented daily with an emulsion of secondary DCA and LCA microbial bile acid metabolites or the vehicle (corn oil) for four weeks (n=6 mice per group).

Our RT-qPCR data showed an increased expression of BAMs target receptors in the intestinal mucosa of C57BL/6 mice that increased with BAMs administration, particularly significant for TGR5 receptor, thus suggesting that the gut tissue responded to BAMs supplementation (**Figure 10**).

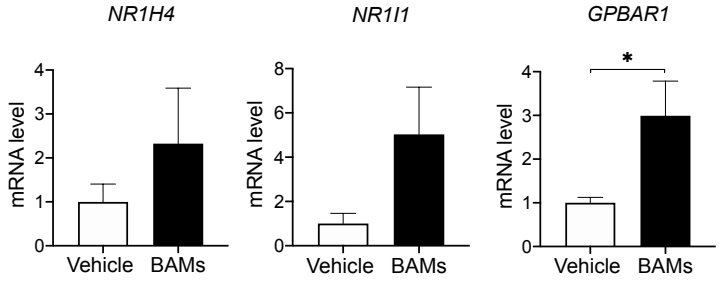


Figure 10. Relative gene expression of NR1H4 (FXR), NR1I1 (VDR) and GPBAR1 (TGR5) in the large intestine of C57BL/6 mice supplemented with BAMs or vehicle.
*Samples were collected after 28 days of treatment. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=6 per group). Bars represent the mean ± S.D. of fold-change induction over the vehicle group. *p<0.05*

Quantitative RT-qPCR analysis of the cytokine profile revealed no differences in the expression of pro-inflammatory cytokines including IL-6, IL-18, IL-1β and TNF-α but rather we detected increased mRNA expression of anti-inflammatory IL-10 after BAMs supplementation (**Figure 11**). Accordingly, when looking at the immunological profile of the large intestinal mucosa we did not detect changes in the frequency of effector Th17 and Th1 cells (**Figure 12**). Instead, we confirmed that oral administration of DCA and LCA promoted Foxp3^{+} Treg cell differentiation in the large intestine of C57BL/6 mice (**Figure 12**). Additionally, we found that IL-10 secreting Tr1 cells, another important regulatory T cell population that differentiate in the intestine, was expanded in the large intestine of BAM-treated mice (**Figure 12**).

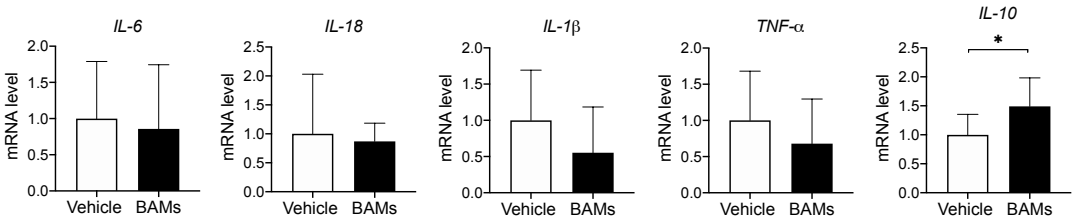


Figure 11. Relative gene expression of IL-6, IL-18, IL-1β, TNF-α and IL-10 in the large intestine of C57BL/6 mice supplemented with BAMs or vehicle. *Samples were collected after 28 days of treatment. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=6 per group). Bars represent the mean ± S.D. of fold-change induction over the vehicle group. *p<0.05.*

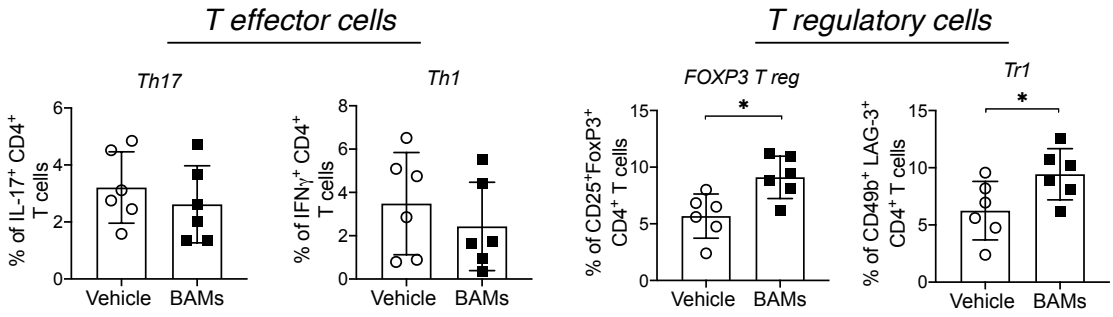


Figure 12. T helper cell subsets in the large intestine of C57BL/6 mice supplemented with BAMs or vehicle. *Percentages of CD4$^+$ T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the colonic lamina propria of C57BL/6 mice treated with BAMs or vehicle (corn oil). (n=6 mice per group). Samples were collected after 28 days of treatment. Bars represent the mean ± S.D. *p<0.05*

1.3. Bile Acid metabolites DCA and LCA prevent EAE

EAE is the most commonly used experimental model for resembling the human MS, since the immunopathological and neuropathological mechanisms which characterize this model allow the establishment of the key pathological features of the disorder, such as inflammation, demyelination, axonal loss and gliosis (Constantinescu *et al*., 2011). To assess whether a BAM-induced tolerogenic gut environment could influence the onset and the clinical course of EAE, we orally administered DCA and LCA starting one week prior to EAE induction through a subcutaneous immunization with a peptide of MOG_{35-55} emulsified in complete Freund's Adjuvant (CFA) with the addiction of pertussis toxin (PT). The immunization procedure triggers the immune response of myelin-reactive T cells at DLN level (i.e., inguinal and axillar lymph nodes), from where these cells migrate to the CNS thus mediating axonal loss and demyelination (Lee *et al*., 2011). The treatment with DCA and LCA was continued for three weeks after immunization and mice were sacrificed at day 24 post immunization as shown in **Figure 13**.

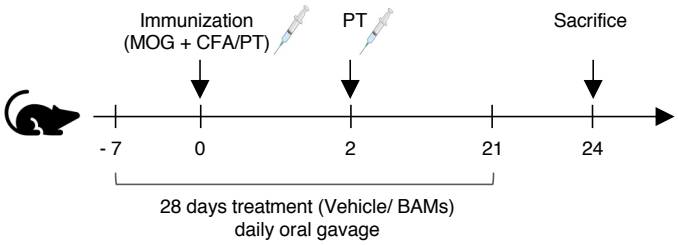


Figure 13. Scheme of EAE induction and treatment.
Seven weeks of age C57BL/6 female mice were orally supplemented daily with an emulsion of secondary DCA and LCA microbial bile acid metabolites or the vehicle (corn oil) starting one week before EAE induction. The treatment has been protracted for four weeks. At day 0 mice were subcutaneously immunized with MOG_{35-55} and CFA together with intraperitoneal injection of PT at day 0 and day 2. Mice were then daily monitored for clinical signs of EAE until day 24 post-immunization.

Interestingly, we found that BAM-treated mice showed attenuated signs of EAE with significantly reduced clinical scores compared to the untreated EAE mice (**Figure 14A**). Moreover, while 100% of vehicle-treated mice developed EAE by day 16 post-immunization, about 30% of BAM-supplemented mice was completely protected and showed no sign of disease up to 24 days post-immunization (**Figure 14B**).

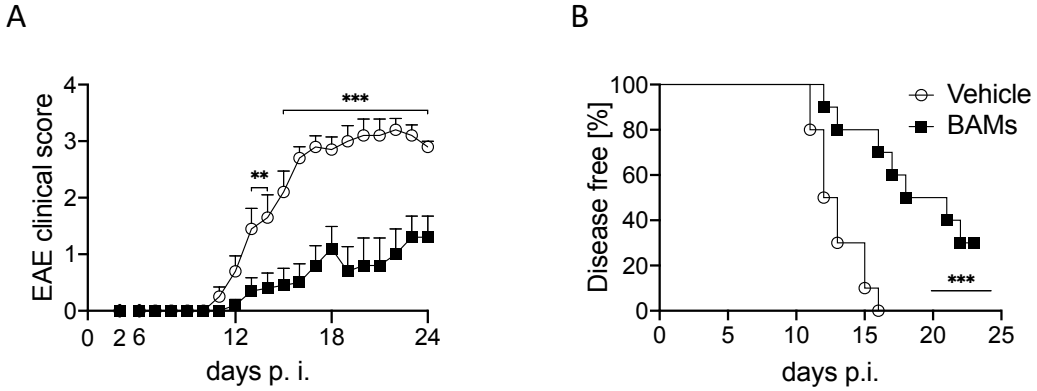


Figure 14. BAM-mediated EAE protection.
*(**A**) Clinical score of MOG_{35-55} mice treated with BAMs or vehicle (corn oil) until* day *24 post-immunization (n=10 mice per group). Scores were recorded daily. Data are expressed as mean ± SEM and were analysed with two-way ANOVA test followed by Bonferroni post-testing for multiple comparisons; (**B**) Percentage of EAE free mice treated with BAMs or vehicle (corn oil) until day 24 post-immunization (n=10 mice per group). Disease incidence was evaluated by log rank [Mantel-Cox] test. *p<0.05, **p<0.01, ***p<0.001.*

In line with clinical protection, our neuro-histopathological analysis showed protection from CNS damage in EAE BAM-treated mice with significant reduced immune cell infiltration in the spinal cord, as well as reduced percentage of demyelination and axonal loss (**Figure 15**). All together those results suggest that BAMs administration protects from CNS autoimmunity.

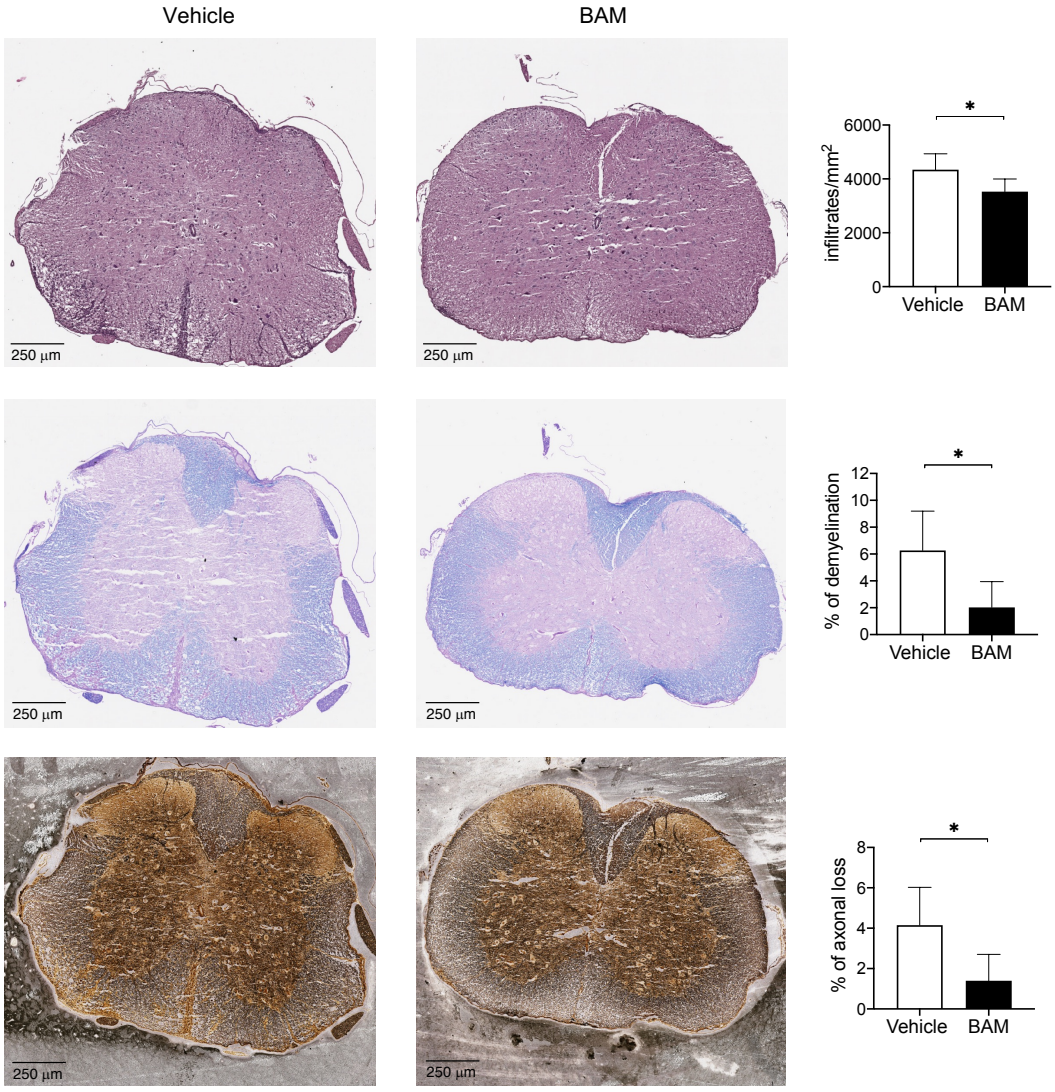


Figure 15. Neuropathology of EAE mice supplemented with BAMs or vehicle.
*Histological analysis of the spinal cord of EAE mice supplemented with vehicle (corn oil) or BAMs (n=5 mice per group). Adjacent sections are stained to detect inflammatory infiltrate (Haematoxylin and Eosin staining), axonal loss (Bielschowsky staining) and demyelination (Kluver-Barrera staining). Samples were collected at day 18 post-immunization. Magnification 20x; scale bar 250 µm. Data were evaluated by two-tailed unpaired t test. Bars represent the mean ± S.D. *p<0.05.*

1.4. DCA/LCA supplementation reduces the pro-inflammatory gut environment and promotes immune tolerance in EAE mice

Since oral administration of secondary DCA and LCA was associated with significant protection of CNS autoimmunity and neurological damage, we asked how BAMs modulated myelin-reactive T cells. Our analysis in untreated EAE mice contributed to further characterize the pro-inflammatory state at large intestinal level occurring during the disease, thus suggesting that intestinal dysfunctions may act to support EAE progression. Hence, we evaluated whether BAMs supplementation promotes a tolerogenic gut environment by reducing the pro-inflammatory response and the Teff/Treg cell ratio at intestinal level in EAE immunized mice. Our data showed that the expression of inflammatory cytokines including IL-6, IL-18, 1L-1β and TNF-α was significantly reduced in BAM-treated EAE mice, while the mRNA expression of tolerogenic IL-10 was significantly enhanced (**Figure 16**).

