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Gut microbiota crosstalk with conventional and non-conventional T cells: a game of many players.

Claudia Burrello

IEO, Milan

Matricola n. R11126

Supervisor:

Prof. Saverio Minucci

IEO, Milan

Co-supervisors:

Dr. Federica Facciotti

IEO, Milan

Prof. Maria Rescigno

Humanitas University, Milan

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“Dai diamanti non nasce niente.

Dal letame nascono i fiori.”

F. De Andrè

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List of Abbreviations

ABX Antibiotics

AJs Adherens junctions

AMPs Antimicrobial peptides

APC Antigen Presenting Cell

CD Crohn's Disease

CDI *Clostridium difficile* infection

DCs Dendritic cells

dFMT dysbiotic FMT

DSS Dextran Sodium Sulphate

FMT Faecal Microbiota Transplantation

GALT Gut-associated lymphoid tissue

GF Germ free

HD Healthy Donor

IBD Inflammatory Bowel Disease

IL- Interleukin-

IL-10R Interleukin-10 Receptor

iNKT invariant Natural Killer T

JAM Junctional adherent molecules

LP Lamina propria

LPMC Lamina Propria Mononuclear Cells

MAMPs Molecular-associated Molecular Patterns

mLN Mesenteric lymph node

nFMT normobiotic FMT

NLR Nod-like receptor

PB Peripheral Blood

PRRs Pattern Recognition Receptors

RCTs Randomized Clinical trials

SCFA Short Chain Fatty Acid

SFB Segmented Filamentous Bacterium

SPF Specific Pathogen Free

TCR T cell receptor

Th1 T helper 1

Th17 T helper 17

Th2 T helper 2

TJs Tight junctions

TLRs Toll-like receptors

TNBS 2,4,6-trinitrobenzene sulfonic acid

Treg T regulatory cells

UC Ulcerative Colitis

αGalCer α-galactosylceramice

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Abstract

The presence of microbial commensals in the gut requires the establishment of a complex network of reciprocal interactions between the microbiota and the host immune system to allow nutrient absorption while preventing undesired mucosal immune responses. Despite these homeostatic mechanisms, during intestinal inflammation alterations of the microbiota composition, namely dysbiosis, trigger abnormal immune responses. Indeed, an exaggerated T cell response against dysbiotic microbial antigens has been causally linked to the onset of Inflammatory Bowel Diseases (IBD), a family of intestinal autoimmune disorders. Moreover, also iNKT cells, a subset of non-conventional T cells that can recognize bacterial lipidic antigens and shape the gut microbial community, have been associated to IBD pathogenesis.

Here, we aimed at investigating the functional crosstalk between gut microbiota and the mucosal immune system during inflammation and upon induction of microbial dysbiosis. Ultimately, the goal of this project was to evaluate the therapeutic potential of gut microbiota manipulation on the mechanisms contributing to the resolution of intestinal inflammation.

We observed that inflammation-induced and antibiotic-driven types of dysbiosis are phenotypically and functionally modifying $CD4^+$ T and iNKT cells activity. Moreover, during intestinal inflammation, the experimental manipulation of the microbiota community through Faecal Microbiota Transplantation (FMT) reduces colonic inflammation and initiates the restoration of intestinal homeostasis through a modulation of the whole mucosal immune response. Indeed, FMT leads to an increase in IL-10 production by innate

and adaptive immune cells, including $CD4^+$ T cells, iNKT cells and Antigen Presenting Cells, and reduces the ability of these cells to present MHCII-dependent bacterial antigens to colonic T cells.

Further, we performed a comprehensive analysis on intestinal iNKT cells isolated from surgical specimens of active IBD patients and non-IBD donors, generating stable cell lines and clones for *in vitro* functional assays. We here report that iNKT cells with a defined pro-inflammatory profile are enriched in the intestinal lamina propria of IBD patients and that exposure to the mucosa-associated microbiota drives their pro-inflammatory activation, inducing direct pathogenic activities against the epithelial barrier integrity.

Collectively, we provided solid evidence that a strict crosstalk between the gut microbiota and the intestinal conventional and non-conventional T cells exists. Antibiotic-associated dysbiosis has immunostimulatory functions, indicating the need of a careful evaluation of antibiotic administration to patients suffering from autoimmune disorders, such as IBD. Moreover, our results demonstrate the capability of FMT to therapeutically control intestinal experimental colitis and pose FMT as a valuable therapeutic option in immune-related pathologies. In addition, we generated fundamental knowledge about the pathogenic functions exerted by human intestinal iNKT cells as a consequence of the interaction with mucosa-associated microbiota communities. This suggests that the selective targeting of iNKT cells or the modulation of their functions may be explored as a potential therapeutic tool in IBD.

1 Introduction

In the gut, the divergent needs of nutrient absorption and the maintenance of a strong host immune defense coexist [1][2]. The intestine is indeed a unique organ, being the most comprehensive reservoir of commensals of the human body, and a vast potential portal for pathogen entry [3]. To comply its duties while overcoming the obstacles that originate from them, the mucosal immune system has developed a complex system to counterbalance immunity, at risk of overstimulation by the microorganisms, and tolerance, required for food intake [4]. To this aim, the intestine has established a network of interactions among different players, such as the specialized epithelial cells, the intraepithelial and lamina propria immune cells and the gut microbiota.

In this Chapter the unique features orchestrating the mucosal immune system will be firstly addressed, focusing on physical and immunological barriers. Then the emerging role of gut microbiota in the shaping of the host response will be unravelled. Finally, the effects of a pathological dysregulation of such fine-tuned mechanisms of crosstalk in Inflammatory Bowel Diseases will be described.

1.1 The intestinal epithelial barrier

In contrast to secondary lymphoid organs, including lymph nodes and spleen, a distinctive characteristic of mucosal tissues like the gut is the close, extensive and bidirectional collaboration between the immune system and epithelial cells [4]. The human intestinal epithelial barrier is composed by a monolayer of approximately 20 billion tightly joined enterocytes that represents the first line of defense of the host [1], [5]. The surface area of this epithelium is to the order of 400 m^2 since it is formed into millions of fingerlike villi in the small bowel and crypts in the colon [2]. This spatial organization optimizes the absorption of nutrients.

The intestinal epithelial barrier function is essential for health and relies on intercellular junctions that maintain epithelial cohesiveness while regulating its selective permeability [5]. These specialized cell membrane structures are referred to as tight junctions (TJs) and adherens junctions (AJs). These structures are composed by multimeric transmembrane protein complexes that in TJs include proteins of the claudin, occludin and junctional adhesion molecules (JAM) families and in AJs include nectins and e-cadherin [6]. They seal epithelial cells forming homo- or etero- dimers with the corresponding structures on the adjacent cells. Junctions have also a cytosolic plaque in charge of intracellular signal transduction, whose main components are adaptor proteins, such as ZO-1, that contain several protein-protein interaction motifs [1], [6]. While the transcellular passage across the epithelium requires an active transport, paracellular spaces between cells allow passive diffusion. Intestinal epithelial TJs permit the diffusion of water and electrolytes but they largely limit the absorption of water-soluble proteins that exceed 0.4 nm in diameter [6].

For decades the intestinal epithelium has been considered to play a passive role of physical barrier. We now know that enterocytes are a family of specialized and highly dynamic cells with active functions in the maintenance of the organ homeostasis [2]. They originate from pluripotent highly proliferative cells, called transit amplifying cells, derived from Lgr5+ stem cells located at the basis of the crypts. Throughout their migration towards the top of the villous the cells decrease their proliferation rate depending on a Notch and Wnt signaling gradient and differentiate into absorptive enterocytes, goblet cells, Paneth cells or M cells [4]. Absorptive enterocytes have the task to absorb nutrients and are the most abundant cell type [4]. Goblet cells are the main producers of mucin glycoproteins, components of the glycocalix and mucous layers lying on top of the epithelium [7]. Paneth cells secrete anti-microbial peptides in response to gut microbiota in a MyD88 dependent manner [8]. M cells play an important role in antigen uptake and transfer to the Peyer's patches, small lymphoid follicles lying underneath the epithelium [2].