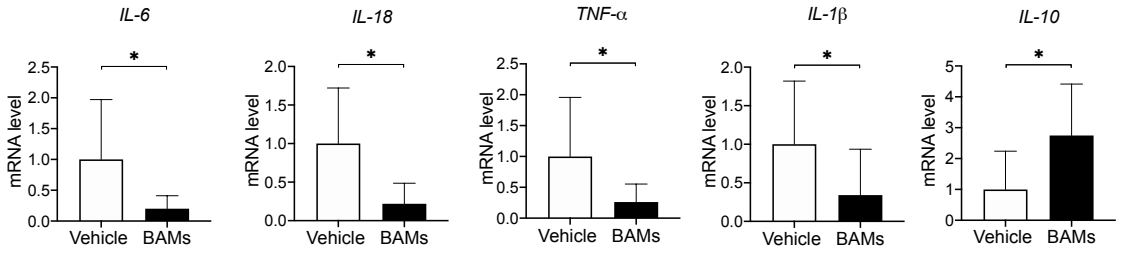


Figure 16. Relative gene expression of IL-6, IL-18, TNF-α, IL-1β, and IL-10 in the large intestine of EAE mice supplemented with BAMs or vehicle. *Samples were collected at day 24 post-immunization. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=10 mice per group). Bars represent the mean ± S.D. of fold-change induction over the vehicle group. *p<0.05.*

A condition of altered gut immune homeostasis has been previously observed in the small intestine of EAE mice (Nouri et al., 2014), thus we asked whether the beneficial effect of DCA/LCA supplementation could extend also in the small intestine. Our data showed a similar trend towards a reduced pro-inflammatory cytokine profile in BAM-treated EAE mice, although the effect was weaker compared to what we observed the in the large intestine (**Figure 17**).

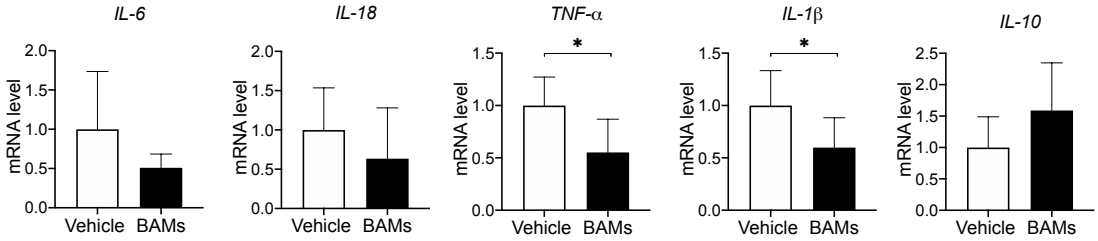


Figure 17. Relative gene expression of IL-6, IL-18, TNF-α, IL-1β, and IL-10 in the small intestine of EAE mice supplemented with BAMs or vehicle. *Samples were collected at day 24 post-immunization. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=10 mice per group). Bars represent the mean ± S.D. of fold-change induction over the vehicle group. *p<0.05, **p<0.01.*

Importantly, BAMs supplementation significantly reduced the relative percentage of effector inflammatory Th17 and Th1 cells while increasing the frequency of both FoxP3$^+$ Treg and IL-10 producing Tr1 cells in the colon of EAE induced mice (**Figure 18A**), and to a less extent in the small intestine (**Figure 18B**). In addition, DCA and LCA enhanced the expression of the intestinal tight junction-related proteins ZO-1 and JAM-A, thus restoring GB integrity in EAE mice (**Figure 19**). Collectively, those data confirmed that oral BAMs supplementation promotes a tolerogenic intestinal environment thus counteracting the pro-inflammatory mucosal immune response and T effector cells expansion related to EAE immunization.

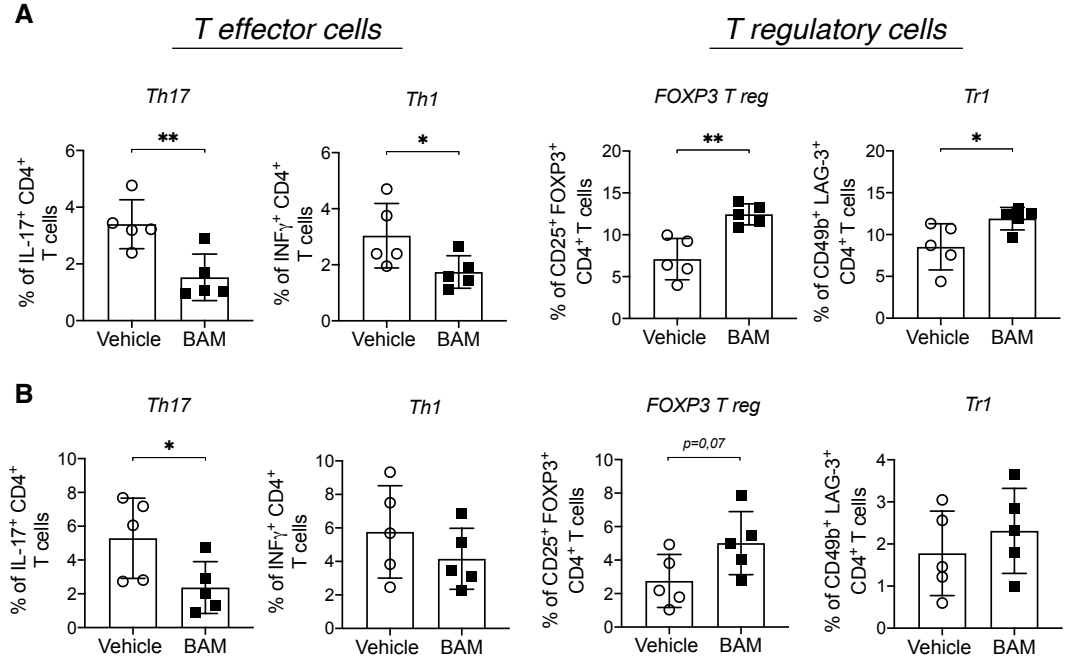


Figure 18. T helper cell subsets in the intestine of EAE mice supplemented with BAMs or vehicle. *Percentages of $CD4^+$ T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the lamina propria of the large intestine (**A**) and small intestine (**B**) of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

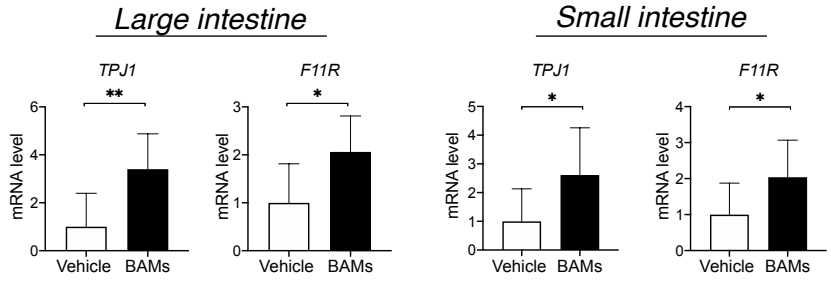


Figure 19. Relative gene expression of TPJ1 (ZO-1) and F11R (JAM-A) in the intestine of EAE mice supplemented with BAMs or vehicle.
*Samples were collected at day 24 post-immunization. Data were normalized to the housekeeping gene RPL32. All samples were quantified in duplicate (n=10 per group). Bars represent the mean ± S.D. of fold-change induction over the vehicle group. *p<0.05, **p<0.01.*

1.5. Secondary DCA/LCA regulates the functional phenotype of myelin-reactive T cells

Our data in the BAM-mediated protection from EAE provide further evidence that alteration of the gut environment is linked to the disease in pre-clinical models and that the modulation of immune response at intestinal level has direct implications on the CNS. Thus, we asked whether the BAM-mediated immune modulation extends beyond the intestinal district thereby impacting the myelin-specific T cells response. To answer this question, we evaluated the immunological profile of T cells isolated from inguinal and axillary DLN as well as from the CNS (i.e., brain and spinal cord). Our data revealed a statistically significant reduction in the relative percentage of effector Th17 cells together with an increase frequency of $FoxP3^+$ Treg cells and Tr1 cells in the DLN of BAMs-treated mice (**Figure 20**). Of note, a subpopulation of effector Th1 and Th17 cells, as well as $FoxP3^+$ Treg and Tr1 cells expressed the gut homing marker $\alpha4\beta7$, thus revealing their intestinal origin (**Figure 21**). Most importantly, BAM-supplemented EAE mice showed

higher percentage of regulatory T cells of intestinal origin ($\alpha4\beta7^+$ $FoxP3^+$ Treg and $\alpha4\beta7^+$ Tr1 cells) (**Figure 21**).

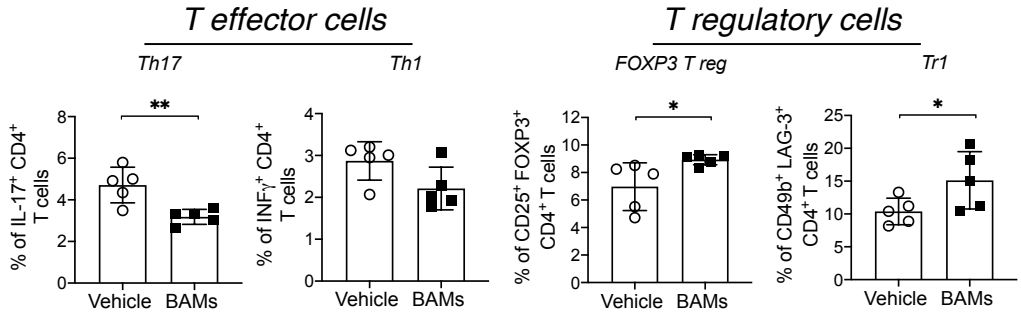


Figure 20. T helper cell subsets in the DLN of EAE mice supplemented with BAMs or vehicle. *Percentages of $CD4^+$ T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in DLN of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

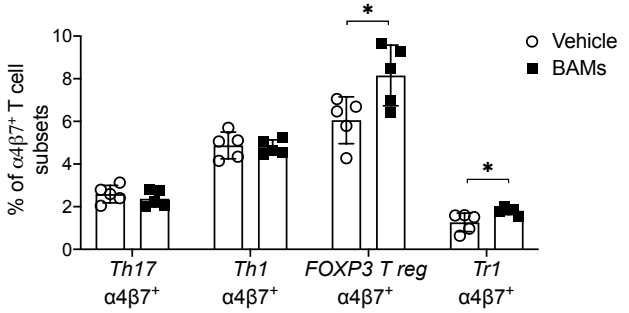


Figure 21. T helper cell subsets expressing the gut homing marker α4β7 in the DLN of EAE mice supplemented with BAMs or vehicle. *Percentages of $CD4^+$ $\alpha4\beta7^+$ T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the DLN of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

When looking at the CNS of BAM-treated mice we did not observe an increase in the relative percentage of $Foxp3^+$ Treg cells and Tr1 cells as in the intestinal mucosa and DLN, but a significant reduction of pro-inflammatory Th1 and Th17 cells (**Figure 22**). A subfraction of T effector and T regulatory cells migrating from the intestine was present even in the CNS, although we did not detect any difference in the relative percentages (**Figure 23**).