1.2 The mucus layers

The mucus layers oversee the containment of the gut microbiota in the intestinal lumen and attenuate the friction of the food bolus against the epithelium [5].

The specialized Goblet cells secrete high amounts of mucus, which is composed by mucin protein backbones with O-linked glycosilation. Once secreted, mucins become hydrated and expand forming a net-like gel sub-structured in three layers [5]. The first one is formed by membrane-bound mucins associated with the colonic epithelial cells and it is referred to as glycocalyx. The second layer is the tightly crosslinked inner layer which is primarily

composed by the secreted mucin MUC2 [7]. This layer is maintained sterile thanks to the high gel density and the presence of high doses of antimicrobial peptides produced by the close epithelium [9]. The outermost layer is less dense and viscous since it is produced by proteolysis of the inner layer. The outer layer allows the gut microbiota to create a specialized niche for mucus-associated bacteria [7]. The mucus turnover is fast and takes place in a few hours. As it is proteolyzed and extruded in the lumen, it binds bacteria and gets expulsed with the peristalsis. This mechanism limits the bacteria approaching the epithelium, without precluding access to metabolites and, in some cases, toxins [5]. This observation is demonstrated by the evidence that mice lacking Muc2 expression ($Muc2^{-/-}$) experience a reduction of the mucus thickness, an enhanced access of bacteria to the epithelium and the spontaneous development of intestinal inflammation [10].

Mucins can be either secreted or be transmembrane and, besides their structural function, they can also actively signal intracellularly and have immunomodulatory activities [11]. For instance, MUC1 has been shown to have potent anti-inflammatory functions [12].

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are one of the evolutionarily most ancient mechanism of defense against microbes developed by multicellular organisms [9]. Albeit many gut microbiota components are commensals that establish mutualistic relationships with the host, the huge numbers of bacteria present in the intestinal lumen represents a continuous threat for barrier integrity. To overcome this threat, Paneth cells secrete abundant AMPs which rapidly kill or inactivate microorganisms. The vast variety of microorganisms that the

host can encounter reflects the complexity and diversity of the AMPs it produces. Intestinal AMPs include defensins lysozymes, C-type lectins and cathelicidins [4]. They use different molecular mechanisms to kill bacteria but many of them target basic and essential cell wall structures of bacteria, such as peptidoglycan. In this way they act specifically against bacteria and they reduce the probability of resistance development [9]. Some of them, like lysozymes, have enzymatic activity to degrade peptidoglycan or cell wall components, others, like defensins and cathelicidins, physically insert into bacterial membranes leading to disruption of membrane potential and osmotic lysis [13]. Being a potent response against bacteria, they have been shown to have also anti-inflammatory and potential therapeutic activity during intestinal inflammation [14].

1.4 Intestinal antigen transport and presentation

Besides exerting barrier functions, intestinal epithelial cells attend the important task to orchestrate mucosal immunity by modulating the immune response through tolerance induction as well as by directing the inflammatory response in the lamina propria. The huge surface area of the intestine is continuously in contact with a wide variety of antigens such as dietary proteins and carbohydrates, lipids, bacteria, fungi and viruses [2]. The fact that 2% of dietary proteins can be found intact in blood circulation suggests the existence of breaches in the epithelial barrier. Indeed, several mechanisms of transcellular transport of antigens have been described.

M (microfold) cells are specialized epithelial cells that reside in the follicle-associated epithelium (FAE) (***Figure 1.1***). The glycocalyx above M cells is thinner as compared to the

one on top of absorptive enterocytes [4]. This allows the access of particulate antigens that are taken up by M cells through various mechanisms including pinocytosis and receptor-mediated endocytosis without any processing. It has been shown that transepithelial transport by M cells is facilitated by toll-like receptors engagement in the presence of bacteria [15]. Antigens transported by M cells are passed on to immature Dendritic cells (DCs) located close to the basolateral membrane of M cells. Loaded immature DCs migrate to the near Peyer's patches and here they present antigens and trigger the adaptive response [15].

In addition, upon mucus secretion, Goblet cells are also able to sense bacterial luminal antigens and take them up. The transport of antigens by Goblet cells is referred to as goblet cell-associated passage (GAP) and it remains still largely unknown [7]. Experiments in mice challenged with fluorescently labelled dextran showed that the dye was primarily taken up by Goblet cells [16]. The size of the antigen that can be transported through this route is determined by mucus filtering and it is limited to low molecular weight (70kDa) soluble antigens. GAP specifically deliver antigens to $CD103^{+}$ $CX3CR1^{-}$ DCs that engage Tregs cells and mediate tolerogenic functions [16].

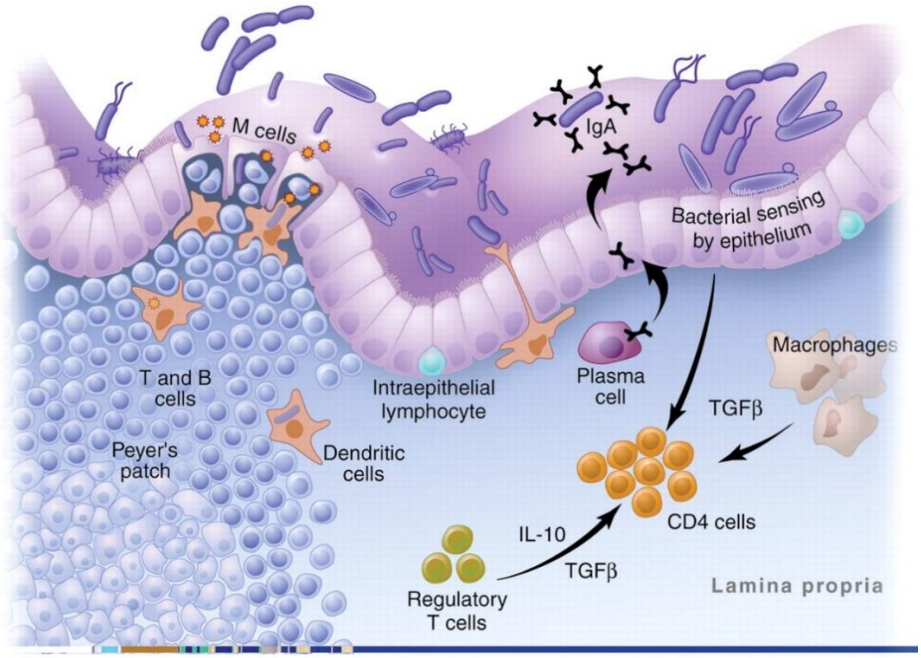

Figure 1.1 Intestinal antigen transfer from gut lumen to lamina propria. The intestinal epithelium orchestrates the activity of gut-associated lymphoid tissue (GALT) through different mechanisms. Specialized M cells constantly transport gut bacteria and antigens from the gut lumen into the Peyer's patches. In the lamina propria Dendritic cells take up the antigens transported or directly sample them from the lumen. Lamina propria contains many CD4 T cells, macrophages, and IgA antibody–producing plasma cells. Potentially tissue-damaging T cell responses may be inhibited by immunosuppressive cytokines and regulatory T cells. Adapted from TT MacDonald and G Monteleone, Science, 2005 [17].

As stated in Section 1.1, also tight junctions between epithelial cells can modulate the passage of small molecular weight antigens through paracellular diffusion. These antigens are thought to diffuse until the mesenteric lymph node (mLN) where they meet professional antigen presenting cells (APCs) that initiate an immune response.

Finally, dendritic cells have been shown to be able to intercalate their dendrites between epithelial cells by expressing tight junction proteins (i.e. ZO-1) thus directly sampling the

antigens from the lumen [18] (***Figure 1.1***). These DCs express CX3CR1 chemokine receptor and trigger a pro-inflammatory adaptive immune response [18].

Dendritic cells are the most potent subset of APCs. Nonetheless, in the intestinal lamina propria antigens can encounter several types of professional antigen presenting cells such as monocytes, macrophages and B cells. These cells share the capability to internalize antigens in endocytic vacuoles, process them and load them on antigen presenting molecules. B lymphocytes can directly bind antigens on Ig surface molecules, whereas T cells require the presentation of a membrane-bound antigen to trigger their activation through T cell receptor (TCR) signaling cascade. Major Histocompatibility Complex (MHC) I and II present peptidic antigens of endogenous and exogenous origin to $CD8^+$ T and $CD4^+$ T cells respectively. More recently it has been identified a pathway for lipidic antigen presentation by members of the CD1 family [19].