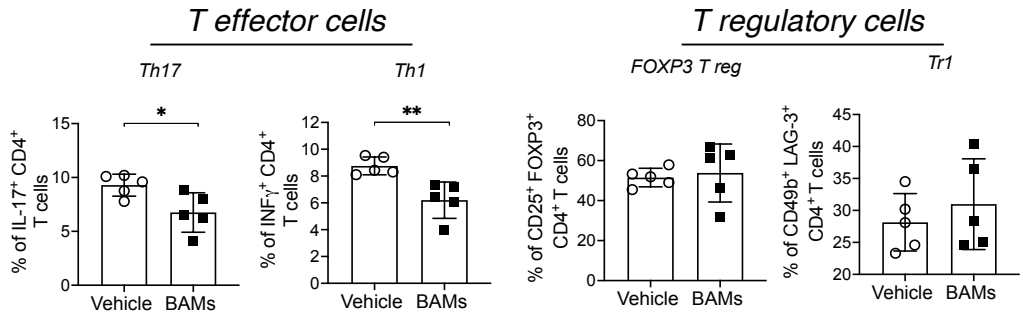


Figure 22. T helper cell subsets in the CNS of EAE mice supplemented with BAMs or vehicle. *Percentages of CD4$^+$T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the CNS (pull of brain and spinal cord tissues) of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Due to the limited number of lymphocytes that are normally isolated from the CNS samples, the absolute cell number was not evaluated. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

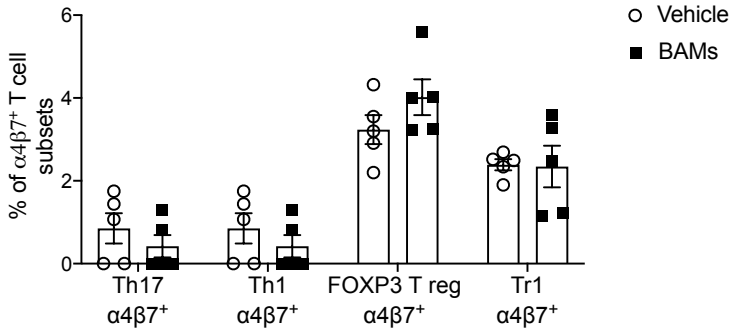


Figure 23. T helper cell subsets expressing the gut homing marker α4β7 in the CNS of EAE mice supplemented with BAMs or vehicle. *Percentages of CD4$^+$ α4β7$^+$ T cells expressing IL-17 (Th17), IFN-γ (Th1), FoxP3 and CD25 (T reg cells) or LAG-3 and CD49b (Tr1 cells) in the CNS (pull of brain and spinal cord tissues) of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Due to the limited number of lymphocytes that are normally isolated from the CNS samples, the absolute cell number was not evaluated. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

Importantly, Th17 and Th1 cells have been identified as a crucial source of GM-CSF which has an essential role in encephalitogenicity and CNS autoimmunity (El-Behi *et al*., 2011)(Restorick *et al*., 2017). Remarkably, while we did not detect differences at intestinal level, we found that the frequency of GM-CSF$^+$ Th17 cells and GM-CSF$^+$ Th1 cells was significantly decrease in the DLN and CNS of BAM-treated EAE mice (**Figure 24**). Collectively, those results suggest that BAMs supplementation enhanced the

differentiation of intestinal T regulatory cells that modulate the functional phenotype and reduce the frequency of pathogenic effector Th17 and Th1 cells as well as GM-CSF$^+$ encephalithogenic T cells in the DLN (i.e., the site of EAE immunization), thus reducing the aggressiveness of auto-reactive T cells in the CNS.

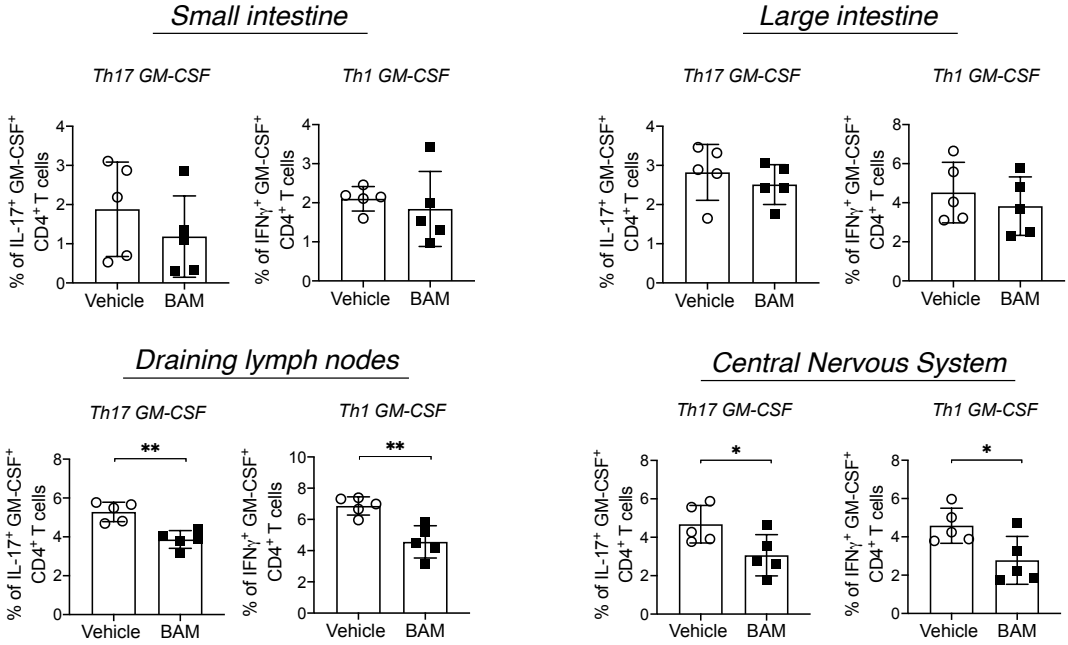


Figure 24. T effector cell subsets releasing the GM-CSF in the intestine, DLN and CNS of EAE mice supplemented with BAMs or vehicle. *Percentages of CD4$^+$ IL-17$^+$ (Th17) and CD4$^+$ IFN-γ$^+$ (Th1) cells releasing the factor GM-CSF in the small and large intestinal mucosa, DLN and in the CNS (pull of brain and spinal cord tissues) of EAE mice supplemented with BAMs or vehicle (corn oil). (n=5 mice per group). Samples were collected at day 24 post-immunization. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

Furthermore, we asked whether FoxP3$^+$ and Tr1 regulatory T cells travelling from the intestine of BAM-treated EAE mice to DLN modulate the functional phenotype of myelin-reactive T cells. To test this hypothesis, we isolated lymphocytes from DLN of BAM-treated and vehicle-treated EAE mice and *in vitro* re-stimulated them with 10μM of MOG peptide for five days (**Figure 25A**). Following cell recover and analysis, we observed a statistically significant reduction of MOG-specific T cells with a decreased percentage of MOG-specific Th17 and Th17 GM-CSF-releasing cells in BAM-treated mice compared to vehicle-treated counterparts (**Figure 25B**). The reduction of myelin-reactive T cells with a GM-CSF$^+$ Th17 phenotype is particularly relevant for protection

from EAE in BAM-treated mice considering that GM-CSF-deficient Th17 cells are uncapable to trigger EAE in recipient mice (El-Behi *et al*., 2011). Hence, BAMs modulate the functional phenotype of myelin-reactive T cells by reducing their pathogenic features, thereby mediating protection against autoimmunity and CNS damage.

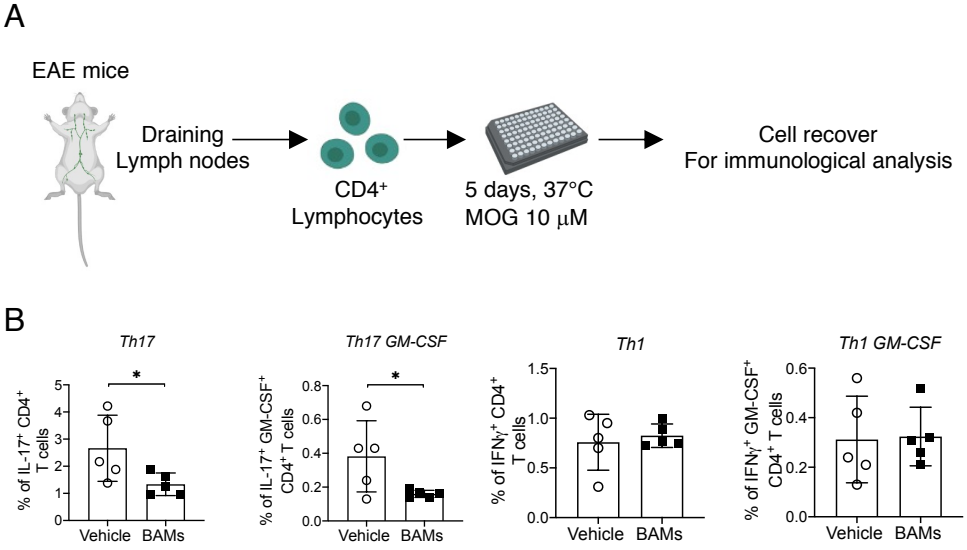


Figure 25. MOG-specific T cells phenotype in the DLN of EAE mice supplemented with BAMs or vehicle. *(**A**) Schematic representation of MOG-recall assay. $CD4^+$ lymphocytes were isolated from DLN (pull of inguinal and sub axillary) of EAE mice treated with BAMs or vehicle (corn oil) at day 18 post-immunization and in vitro re-stimulated for 5 days at 37°C with 10 µM of MOG_{35-55} peptide. After five days of incubation cells were recovered for staining and flowcytometric analysis. The representation was created with BioRender.com. (**B**) Percentages of MOG_{35-55}-specific $CD4^+$ T cells among Th17, Th1, Th17 GM-$CSF^+$and Th1 GM-CSF^+ cells in the DLN of EAE mice supplemented with BAMs or vehicle. (n=5 mice per group). Bars represent the mean ± S.D. *p<0.05*

2. HUMAN STUDIES

2.1. Defective BAMs production in RRMS patients

We previously demonstrated that RRMS patients have alterations of gut mucosal immunity characterized by an higher frequency of intestinal Th17 cells which correlates with gut microbial dysbiosis and high disease activity (Cosorich *et al*., 2017). Moreover, alterations of GB integrity have been observed in RRMS patients (Buscarinu *et al*., 2017). Those findings strongly suggest the presence of an inflammatory intestinal environment in RRMS patients similar to what we observed in the pre-clinical model. Thus, we asked

whether impairments of intestinal immune homeostasis in RRMS patients is associated with alterations of the metabolic environment and, specifically, with reduction of immune regulatory BAMs and BAM-producing bacteria. To investigate this hypothesis, we performed an observational study collecting fecal and blood samples from 20 healthy controls and 20 RRMS patients and measured primary and secondary bile acids concentration together with the metagenomic profile related to BAM-producing bacteria. Simultaneously, we performed immunological profiling of effector and T regulatory T cells subsets in these two cohorts (**Figure 26**). Demographics of study population as well as clinical features of RRMS patients are summarized in **Table 1** and **Table 2**, respectively.

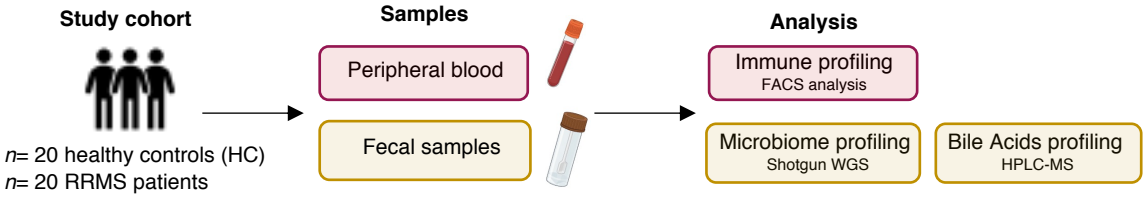


Figure 26. Schematic representation of the study design.
20 healthy controls and 20 patients suffering from RRMS have been enrolled in the clinical study. Patients donated peripheral blood samples together with fecal samples for research purposes. Peripheral blood samples have been processed for immune profiling through multiparametric FACS analysis. Fecal samples have been processed for Bile Acids metabolomic profiling through HPLC-MS and microbiome profiling via shotgun Whole Genome Sequencing (WGS).

Cohort	*n*	Age (mean) ± ST	Sex (Females/ Males)	Other Autoimmune Diseases
Healthy donors	20	39,2 ± 10,9	8/12	NA
RRMS	20	43,8 ± 12	17/3	NA

Table 1. Demographics of study population.
All recruited individuals donated biological samples for research purposes and each subject signed an informed consent. Our RRMS patients cohort was mainly composed by females compared to the HC cohort; however, our correlative analysis did not reveal association between gender and the concentration of DCA and Th17 cell percentage as well as bacteria relative abundance. Data are expressed as mean ± S.D.

MS patient ID	Sex	Age (years)	Disease activity (12 months follow up)	EDSS	Disease Duration (years)	Current Therapy
MS-6	F	38	NEDA	1,5	6	GA
MS-11	F	36	NEDA	1,5	14	Ocrelizumab
MS-13	F	53	NEDA	4	4	TFU
MS-58	F	74	NEDA	2	26	-
MS-76	F	41	NEDA	1,5	16	BG12
MS-86	F	39	NEDA	3,5	1	BG12
MS-87	F	40	EDA	0	1	TFU
MS-88	F	24	NEDA	1	18	-
MS-96	F	42	NEDA	2	20	GA
MS-98	F	43	NEDA	1	2	AZA
MS-99	F	41	NEDA	1	20	BG12
MS-100	F	41	NEDA	1	8	BG12
MS-101	F	70	NEDA	6,5	27	BG12
MS-102	F	41	EDA	1	18	-
MS-103	F	58	NEDA	2	25	TFU
MS-104	F	39	NEDA	1	23	IFN
MS-105	M	45	NEDA	1,5	19	CLA
MS-106	F	30	NEDA	1	10	FTY720
MS-107	M	46	NEDA	2	17	FTY720
MS-108	M	35	NEDA	1,5	8	FTY720

Table 2. Clinical data.
All patients enrolled in this study have a diagnosis of RRMS. None of the patients received corticosteroids in the 3 months before the date of enrolment (time of collection of biological samples). RRMS patients were classified based on the presence or absence of disease activity at 1-year follow up: patients with no evidence of disease activity (RR-NEDA, n=18) and patients with evidence of disease activity (RRMS/EDA, n=2). The population was highly heterogeneous for clinical characteristics with disease duration ranging from 2 to 27 years and different disability (EDSS ranging from 0 to 6.5). Almost all RRMS patients recruited in this study were undergoing different therapeutic immunomodulatory regimens (GA, Ocrelizumab, TFU, BG12, AZA, IFN, CLA, FTY729) at the time of biological samples collection.