Besides professional APC, intestinal epithelial cells have also been shown to be able to present antigens of both peptidic [20] and lipidic [21] nature and initiate an adaptive immune response. To note, the type and origin of the APC is critical to induce specific outcomes [22]. Indeed, for instance, it has been shown that lipidic antigen presentation by intestinal epithelial cells elicits a protective and regulatory response [23], while several evidences show that lipid specific intestinal T cells can exert pro-inflammatory and pathogenic functions in response to antigen presentation [24].

1.5 T cell mediated mucosal immune response

Initially, mucosal immunology was dominated by a focus on secretory IgA mediated immunity. We now know that T cells form an equally relevant component of cell-mediated immunity at mucosal sites [25]. During homeostasis, the most abundant subsets of intestinal T cells are $CD8^+$ αβ T cells and intraepithelial γδ T cells [26]. The former form a population of tissue resident long-lived memory cytotoxic T cells that can be promptly reactivated in case of infections, thus securing the tissue with immunesurveillance activity. The latter exert both innate and adaptive cytotoxic functions, besides having a role in epithelial repair [26]. MHCII restricted $CD4^+$ T cells are less frequent in the intestinal mucosa, but they can massively infiltrate the gut lamina propria in case of inflammation and contribute significantly to the pathology [27], [28].

In the mid-1980s, *in vitro* cloning of $CD4^+$ T cells showed the existence of two distinct effector subsets [29]. One was referred to as T helper 1 (Th1), secreting pro-inflammatory cytokines such as IFNγ and TNF and is employed for defense against viruses and intracellular bacteria. The other was called T helper 2 (Th2) because it differentially secreted cytokines such as IL-4, IL-5 and IL-13 in response to parasitic infections. These two opposed subsets were shown to reciprocally suppress their development [30].

The development of Th1 cells is induced by the production of IL-12 by APCs. This drives the activation of the signaling cascade of STAT1 leading to the expression of Tbet (T-box transcription factor expressed in T cells), the master regulator transcription factor of Th1 subset [31]. IL-12 also induces STAT4 phosphorylation that, together with Tbet, triggers the production of IFNγ and TNF. Downstream, Th1 cells are driving B cell class switching to

opsonizing IgG and the activation of intracellular killing by macrophages. This way Th1 cells respond to intracellular bacteria and virus infections [25].

Th2 cells differentiate mainly in response to helminth parasitic infections. Their differentiation is induced by IL-4 expression by innate immune cells and it is characterized by the phosphorylation of the STAT6 signaling cascade that, in turn, activates GATA3 transcription factor [32]. Via the secretion of IL-4, IL-5 and IL-13 they orchestrate B cell class switching to IgE and the recruitment of granulocytes like mast cells, basophils and eosinophils [25].

In 2005, a third T helper cell subset, called Th17, was added to the Th1/2 paradigm [33]. Th17 cells are characterized by the production of IL-17A and IL-17F. Their differentiation is induced upon TGFβ, IL-23 and IL-6 secretion. These cytokines drive the phosphorylation cascade of STAT3 pathway that determines the expression of RORγt transcription factor. IL-17 is actively recruiting neutrophils that contribute to the elimination of extracellular bacteria and fungi. Intestinal Th17 cells have been clearly shown to contribute to Crohn's disease pathogenesis [34],[35].

More recently, many mucosal immunologists are focusing their research on the characterization of several additional Th subsets, including IL-9 producing Th9 cells ad IL-22 producing Th22.

To deal with this arsenal of effector $CD4^+$ T helper cells, the intestinal immune response deploys another subset of $CD4^+$ T cells with potent immune suppressive functions, i.e. the regulatory T cells (Treg). This population was firstly described in 1995 by Sakaguchi et al. and it was initially identified by its expression of the IL-2 receptor α chain (CD25) [36]. Mice

transferred with thymocytes depleted of $CD4^+CD25^+$ T cells developed severe autoimmune diseases in several organs [36]. Later, Foxp3 was recognized as the master transcriptional regulator of Treg cells [37].