Metabolomic analysis via HPLC-MS revealed no differences in the colonic concentration of primary bile acids such as cholic acid, glycocholic acid, taurocholic acid, chenodeoxycholic acid and glycochenodeoxycholic acid between the two cohorts (**Figure 27**). This finding suggests a normal capacity of hepatocytes to produce primary bile acids in RRMS patients. Conversely, we found a highly statistically significant reduction of immune regulatory DCA in RRMS patients (**Figure 27**). Intestinal levels of other secondary bile acids like LCA, taurolithocholic acid, glycoursodeoxycholic acid and taurohyodeoxycholic acid were unaltered in RRMS patients compared to healthy controls (**Figure 27**).

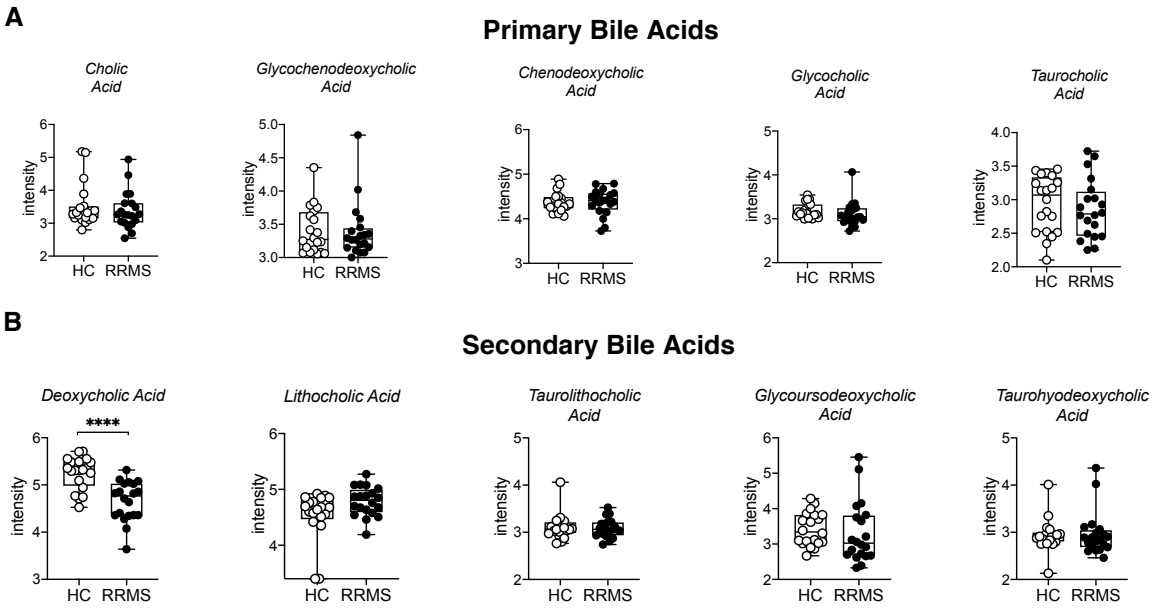


Figure 27. Pool of primary and secondary bile acids detected in fecal samples of RRMS patients and healthy controls.
*(**A**) Box plots of mass-spectrometry peak intensity (logarithmic scale) for 20 healthy controls (HC) and 20 RRMS patients show the concentration of primary bile acids non significantly altered. (**B**) Box plots of mass-spectrometry peak intensity (logarithmic scale) for 20 healthy controls (HC) and 20 RRMS patients show the concentration of secondary bile acids and a significant down-representation of DCA in RRMS patients compared to healthy controls. Data were analysed upon t test among the two experimental sample group. ****p<0.0001.*

Our multivariate analysis considering possible confounding factors due to the heterogeneity of the population demonstrated that the intestinal concentrations of DCA were due to the RRMS condition and were not impacted by age, gender, disease duration

and neurological deficits (**Figure 28**). Similarly, DCA levels did not depend on altered hepatic functions, including bilirubin levels and hepatic enzymes such as gamma-glutamyl transferase (GGT), aspartate aminotransferase (ATS), alanine transaminase (ALT) in our cohort of RRMS patients (**figure 28**).

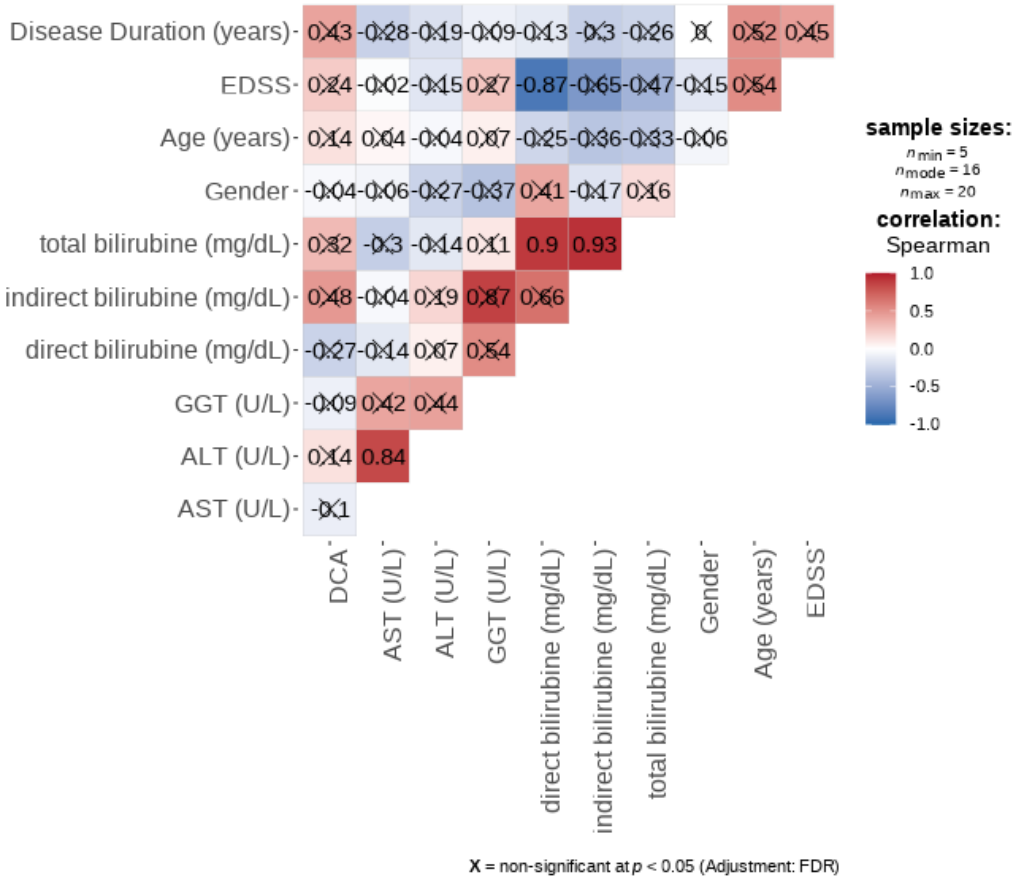


Figure 28. Multivariate analysis among the concentration of DCA, hepatic functions and clinical features of RRMS patients. *Multivariate analysis was performed to find any correlation among the concentration of DCA, hepatic functions (AST, ALT, GGT, direct bilirubine, indirect bilirubine, total bilirubine), clinical data (disease duration and EDSS) and potential confounders such as age and gender. Our data show no correlation between DCA and any of the tested variables related to RRMS patients. Multivariate analysis was performed by Spearman correlation coefficients and adjusted by FDR.*

2.2. Impairments of BAM-producing bacteria in RRMS patients

Since secondary BAMs are generated by microbiota-induced modifications of primary bile acids in the large intestine, we asked whether DCA reduction in RRMS patients was due to a condition of dysbiosis and, specifically, to a lower abundance of BAMs-producing bacteria in the colonic commensal community. The WGS analysis of bacterial DNA extracted from fecal samples revealed no significant difference in the alpha diversity between the two cohorts (**Figure 29**). However, we found significant differences at species level differentially represented in the intestine of RRMS patients and healthy controls (**figure 30A**). Remarkably, our functional analysis of the gut microbiota (analysis of the microbial biochemical pathways based on the Kyoto Encyclopaedia of Genes and Genomics-KEGG*)* revealed that among the bacterial species which we found to be down-regulated in RRMS patients, about 10% was represented by BAM-producing bacteria (**figure 30A**). Accordingly, we found that the abundance of BAM-producing bacteria is significantly reduced in RRMS patients (**Figure 30B**). A list of BAM-producing bacteria which we found to be decrease in our RRMS cohort is shown in **Table 3**.

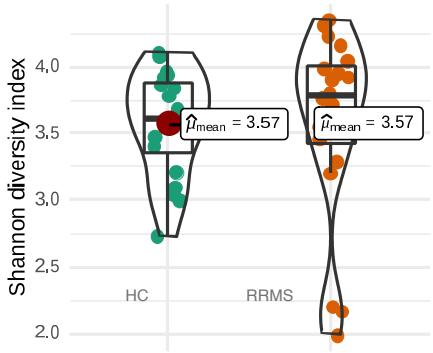


Figure 29. Shannon diversity index.
Violin plots showing alpha diversity in RRMS patients and healthy controls based on Shannon index.

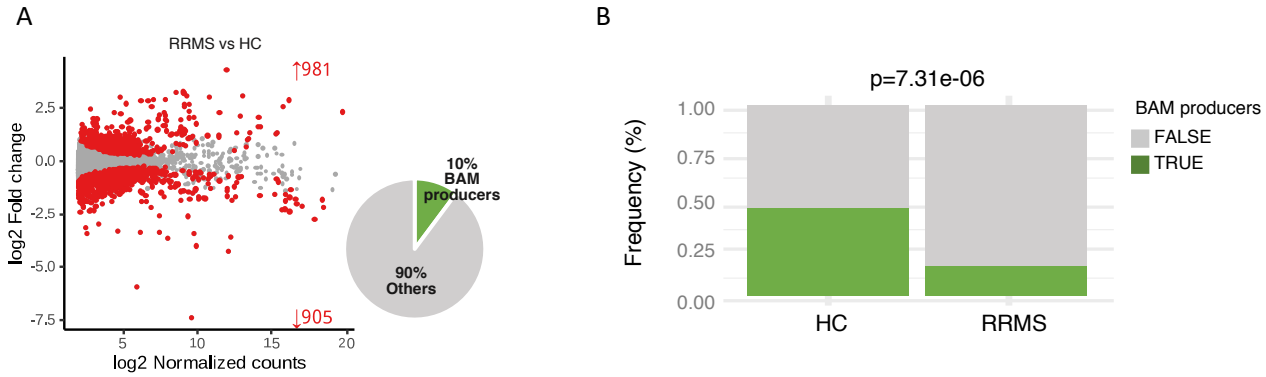


Figure 30. Differential abundance of bacterial species between RRMS patients and healthy controls reveal a decrease percentage of BAM-producing strains. *(**A**) MA plot showing the differential abundance of bacterial species between RRMS patients and healthy controls. Functional analysis of the microbial biochemical pathways based on the Kyoto Encyclopaedia of Genes and Genomics (KEGG) revealed that among the decreased bacterial species, 10% is represented by BAM-producing bacteria. (**B**) Percentage of reads belonging to BAM-producing bacteria in RRMS patients and healthy controls.*

Gene	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Bifidobacterium catenulatum	4291,09659	-4,273958598	0,590997634	-7,231769392	4,76741E-13	7,03289E-10
Amedibacterium intestinale	4821,610963	-3,581256355	0,396181402	-9,039435812	1,57483E-19	3,87197E-16
Bacteroides eggerthii	237389,0186	-2,743431121	0,753842338	-3,639263787	0,000273419	0,005171117
[Ruminococcus] torques	74184,34714	-2,388168276	0,39750707	-6,007863643	1,87984E-09	9,24379E-07
[Ruminococcus] gnavus	58897,45928	-2,271520147	0,472815058	-4,804246623	1,55335E-06	0,000154741
Phocaeicola dorei	357361,5745	-2,177554807	0,687736916	-3,166261337	0,00154412	0,016247402
Bifidobacterium longum	102289,6624	-2,039181585	0,555128225	-3,673352375	0,000239389	0,004696099
Sulfurospirillum sp. ACSDCE	4,528130134	-1,951982422	0,470698923	-4,146987225	3,36879E-05	0,001294176
Lactobacillus paragasseri	18,82337073	-1,931822542	0,377171422	-5,121868808	3,02522E-07	4,55389E-05
Bifidobacterium angulatum	475,9381744	-1,874193121	0,533109378	-3,515588353	0,000438781	0,007051082
Bifidobacterium breve	1461,958327	-1,77900348	0,406227776	-4,379325065	1,19047E-05	0,000605582
Bacteroides xylanisolvens	73186,26944	-1,71352543	0,437488347	-3,916733878	8,97567E-05	0,002418673
Escherichia sp. SCLE84	4,543546299	-1,695882291	0,507332631	-3,342742387	0,000829549	0,010734652
Bifidobacterium pseudocatenulatum	5926,907798	-1,688238612	0,65312939	-2,584845572	0,009742262	0,053140962
Escherichia sp. TC-EC600-tetX4	4,17690953	-1,516191493	0,493056675	-3,075085625	0,002104421	0,019798739
Lactiplantibacillus plantarum	187,9847127	-1,490465938	0,329114415	-4,528716675	5,9343E-06	0,000370944
Latilactobacillus sakei	77,10320998	-1,489428036	0,388517486	-3,833619048	0,000126272	0,003019673
Cloacibacillus porcorum	539,4298972	-1,42550207	0,409897324	-3,477705232	0,000505726	0,00772305
Pedobacter sp. D749	37,39778929	-1,362547133	0,351715002	-3,874009137	0,000107059	0,00269512
Romboutsia sp. CE17	377,4012101	-1,200414292	0,331254381	-3,62384427	0,000290256	0,0053257
Helicobacter mustelae	20,93993616	-1,166214091	0,258404354	-4,513136374	6,38759E-06	0,000379959
Gordonia amicalis	7,17323935	-1,149780291	0,291474836	-3,944698298	7,99005E-05	0,002228385
Turicibacter sanguinis	371,7644	-1,091642483	0,472486008	-2,31042288	0,020864754	0,087194574
Planktomarina temperata	15,92011045	-1,074723005	0,371966771	-2,889298423	0,003861025	0,02906012
Campylobacter sp. RM16192	10,75807769	-1,065963468	0,25361656	-4,203051515	2,63341E-05	0,001055652
Escherichia sp. E4742	30,97495344	-1,056614948	0,369603378	-2,858780551	0,004252729	0,030965574
Lactobacillus gasseri	30,37411506	-1,039255165	0,253511149	-4,099445602	4,14141E-05	0,001482866
Cognaticolwellia beringensis	15,58142919	-1,012565079	0,306526966	-3,303347472	0,000955379	0,011899525
Campylobacter subantarcticus	10,92408563	-1,012105004	0,356116449	-2,842061934	0,004482278	0,031912437
Campylobacter armoricus	14,93395347	-1,010231024	0,286320822	-3,528318401	0,000418209	0,006794511
Arcobacter venerupis	22,32237799	-1,009226968	0,275052332	-3,669218009	0,000243294	0,004747442
Legionella adelaidensis	12,57683442	-1,008843535	0,393034078	-2,566809323	0,010263904	0,055381531
Cellulosilyticum sp. WCF-2	119,4729565	-1,006478894	0,2415506	-4,16674144	3,08985E-05	0,001199511
Paraclostridium bifermentans	195,8562939	-0,972398365	0,238114985	-4,08373444	4,43177E-05	0,001556606
Ligilactobacillus murinus	35,93068337	-0,964363396	0,241619675	-3,991245311	6,57272E-05	0,001939217
[Arcobacter] porcinus	15,66306838	-0,93763817	0,304628441	-3,077973179	0,002084137	0,019678872
Arcobacter ellisii	23,56529989	-0,923068821	0,302576847	-3,050692175	0,002283145	0,020739506
Halarcobacter bivalviorum	33,86166892	-0,917598783	0,302255022	-3,035842966	0,002398643	0,021471344
Sphingorhabdus sp. SMR4y	22,82030683	-0,908615522	0,190915199	-4,759262359	1,94302E-06	0,000168464
Peptacetobacter hiranonis	261,2908936	-0,908094028	0,194411614	-4,670986516	2,99757E-06	0,000212597
Vibrio rotiferianus	16,92403757	-0,89575906	0,30100643	-2,975880144	0,00292149	0,024376597
Escherichia marmotae	40,31516428	-0,873648569	0,376428897	-2,320886034	0,020292996	0,085592667
Psychrobacter sp. P2G3	8,503058026	-0,852809067	0,296508977	-2,8761661	0,004025378	0,029900494
Aliarcobacter skirrowii	23,17049825	-0,847861342	0,270696558	-3,13214674	0,001735331	0,017654901
Legionella spiritensis	30,09956482	-0,843531428	0,272221975	-3,098689695	0,001943785	0,01891472
Flammeovirga yaeyamensis	40,45306481	-0,835897568	0,289535682	-2,887027812	0,003888999	0,029240829
Oceanihabitans sp. IOP_32	26,31775029	-0,835268062	0,213135203	-3,918958723	8,89323E-05	0,002411636
Malaciobacter marinus	21,85854724	-0,817763114	0,307750885	-2,657224252	0,007878701	0,046053987
Sulfitobacter sp. SK011	18,32392675	-0,800312116	0,219883038	-3,639717388	0,000272937	0,005171117
Campylobacter insulaenigrae	13,45638688	-0,787185582	0,350623726	-2,24510073	0,024761667	0,09760235
Mycobacterium shottsii	5,324062391	-0,776487384	0,346932092	-2,238153811	0,025211027	0,098860463
Winogradskyella sp. PC-19	19,98684357	-0,77191324	0,305756996	-2,524597144	0,011583099	0,059662664
Ornithobacterium rhinotracheale	71,96787855	-0,726019507	0,252149113	-2,87932604	0,003985261	0,029662245
Lactobacillus johnsonii	58,96091581	-0,724858127	0,231285172	-3,134044955	0,001724144	0,01758363
Bacteroides caecimuris	4217,956729	-0,714817813	0,267127055	-2,675946895	0,007451845	0,044122749
Bifidobacterium choerinum	70,04458969	-0,70814269	0,28845193	-2,454976428	0,014089383	0,068057162
Enterococcus hirae	53,93231837	-0,707718238	0,285227061	-2,481245063	0,013092433	0,064802011
Limosilactobacillus mucosae	51,140173	-0,696328385	0,189821363	-3,668335182	0,000244135	0,004751292
Prosthecochloris sp. HL-130-GSB	43,60296193	-0,6927757	0,175315552	-3,951592944	7,76327E-05	0,002201019
Fusobacterium mortiferum	42,92499882	-0,690896091	0,200409451	-3,447422698	0,000565962	0,00820145
Planococcus faecalis	9,656371186	-0,675987454	0,301571237	-2,241551484	0,024990375	0,098204051
Aequorivita sp. H23M31	28,04281261	-0,661001208	0,275182737	-2,4020446	0,016303721	0,074879354
Falsihalocynthiibacter arcticus	23,32214075	-0,650279726	0,236221402	-2,752840001	0,005908076	0,037993
Sphingomonas paeninsulae	15,9819758	-0,646016588	0,267691284	-2,413289582	0,015809253	0,073477664
Christensenella minuta	1759,509361	-0,642487148	0,22944734	-2,800150782	0,005107874	0,034852618
Vagococcus fluvialis	57,70540973	-0,637350872	0,201497529	-3,163070413	0,001561146	0,016385866
Wolinella succinogenes	13,51738571	-0,629083581	0,233336863	-2,69603171	0,007017099	0,042482728
Parasedimentitalea marina	19,03692732	-0,62468328	0,219247446	-2,849215767	0,004382715	0,031554672
Campylobacter lari	30,41120784	-0,617671314	0,206049427	-2,997685182	0,002720385	0,023170395
Arcobacter cloacae	18,05321059	-0,616115394	0,251912164	-2,445754838	0,014454925	0,069323492
Sulfurimonas sp. H1576	19,24328169	-0,615267523	0,210263345	-2,926175855	0,003431569	0,027234073
Campylobacter avium	10,79653467	-0,592977794	0,244968172	-2,420631997	0,015493553	0,072559015
Mycobacteroides saopaulense	19,47904024	-0,588240977	0,247339816	-2,378270462	0,017394063	0,077662594
Brucella anthropi	43,58105044	-0,584827287	0,127526179	-4,585939066	4,5195E-06	0,000297642
Lactobacillus ultunensis	21,59274725	-0,583435024	0,190566512	-3,061582105	0,002201706	0,020279708
Cellulosilyticum lentocellum	61,45478963	-0,56657065	0,216615216	-2,615562566	0,00890806	0,050042536
Sulfurospirillum deleyianum	12,75060918	-0,564154161	0,220102372	-2,5631444	0,01037289	0,05567999
Hydrogenovibrio thermophilus	13,57821016	-0,557972864	0,235609137	-2,368214032	0,017874192	0,079088208
Enterococcus cecorum	178,1546818	-0,5496677	0,241649249	-2,274650981	0,022926875	0,092662264
Clostridium bornimense	160,5900332	-0,540521186	0,164983658	-3,276210457	0,001052101	0,01274526
Maribius sp. THAF1	17,37035912	-0,529610515	0,220139614	-2,405793782	0,016137371	0,074271
Sphingorhabdus lacus	22,63655172	-0,513126694	0,183550912	-2,795555131	0,005181066	0,03511649
Clostridium sporogenes	116,5898461	-0,502376423	0,167501607	-2,999233457	0,002706598	0,023079617
Microbacterium wangchenii	24,34015979	-0,501783261	0,220328848	-2,277428791	0,022760632	0,092293801
Listeria ivanovii	42,18919163	-0,499681842	0,191154767	-2,614017162	0,008948454	0,050184323
Aliarcobacter cryaerophilus	41,81980848	-0,498443956	0,18969748	-2,627572897	0,008599641	0,048981432
Lactobacillus amylovorus	45,16006803	-0,483263547	0,212787104	-2,271112948	0,023140141	0,09333066
Nocardia mangyaensis	54,87209211	-0,464901708	0,16483417	-2,820420721	0,004796072	0,033341969
Campylobacter sputorum	25,26939528	-0,46133395	0,170919486	-2,699130214	0,006952097	0,0422634
Nitratifractor salsuginis	35,3610191	-0,413791485	0,173850888	-2,380151698	0,017305512	0,077407798
Martelella sp. AD-3	25,92311159	-0,405129844	0,178396773	-2,270948276	0,023150109	0,09333066
Paraburkholderia xenovorans	30,19676383	-0,384148543	0,158289563	-2,42687221	0,015229619	0,071962634

Table 3. List of BAM-producing bacteria found decreased in RRMS patients.
BAM-producing bacterial strains decreased (-Log2FoldChange) in RRMS patients. Statistically significant differences are shown. p<0.05, FDR<0.1.

2.3. Th17 cells are increased in the peripheral blood of RRMS patients and inversely correlate with the relative abundance of BAM-producing bacteria

Our pre-clinical studies demonstrated that the ability of secondary DCA and LCA in shaping the immune response at intestinal level has beneficial effects also outside the gut, resulting in a reduced expansion of effector Th1/Th17 cells at peripheral level and in the CNS, thus ameliorating the clinical course of EAE. The reduction in the intestinal concentration of DCA together with the decreased abundance of secondary BAM-producing bacteria drove us to test whether the same RRMS patients have alterations in the peripheral immune profile and, specifically, an increased in effector T cells. To explore this hypothesis, peripheral blood mononuclear cells (PBMCs) were isolated from the same patients starting from whole blood by gradient centrifugation and stained for different markers in multiparametric FACS analysis. The immunological profiling revealed a significant increase of effector Th17 cells in RRMS patients, while other T cell subsets including Th1, Th2, $Foxp3^+$ T reg cells, and γδT cells were not altered in PBMCs of RRMS patients (**Figure 31**). Of note, circulating mucosal-invariant associated T (MAIT) cells, a T cell subset with intestinal tissue homeostasis maintaining-properties, were reduced in RRMS patients in accordance with previous findings (**Figure 31)** (Mechelli *et al*., 2021)(Carnero Contentti *et al*., 2019).

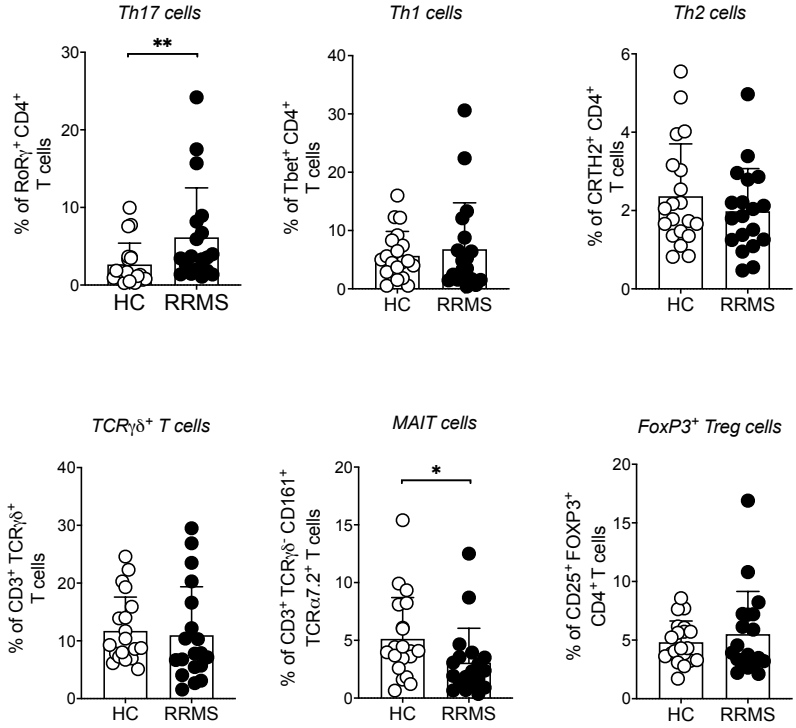


Figure 31. T cell subsets in the peripheral blood of RRMS patients and healthy controls. *Percentages of Th17 ($CD4^+$ $RoR\gamma^+$), Th1 ($CD4^+Tbet^+$), Th2 ($CD4^+CRTH2^+$), Foxp3 T reg cells ($CD4^+$ $CD25^+$ $Foxp3^+$) together with TCRγδ T cells ($CD3^+$ $TCR\gamma\delta^+$) and MAIT cells ($CD3^+$ $TCR\gamma\delta$ $CD161^+$ $TCR\alpha7.2^+$) in the peripheral blood of RRMS patients and healthy controls. (n=19-20 per group). Data were evaluated by Mann-Whitney U test. Bars represent the mean ± S.D. *p<0.05, **p<0.01.*

Importantly, we found that the increased expansion of inflammatory Th17 cells in RRMS patients inversely correlated with the lower abundance of specific BAM-producing bacteria in a specific manner (**Figure 32, Figure 33**). Indeed, such inverse correlation between immune cell subsets and BAM-producing was undetectable for all the other analyzed immune cell populations, which instead showed a direct correlation only with few BAM-associated bacterial strains (i.e., Th1 cells with *Microbacterium wangchenii*, Th2 cells with *Gordonia amicalis*, and MAIT cells with *Brucella anthropi*, *Hydrogenovibrio thermphilus*, *Ligilactobacillus murinus* and *Microbacterium wangcheni*) (**Figure 32**). Those findings suggest that the metabolic intestinal environment and, specifically, the presence of BAM-producing bacteria regulate the relative percentage of effector Th17 cells outside the gut in humans as previously demonstrated in mice.

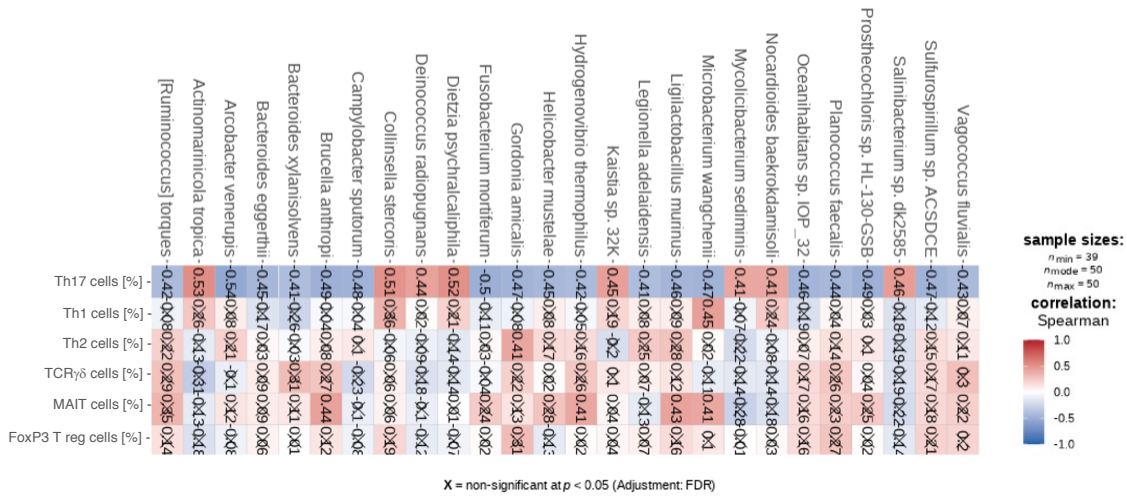


Figure 32. Multivariate analysis between the percentage of different peripheral immune cell subsets and BAM-producing bacteria in RRMS patients and healthy controls.

Multivariate analysis was performed to find correlation between the differential presence of peripheral immune cell subset (Th17, Th1, Th2, TCRγδ, FoxP3 Treg and MAIT cells) and bacterial species associated with BAMs production. The analysis shows an inverse correlation between the increasing percentage of Th17 cells in RRMS patients and the decreased abundance of specific BAM-producers. Multivariate analysis was performed by Spearman correlation coefficients and adjusted by FDR.

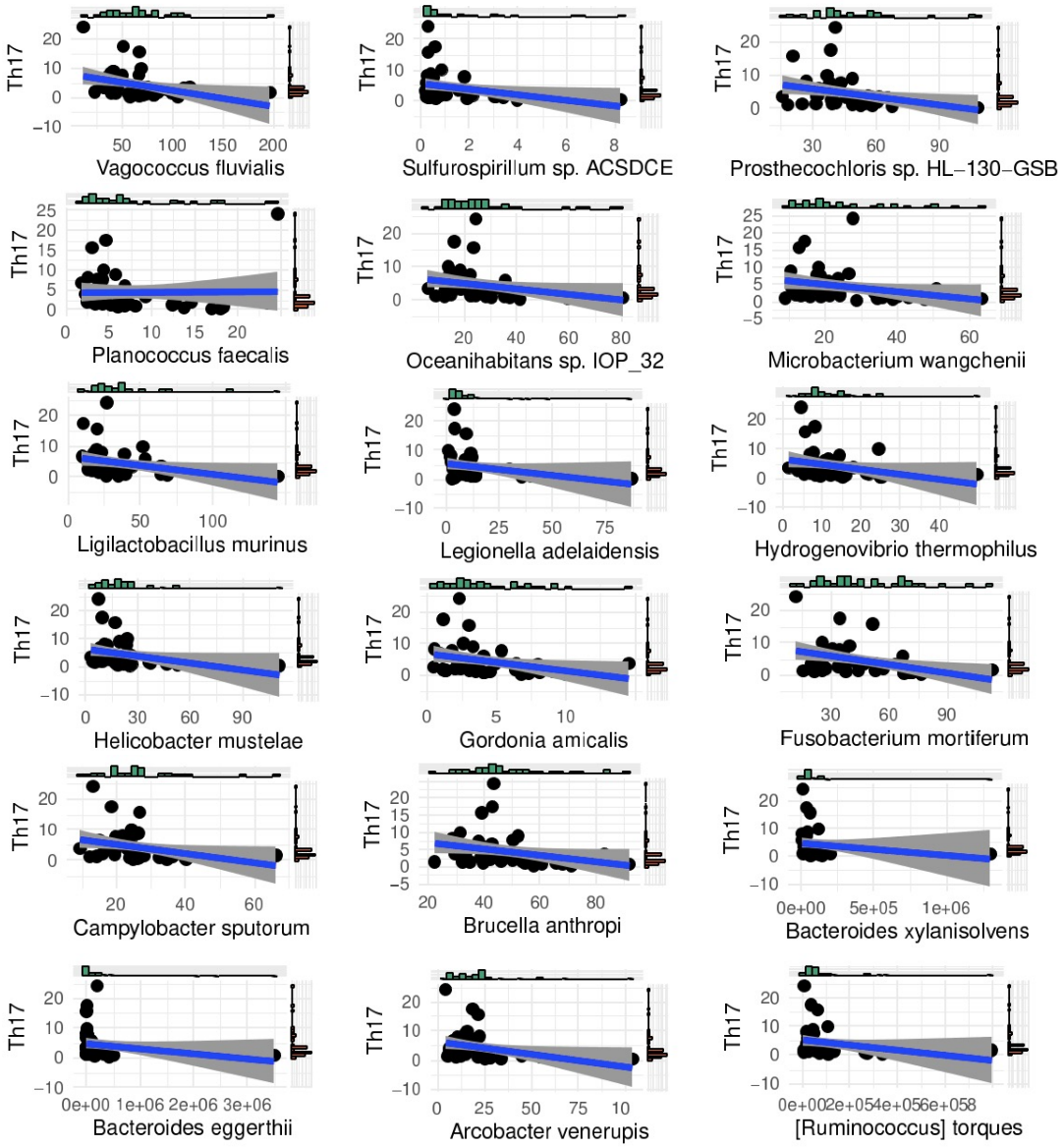


Figure 33. Correlation between the increased percentage of circulating Th17 cells in RRMS patients and the decreased abundance of specific BAM-producing species. *Dispersion plots showing the inverse correlation between the increased percentage of pro-inflammatory Th17 in the peripheral blood of RRMS patients and decreased BAM-producing bacteria.*

DISCUSSION

MS is an autoimmune disease affecting the CNS sustained by an inflammatory process characterized by the breakdown of the BBB and resulting infiltration of myelin-specific T cells into the CNS. Evidence in humans and preclinical models indicate that myelin-reactive T cells are activated and acquire an effector Th17 cell phenotype at peripheral level. The regulation of the balance between pathogenic Th1/Th17 cells and tolerogenic Treg cells is fundamental to modulate the pathogenic potential and aggressiveness of myelin-specific T cells. In support, the rational of the current therapeutic strategies aims at dampening the differentiation of pathogenic effector cell subsets, thereby favoring the mechanisms of immune tolerance. In the past decades, it has been largely established that the intestinal microenvironment plays a fundamental role in modulating the immune system through the presence of the gut microbial community and microbial metabolites, having impact in regulation of extra-intestinal immunity and autoimmunity and Teff/Treg cell ratio. Thus, impairments in the intestinal milieu may support both the pathogenesis and the progression of autoimmune diseases such as MS by affecting the functional phenotype of myelin-specific autoreactive T cells. In support, changes in the gut microbiota composition and gut mucosal immunity, i.e., high frequency of Th17 cells, are detected both in pre-clinical model and RRMS patients and correlate with disease activity (Cosorich *et al*., 2017). However, the mechanisms by which commensal gut microbiota affects the autoimmune response in MS remain to be clarified. Our data indicate that the commensal gut microbiota could regulate CNS autoimmunity by changing the metabolic milieu in the gut. Emerging evidence identify microbial BAMs as key regulators of intestinal homeostasis and gut mucosal immunity, being capable to increase Treg cell differentiation and restore a beneficial Teff/Treg cell ratio. In this context, secondary DCA and LCA have recently drawn the attention of the scientific community for their ability to shape the immune response at both intestinal and extra-intestinal level. Indeed, by binding the immunomodulatory TGR5 receptor, they promote the differentiation of intestinal $Foxp3^+$ Treg cells and the establishment of an anti-inflammatory environment. Secondary BAMs and, specifically, DCA was found reduced in the plasma of a spontaneous model of RRMS, thus suggesting that they could play an important role in CNS autoimmunity (AK, 2013). Nevertheless, their mechanism of action and effect on the regulation of autoimmune response in MS and preclinical models

were never explored. In our experiments we demonstrated that by shaping the mucosal immunity, BAMs can directly affect the CNS autoimmunity in a preclinical model of MS. We observed that oral administration of secondary DCA and LCA significantly protected mice from EAE, as demonstrated by the reduced clinical scores and the increased percentage of disease-free mice. Accordingly, in the spinal cord of BAM-treated mice, we detected fewer immune infiltrates, together with a reduced demyelination area and diminished percentage of axonal loss. We also characterized the mechanism through which microbial BAMs prevented EAE. Specifically, we demonstrated that BAMs administration modulate the gut environment and reduce mucosal immune alterations that normally occur in EAE-immunized mice. Indeed, previous studies demonstrated that EAE mice are characterized by alterations of gut permeability and changes in mucosal immunity including increased intestinal infiltration of Th17 cells concomitantly with a reduction of Treg cell number in the small intestinal lamina propria, and that such alterations correlate with clinical score progression (Nouri *et al*., 2014). Here, we contributed to better characterize the pro-inflammatory events whichoccur in the intestine of EAE immunized mice and found alteration of the GB function (i.e., reduced expression of TJs ZO-1 and JAM-A), abnormal Teff/Treg cell ratio as well as the activation of pro-inflammatory cytokines also in the large intestine. Most importantly, we demonstrated that oral administration of DCA/LCA in EAE mice corrected both the GB damage and intestinal immune dysregulation. In accordance with previous literature we found a significative reduction in the colonic frequency of effector Th17 and Th1 cells and an enhancement of $FoxP3^+$ Treg cells (Hu *et al*., 2021)(Biagioli *et al*., 2017) (Sun et al., 2023). Likewise, we detected reduced Th17 cell percentage in the small intestine and, although with a weaker effect, we observed a similar trend toward an expansion of $Foxp3^+$ Treg cells. Furthermore, we showed that secondary BAMs DCA/LCA downregulate the expression of pro-inflammatory cytokine while increasing tolerogenic IL-10 and Tr1 cell differentiation in the large intestine. Moreover, we demonstrated that BAMs administration leads to amelioration of GB function with increased expression of TJs ZO-1 and JAM-a, an effect possibly related to the correction of the pro-inflammatory gut environment. Indeed, in both small and large intestine we detected decreased expression of TNF-α, a pro-inflammatory cytokine mainly released by Th1 cells and macrophages responsible for increasing paracellular intestinal permeability via a damaging effect on

TJs (Jang et al., 2021)(Gibson, 2004). Of note, despite we observed a reduced frequency of intestinal Th1 cells in BAM-supplemented EAE mice, we cannot exclude a BAM-related anti-inflammatory effect also on mucosal macrophages. In fact, activation of TGR5 on intestinal macrophages determinates their polarization from pro-inflammatory M1 to anti-inflammatory M2 phenotype, which might explain the decreased expression of TNF-α and the upregulation of TJs protein ZO-1 and JAM-a which we detected in the gut mucosa (Biagioli et al., 2017). Previous studies showed that the tolerogenic effect of BAMs extend outside the gut. For example, DCA and LCA can reduce the expansion of peripheral Th17 and Th1 cells, thus protecting against an experimental model of autoimmune uveitis (Hu *et al*., 2021). Here, we confirmed that the BAM-mediated immunomodulatory effects extend beyond the gut thus affecting the autoimmune response in preclinical model of MS. By evaluating the immunological profiling in the DLN of BAM-treated mice, we found an increased percentage of $Foxp3^+$ Treg and Tr1 cells and established that in BAM-treated mice there was an increase of Treg cell subpopulation of intestinal origin, expressing the α4β7 integrin. Importantly, in both DLN and CNS we detected decrease pro-inflammatory Th17 and Th1 cells, together with reduced frequency of encephalitogenic Th17 and Th1 GM-CSF-releasing cells. Our conclusion is that $FoxP3^+$ and Tr1 cells coming from the large intestine reduced the acquisition of a pathogenic Th1/Th17 $GM\text{-}CSF^+$ phenotype by myelin-reactive T cells triggered by MOG immunization. As a result, a reduced number of effector myelin-reactive T cells reached the CNS and triggered myelin destruction. In support to this idea, we found a statistically significant reduction of MOG-specific Th17 and Th17 GM-CSF-releasing cells in the DLN of BAM-treated mice compared to vehicle-treated counterparts. Of note, Th17 cells exhibited a higher proliferative response than Th1 cells upon *in vitro* stimulation with MOG peptide. However, for unknown reasons, our defined culture conditions did not allow expansion of $GM\text{-}CSF^+$ Th1 cells. As we detected Th1 and Th1 $GM\text{-}SCF^+$ expansion *in vivo* in EAE-immunized mice, we cannot exclude that induction of these cell subsets might come from additional inflammatory/antigenic stimuli. Collectively, our findings contribute to reveal the impact that a microbially-related intestinal immune modulation can have on EAE pathogenesis by altering the functional phenotype of myelin-reactive T cells in the periphery. Simultaneously, BAMs could directly reach the CNS and modulate inflammation and tissue destruction locally.

Indeed, other microbial metabolites such as tryptophan derivatives have been found capable to spread from the gut to the systemic circulation and reach the CNS where they direct modulate inflammation and tissue damage. For example, tryptophan derivatives which act as AHR ligands can directly reach the CNS and dampen inflammation by exerting immunomodulatory properties on the resident microglia (Rothhammer *et al*., 2016). In the context of BAMs, those unconjugated metabolites can passively diffuse across the GB and thus, similar to tryptophan derivatives, they could reach the CNS and bind target receptors such as TGR5, thereby locally reduce neuroinflammation. Expression of TGR5 receptor in the BBB, neurons and microglia has been already described and associated to neuroprotection (McMillin *et al*., 2015). Accordingly, *in vitro* activation of TGR5 signaling by the secondary bile acid TUDCA, prevented neurotoxicity in astrocytes and pro-inflammatory polarization of microglia (Bhargava *et al*., 2020). Although the oral administration of TUDCA acid ameliorated the clinical course of EAE, authors did not explore the effect on systemic autoimmunity and the possibility of a direct diffusion of such secondary bile acid metabolite from the intestine to the CNS remains to be tested (Bhargava *et al*., 2020).The key role of BAMs in preventing brain autoimmunity is further confirmed by our results collected in RRMS patients. We detected a highly significant reduction of immunomodulatory DCA in our cohort of RRMS patients which was not impacted by confounding factors such as age, gender and neurological deficits. RRMS often have defective hepatic function related to assumption of immune modulatory drugs. However, the hepatic function (bilirubin, AST, ALT and GGT) as well as the concentration of primary bile acids between RRMS patients and healthy controls were unaltered. This observation suggests that the reduced levels of DCA were not linked to hepatic function impairment but rather to the presence of a defective microbial community in the colon of RRMS patients, with decreased abilities to transform primary bile acids into secondary ones. In support to this view, we found changes at taxonomy species level in the microbiota composition of RRMS patients, characterized by a reduction of BAM-producing bacteria (i.e., BSH and 7-α-dehydroxylating bacteria). Most importantly, in RRMS patients the decreased abundance of BAMs producing species inversely correlated with a peripheral significant increase of Th17 cells. Thus, what we conclude is that a reduction of BAM-producing bacteria results in decrease colonic DCA concentration and, in turn, in an expansion of effector Th17

cells in RRMS patients. Our finding could have important therapeutic implications. In fact, intestinal dysbiosis can be reversed in RRMS patients in order to change the microbiota composition and increase the production of secondary BAMs to reduce gut immune alterations and peripheral Th17 cell expansion (that may regard also myelin-reactive T cells). These modifications could have a beneficial impact on the intestinal concentration of BAMs, which by regulating the Teff/Treg cell ratio at intestinal and systemic level, can in turn positively affect CNS autoimmunity in humans, possibly by reducing clinical MS relapses. For example, a preventive oral administration in models of autoimmune arthritis of the single probiotic *B. pseudocatenulatum*, a BAM-producer which we found to be decreased in our cohort of RRMS patients (Table 3), significant increases the concentration of secondary DCA and LCA, thus ameliorating the disease course by suppressing peripheral Th17/Th1 cell population while promoting $FoxP3^{+}$ T reg cell expansion (Zhao *et al*., 2023). Certainly, with a view to a potential therapeutic approach on top on the immunomodulatory drugs currently used, attention should be paid to a possible state of general immune suppression in the gut that could increase host susceptibility to infections. In the contest of CNS autoimmunity, it remains to be elucidated in which disease phase those microbiota modifications aimed at determining a proper concentration of BAMs and a correct balance in mucosal immunity could be relevant. Indeed, in preclinical model of MS, manipulation of the microbiota prior to the development of the disease either by antibiotics or diet modulate disease severity and incidence (Seifert et al., 2018)(Berer et al., 2018). Nevertheless, antibiotic treatment are not beneficial in a therapeutic regimen (Gödel et al., 2020). In such overview, our data in the mouse model showed that supplementation of BAMs applied prophylactically, thereby affecting the initial activation of autoreactive T cells, protects from EAE severity but remains to be clarified whether BAMs also attenuate ongoing EAE. Hence, further studies are required to investigate the effect of BAMs in clinically established neurological disease and, in so doing, to identify whether the anti-inflammatory properties of such microbial metabolites can also impact the immunological processes during established disease. Furthermore, additional investigation into the role of these bile acid microbial metabolites when singularly administered is needed to better understand their ability to mitigate brain autoimmunity. This characterization will be crucial to understand whether the protection we observed in the EAE model is a result of

a synergic effect of BAMs or whether the beneficial effect of one outweighs the other in the modulation of autoimmune response. Our metabolomic analysis revealed a relevant decrease concentration of DCA in RRMS patients while no difference in the level of LCA, thus suggesting that DCA might have a more prominent role in counteracting the autoimmune response against the CNS. Although further studies are required to assess the effect of BAM-producing bacteria on RRMS and pre-clinical models and to eventually optimize a probiotic formula to enhance relative abundance of BAM-producing species in the intestine of RRMS patients, we propose that the modulation of gut immunity can be a valid approach to prevent MS relapses in combination with current disease-modifying treatments (DMT).

MATERIALS AND METHODS

1. PRE-CLINICAL STUDIES

1.1. Mice and treatment

Female C57BL/6 mice, 6 weeks old, were purchased from Charles River Laboratories. All mice were maintained in specific-pathogen free conditions, in the animal facility at San Raffaele Scientific Institute (Milan, Italy), and all experiments were conducted in accordance with the Institutional Animal Care and Use Committee in accordance with the rules of the Italian ministry of Health. After a period of acclimatization (one week), mice were orally gavaged daily with an emulsion of secondary LCA (Sigma) and DCA (Merck) (50 mg/Kg) or the vehicle corn oil (Sigma-Aldrich), starting on day 7 before the immunization. The treatment was continued for four weeks.

1.2. Induction and clinical evaluation of EAE

EAE was induced by subcutaneous injection of an emulsion containing 200 μg MOG35-55 (Espikem) in Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich) supplemented with 4 mg/mL Mycobacterium tuberculosis (strain H37Ra; Difco). Pertussis toxin (500 ng, List Biological Laboratories) was injected i.v. on the day of immunization and again 2 days later. Mice were daily weighed and scored for clinical signs of EAE up to the day of sacrifice. Clinical assessment of EAE was performed according to the following scoring criteria: 0, healthy; 1, limb tail; 2, ataxia and/or paresis of hindlimbs; 3, paralysis of hindlimbs and/or paresis of forelimbs; 4, tetraparalysis; and 5, moribund or death. EAE mice were sacrificed for MOG-antigen recall assay at day 18 post immunization and for the analysis of tissue-infiltrating cells at day 24 post immunization.

1.3. Histopathological evaluation

For neuropathological analysis mice were transcardially perfused with 4% paraformaldehyde. Spinal cords tissues were isolated and post-fixed in the same fixative overnight, washed in PBS and then embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E), Bielschowsky, and Luxol fast blue (Kluver Barrera) staining methods to reveal the perivascular inflammatory infiltrates, the axonal loss and demyelinated areas, respectively. Stained slides were scanned using Aperio's ScanScope CS for digital image acquisition. Neuropathological findings were quantified on an average of 10 complete cross-sections of spinal cord per mouse collected at 9 different levels of the spinal cord by using QuPath softwer (Bankhead *et al*., 2017). The number of inflammatory infiltrates were calculated and expressed as the numbers of inflammatory infiltrates per mm^2; demyelinated areas and axonal loss were expressed as percentage of damaged area per mm^2.

1.3.1 Haematoxylin -Eosin staining

For H&E staining, sections were deparaffinized and rehydrated in xylene (1 hour), ethanol 100%, ethanol 70%, ethanol 50% (5 minutes per step) and H_2O (5 minutes). Sections were then stained with haematoxylin for 30 seconds to colour cell nuclei. After a 10 minutes wash with tap water, Sections were stained for 1 minute in a solution of eosin for cell cytoplasm identification and finally dehydrated following the alcoholic scale (5 minutes per step), after which slides were mounted.

1.3.2 Bielschowsky staining

For Bielschowsky staining, sections were deparaffinized and rehydrated in xylene (1 hour), ethanol 100%, ethanol 70%, ethanol 50% (5 minutes per step) and H_2O (5 minutes). Sections were then places in a pre-warmed (40°C) silver nitrate solution (20 g AgNO3 in 200 mL of deionized H_2O) and stained for 20 minutes. Ammonium hydroxide was added to the silver solution until the precipitate was clear and sections were incubated in the dark for 15 minutes, followed by wash in deionized H_2O. Sections were then incubated in a solution containing 20 mL formalin at 37%, 100 mL deionized H_2O, few microliters

of concentrated HNO_3 and 0.5 citric acid for about 1 minute. Sections were washed and quickly dipped into a 5% solution of sodium thiosulfate to stop the reaction. Finally, sections were de hydrated with a scale of alcohols (ethanol 50%, ethanol 70%, ethanol 100%, xylene; 5 minutes per step) and slides were mounted.

1.3.3 Kluver Barrera staining

For Kluvera Barrera staining, sections were hydrated to 95% EtOH (10 minutes) than incubated in Luxol fast blue solution 0.1% at 60 C° overnight. The following day, after complete cooling, the excess of Luxol Fast Blue was eliminated by washing for 10 minutes in ethanol 96% followed by deionized H_2O. Slides were differentiated in a 0.1% lithium carbonate solution for 30 seconds. Differentiation continued in 70% ethanol for 30 seconds. After a wash in distilled water, slides were incubated in periodic acid 0,8% (10 minutes), followed by washing in distilled H2O (1X5') and incubation for 20' in Shiffs reagent (Merck). Sections were then washed in solution containing 20 ml of 10% potassium disulfite, 5 ml of 37% HCl and 500 ml deionized H2O and then under tap water. Finally, sections were de hydrated with a scale of alcohols and slides were mounted.

1.4. Real-time qPCR

Immediately after the mice were sacrifice, intestinal tissues were flushed with PBS, opened longitudinally, and placed in 500 μL of TRIzol reagent (Life Technologies). Colonic sections were homogenized by using TissueRuptor (QIAGEN) and RNA was extracted by adding 10 μL of chloroform, precipitating the aqueous phase with 300 μL of 70% ethanol, and purifying RNA with RNeasy Mini Ki (QIAGEN). RNA was retrotranscribed with SuperScript III First-Strand Synthesis System following manufacturer's inctructions (Life Technologies). Real-time qPCR assay was performed with SYBR Select Master Mix (Life Technologies) using primers specific for different cytokines, receptors and TJs on a Viia 7 real-time PCR System (Life Technologies). Transcript expression of individual genes was normalized to the expression of Rpl32.

List of primers:

NR1H4 forward: TCACCTGTGAGGGCTGCAAA
NR1H4 reverse: ACACTGGATTTCAGTTAACAAACCT

GPBAR1 forward: ATGGAGCCGGAACCATCAGG
GPBAR1 reverse: CAGCAGATTGGCAAGCAGGG

NR1l1 forward: GCCTCCAATTCGTGCAGACG
NR1l1reverse: GGTCACAGAGGGGTCATCGG

IL-6 forward: ACAAAGCCAGAGTCCTTCAGAGA
IL-6 reverse: AGGAGAGCATTGGAAATTGGGGT

IL-18 forward: CTTGGCCCAGGAACAATGGC
IL-18 reverse: CGGTTGTACAGTGAAGTCGGC

IL-1β forward: TGCCACCTTTTGACAGTGATGA
IL-1β reverse: TGCCTGCCTGAAGCTCTTGT

TNFα forward: CTGTAGCCCACGTCGTAGCA
TNFα reverse: GTGTGGGTGAGGAGCACGTA

IL-10 forward: TGGGTTGCCAAGCCTTATCG
IL-10 reverse: CTCTTCACCTGCTCCACTGC

TPJ1 forward: ACCCGAAACTGATGCTGTGGATAG
TPJ1reverse: AAATGGCCGGGCAGAACTTGTGTA

F11R forward: GTAATGGGCACCGAGGGGAA
F11R reverse: ACACCGAACCCTTGCCTTGT

RPL32 *forward*: AAGCGAAACTGGCGGAAAC
RPL32 *reverse*: TAACCGATGTTGGGCATCAG

1.5. Immune cell isolation

At indicated time of sacrifice, mice were anesthetized and transcardially perfused with a physiological solution and the intestine, DLN (inguinal and axillary), brain and spinal cord tissues were collected. Lymphocytes were isolated from the intestinal lamina propria using the Lamina Propria Dissociation Kit following manufacturer's instructions (Miltenyi Biotec). For CNS cells isolation, both brain and spinal cord tissues were cut into small pieces, homogenized and incubated in HBSS w Calcium and Magnesium) containing 0.4 mg/mL Collagenase Type IV (Sigma) and 10% FBS at 37°C for 30

minutes. The digested tissues were washed and resuspended in 9 mL PBS 1X in a 15 mL-Falcon tubes. A 90% Percoll (GE Healthcare) solution was prepared, and 4,5 mL of such solution were overlaid on 9 mL PBS 1X containing digested tissues. Percoll gradient separation was performed by centrifugation at 10800 rpm for 30 minutes at 4°C. After the centrifuge, the layer of myelin was aspirated and eliminated while the rest of the sample containing CNS immune cells was resuspended in PBS 1X. Immune cells from DLN were isolated by mechanical disruption of the tissue. Cells isolated from each organ were stimulated for 4 hours with Leukocytes Activation Cocktail with BD GolgiPlug™ following manifacturer's instruction (BD Bioscience) and stained for flow cytometry analysis.

1.6. MOG-specific T cell recall assay

Lymph nodes were isolated from EAE mice and smashed using a 70 μm cell strainer. Cells were washed in 1X PBS and counted. 2,5 x 10^5 cells for each condition were plated in a 96 multi-well plate (round bottom) and incubated at 37°C for 5 days in the presence of 10 μM of MOG35-55. Stimulation with Dynabeads™ Mouse T-Activator CD3/CD28 (ThermoFisher Scientific) was used as a positive control. Untreated cells were included in the assay to assess background activation. After 5 days, cells were stimulated for 4 hours with Leukocytes Activation Cocktail with BD GolgiPlug™ following manufacturer's instruction (BD Biosciences) and stained for flow cytometry analysis.

1.7. Flow Cytometry

For the analysis of tissue-infiltrating cells, cells were suspended in PBS and dead cells were stained with the Viakrome 808 Fixable Viability Dye (Beckman coulter). Cells were subsequently washed and resuspended in staining buffer containing PBS, 1% FBS and 0.09% NaN_3. Cells were first stained for surface markers, then fixed and permeabilized using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). After the permeabilization, cells where stained for intracellular cytokines and intracellular Foxp3. The following antibodies were used: BV510 anti-mouse CD45 (30-F11, BioLegend), Alexa Fluor 700 anti-mouse CD4 (RM4-4, BioLegend), FITC anti-mouse CD25 (PC61, BioLegend), PE-Cy7 anti-mouse LAG-3 (C9B7W, BioLegend), APC-Cy7 anti-mouse

CD49b (DX5, Biolegend), BV650 anti-mouse LPAM-1 (DATK32, BD Bioscience), Alexa Fluor 647 anti-mouse IL-17 (TC11-18H10, BD Bioscience), PerCP-Cy5 anti-mouse IFNγ (XMG1.2, BD Bioscience), PE anti-mouse GM-CSF (MP1-22E9, BD Bioscience), Pacific Blue anti-mouse Foxp3 (FJk-16s, Invitrogen). Flow cytometry was performed using CytoFLEX LX and data were analyzed with FlowJo™ Software (v.10.8.1) (BD Life Science) using unstained and single-stained compensation beads as compensation controls.

For the analysis of MOG-specific T cells, after the stimulation cells were suspended in PBS and dead cells were stained with Fixable Viability Dye eFluor 506 (eBioscience). Cells were subsequently washed and resuspended in staining buffer containing PBS, 1% FBS and 0.09% NaN_3 for the staining of cell surface markers. Cells were then fixed and permeabilized by using the BD Cytofix/Cytoperm kit, and finally stained for intracellular cytokines. The following antibodies were used: FITC anti-mouse CD3 (17A2, BD Bioscience), PerCP-Cy5.5 anti-mouse CD4 (RM4-5, BD Bioscience), APC-Cy7 anti-mouse CD45 (30-F11, Invitrogen), Pacific Blue anti-mouse IFNγ (XMG1.2, BD Bioscience), Alexa Fluor 647 anti-mouse IL-17 (TC11-18H10, BD Bioscience), PE anti-mouse GM-CSF (MP1-22E9, BD Bioscience). Flow cytometry was performed using FACSCanto III and data were analyzed with FlowJo™ Software (v.10.8.1) (BD Life Science) using unstained and single-stained compensation beads as compensation controls.

2. HUMAN STUDIES

2.1. Human subjects and sample collection

The study cohort included 20 RRMS patients and 20 healthy controls. Each subject provided fecal samples that were collected in DNA/RNA Shield Fecal Collection Tubes (Euroclone) for subsequent metagenomic analysis or snap-frozen and used for metabolomic analysis. Blood samples from the two groups of patients were collected in EDTA-anticoagulated vacutainers and immediately processed for PBMCs isolation. All individuals signed a written informed consent and were aware that they donated blood and stool samples for research purpose. Analysis of the samples was approved by the

Institutional Ethical Committee of the IRCCS San Raffaele Scientific Institute (Protocol: MS-GUT2019), Milan, Italy.

2.2. Metagenomic analysis

DNA was extracted from fecal samples using the QIAamp powerFecal Pro DNA Kit (Qiagen) following the manufacturer's instructions. Sequencing libraries were prepared using the Nextera DNA Flex Library Preparation Kit (Illumina), following the manufacturer's guidelines. Sequencing was performed on the Illumina NovaSeq 6000 platform following the manufacturer's protocol on an S4 flow cell for 150bp paired-end. For human microbiota analysis, microbial reads were discriminated against human reads with BMTagger (ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/). To perform the analysis at species/family/order levels, reads were mapped to the collection of all available genomes (https://www.ncbi.nlm.nih.gov/genome/) with Kraken2 for exact alignment of k-mers and accurate read classification (Wood & Salzberg, 2014). Species alpha diversity index was calculated with vegan (https://cran.r-project.org/web/packages/vegan). Visualization was performed with ggplot2 and Krona. Before statistical analysis, classified reads were double-checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to confirm quality filtering and adaptor trimming, and then submitted to BLAST (Johnson *et al*., 2008) to exclude possible artifacts resulting from the *in silico* analysis. Read calls will be confirmed by manually aligning Kraken2 classified reads to the respective genomes with Bowtie2, and visualizing the resulting BAMs alignments with the Integrative Genomics Viewer (IGV)(Thorvaldsdóttir *et al*., 2013).

2.3. Metabolomic analysis

300 µl Isopropanol were added to 100 mg of fecal samples along with Internal Standards (IS). 1 µg of each IS (Tryptophan(indole)-d5; Phenylalanine-15N; Stearic Acid-d3; Sphingosine(d18-1)d7; Glucosyl(β)sphingosine-d5; C16-Glucosylcarbamide-d3; Phosphoetanolamine-d4) were added to each sample with 20 µl of Ceramide LIPIDOMIX (Avanti Polar). Samples were vortexed for 2 min to obtain a homogeneous mixture, sonicated on ice for 5 min, then centrifuged at 14000g for 15 min at 4 °C. The

supernatants were filtered with 0.45 µm filter. After filtration the samples were diluted 1:1 with Isopropanol. The metabolomic profile of feces from 20 healthy and 20 RRMS patients was analyzed by UPLC (UPLC 1290 system, Agilent Technologies) directly connected to mass spectrometry (TripleTOF 5600+ mass spectrometer, SCIEX equipped with an electrospray ionization source (ESI). Quality control (QC) samples were prepared as pool of all the samples, by mixing equal volume of each extracted sample and analyzed within the queue. Chromatographic separations occurred on an Acquity BEH AMIDE (100 x 2.1 mm, 1.7 µm, Waters) capillary column. A gradient of solvent A (acetonitrile containing 0.1% formic acid) and B (water containing 0.1% formic acid) was used to achieve separation (600 µl/min as flow rate): 1 minute at 2% B, from 2% B to 60% B in 10 minutes, 2 minutes hold at 60% B, in 0.50 minutes to 2% B, 3 minutes hold at 2% B. The column temperature was set at 40°C, while the autosampler was set at 4°C. 8 µl of samples were injected. Full scan spectra were acquired in the mass range from *m*/*z* 50 to 500 with a SWATH modality acquisition of 10 windows. The source parameters were: Gas 1: 33 psi, Gas 2: 58 psi, Curtain gas: 35 psi, Temperature: 500 °C and ISVF (IonSpray Voltage Floating): 5500 V (-4500 V for negative polarity), DP: 80 V, CE: 35 V with a spread of 15V. The same samples were also analyzed by using reverse phase (RP) Acquity HSS T3 C18 column (100 x 2.1 mm, 1.8 µm, Waters). A gradient of solvent A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) was used to achieve separation (600 µl/min as flow rate): 1 minute at 2% B, from 2% B to 95% B in 14 minutes, 5 minutes hold at 95% B, in 0.50 minutes to 2% B, 5 minutes hold at 2% B. 10 µl of samples were injected. Full scan spectra were acquired in the mass range from m/z 50 to 850 with a SWATH modality acquisition of 16 windows. The source parameters were: Gas 1: 33 psi, Gas 2: 58 psi, Curtain gas: 35 psi, Temperature: 500 °C and ISVF (IonSpray Voltage Floating): 5500 V (-4500 V for negative polarity), DP: 80 V, CE: 35 V with a spread of 15V. The .wiff files acquired on the mass spectrometer were converted to .abf files using Reifycs Analysis Base File Converter and analysed with MS-DIAL v. 4.7 (42) for peak picking, gap filling, alignment. The identification was performed using MS/MS Positive Public library v. 14.0, MS/MS Negative Public library v. 14.0. The MS-DIAL output was manually inspected to verify the annotation of the metabolites.

2.4. Isolation and flow cytometry analysis of human PBMCs

PBMC were isolated from freshly isolated whole blood by density gradient centrifugation using the Lympholyte®-H Cell Separation Media (Cederlane Laboratories) according to the manufacturer's instruction. Following isolation, cell pellet was suspended in FBS supplemented with 10% Dimethyl Sulfoxide (DMSO), aliquoted and cryopreserved in liquid nitrogen for long-term storage. On the day of analysis, cryopreserved PBMCs were thawed, washed with RPMI-1640 containing 10% FBS and counted. Cells were transferred into a 96-well U-bottom plate and incubated for 20 min at RT with 10% heat-inactivated human serum (Human Serum - Type AB male, Euroclone) as Fc receptor blocking agent. Subsequently, cells were washed once in PBS by centrifugation (400 × g, 5 min, RT) and subjected to the following staining procedure. Cells were first stained with eBioscience™ Fixable Viability dye eFluor506 diluted in PBS for live/dead cell discrimination. After a wash with PBS by centrifugation as above, cells were incubated 20 min at 4°C in the dark with a combination of fluorochrome-conjugated surface antibodies in staining buffer. Staining of nuclear transcription factors was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences). In brief, after surface staining, cells were incubated with freshly prepared fixation/permeabilization solution for 45 minutes at 4°C, washed twice with 100µl of Perm/Wash buffer, and then stained for 30 min at RT with 100 µl mixture of fluorochrome-conjugated anti-human antibodies against FOXP3, Tbet and RORgt diluted in a 1:1 mix of 1X Perm/Wash buffer and Brilliant Stain Buffer. Cells were then washed twice with Perm/Wash buffer by centrifugation (400 × g, 5 min, RT) and suspended in 200 µl of FACS buffer for subsequent analysis. For cell detection, the following antibodies were used: BUV395 anti-human CD45 (HI30, BD Bioscence), APC-Cy7 anti-human CD3 (SP34-2, BD Bioscience), BUV496 anti-human CD4 (L200, BD Bioscience), BV786 anti-human CRTH2 (BM16, BD Bioscience), PE-Cy7 anti-human CD25 (M-A251, BD Bioscience), BUV805 anti-human TCRγδ (11F2, BD Bioscience), BV711 anti-human CD161 (DX12, BD Bioscience), BV570 anti-human TCRVα7.2 (OF-5A12, BD Bioscience), PE anti-human RORγt (AFKJS-9, Thermofisher), Pe-Cy5 anti-human Tbet (4B10, Invitrogen), APC anti-human Foxp3 (PCH101, Invitrogen). All samples were acquired on BD FACSymphony A5 flow cytometer (BD Biosciences) and

analyzed using FlowJo software (v.10.8.1) using unstained and single-stained compensation beads as compensation controls.

2.3. Statistical analyis

Graphpad Prism version 8.0 (Graphpad Software) was used for statistical analysis unless noted otherwise and p values < 0.05 were considered to be significant. EAE clinical scores were analysed with two-way ANOVA test followed by Bonferroni post-testing for multiple comparisons. Disease incidence was evaluated using the Kaplan-Meier estimation, whereas statistical significance was evaluated by the by log-rank [Mantel-Cox] test. Other differences were estimated by two-tailed Student t test and Mann Whitney U test. For human microbiota analysisrelative abundance profiling and differential analysis were performed with DESeq2 upon variance-stabilizing transformation (Love *et al*., 2014). For human metabolomic analysis, the annotated metabolites were statistically analyzed by a R pipeline in-house developed by the Proteomics and Metabolomics Facility of IRCCS San Raffaele Hospital and box plots were generated upon t test among the two experimental sample groups ($p<0.05$).

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