Also for Treg cells, a wide number of sub-populations have been defined, including $Foxp3^+$ inducible Treg, $Foxp3^+$ thymic Treg, Tr1 and regulatory Th17. The population described by Sakaguchi is the now called thymus-derived tTreg. Within 3 days after birth, these cells migrate to the periphery and become in charge of peripheric tolerance. Conversely, some $CD4^+$ T cells, with a distinct TCR specificity, can exert regulatory activity once they are in the periphery if properly stimulated by APCs. This is the case of inducible iTreg cells [38].

Recent studies have questioned the idea that the above-described $CD4^+$ T cell subsets represent stable populations. Indeed the Th17 cell lineage displays a marked plasticity having overlapping differentiation programs with Th1 and iTreg [39]. Foxp3 or Tbet expression in Th17 cells can induce a functional transition to Treg or Th1 cell state, respectevely. If the transition to iTreg suggests a clear role in the resolution of the inflammation, the functional meaning of Th17-Th1 plasticity remains elusive. Nonetheless it has been shown to greatly contribute to autoimmune pathogenesis in Crohn's disease patients [35].

1.6 iNKT cells

Invariant natural killer T (iNKT) cells were identified in the mid-1990s as a mature T cell subset with semi-inviariant T cell receptor (TCR) [40], [41]. iNKT cells, also called type 1 NKT

cells, bear a semi-invariantly rearranged αβ TCR which comprises an invariant α chain rearrangement (Vα14-Jα18 in mouse and Vα24-Jα18 in human) paired with a limited β chain repertoire (Vβ7, Vβ8 or Vβ2 in mouse and Vβ11 in human) [40]. However, in both mice and humans, TCR β chain diversity is achieved through the use of variable TCR Dβ and Jβ region that results in high level of complementarity determining region 3 (CDR3) diversity and thus polyclonal repertoire of multiple specificities [42].

iNKT cells recognize lipidic antigens presented by the MHCI-like molecule CD1d that has the unique characteristic to be non-polymorphic and greatly conserved among mammals (***Figure 1.2***). CD1d belongs to the larger family of CD1 complexes that includes CD1a, CD1b and CD1c molecules, all of which present lipid antigens rather than peptides to T cells. The antigens recognized by iNKT cells can be either of exogenous bacterial origin or of self-origin [21], [43]. The best known iNKT TCR agonist is α-galactosylceramice (αGalCer), a bacteria- derived glycosphingolipid originally purified from a marine sponge [44]. Unlike all mammalian glycosphingolipids which have β-linked sugars, αGalCer contains an α linkage of the sugar to the ceramide lipid, that has been shown to be essential for its high affinity with the TCR and its antigenic potency. Thanks to the αGalCer strong and conserved recognition, iNKT cells can be easily detected with αGalCer-loaded tetramers [45]. Similar antigens have been isolated also from gut commensals [21], [46] and from pathogens [47]. iNKT cells are also autoreactive. However, self-antigens are often structurally variable and engage the CD1d with low affinity. Some reported candidate antigens are members of the glycosphingolipid family as well [48]. Also completely different compounds have been described as self-antigens, like a peroxisome-derived lysophosphatidylethanolamine important for positive thymic selection [43].

Besides antigen presentation, iNKT cells can be activated also by innate-like stimuli such as cytokine secretion or surface receptor engagement [45]. For instance, IL-12 produced by APC was sufficient to activate iNKT cells *in vivo*, even without CD1d antigen presentation [45]. Moreover, iNKT cells express a wide range of surface receptors that are characteristics of natural killer cells such as NK1.1 (CD161 in human), NKG2D and NKp46 and that correlate with their functional activation and maturation status. More often, the TCR-mediated and the cytokine-dependent stimulations occur simultaneously and work in synergism [45].

Another feature they share with innate immune cells is that iNKT cells store huge amounts of cytokines in cytoplasmic granules. These vesicles can be quickly degranulated upon stimulation, leading to a massive and prompt immune response [49].

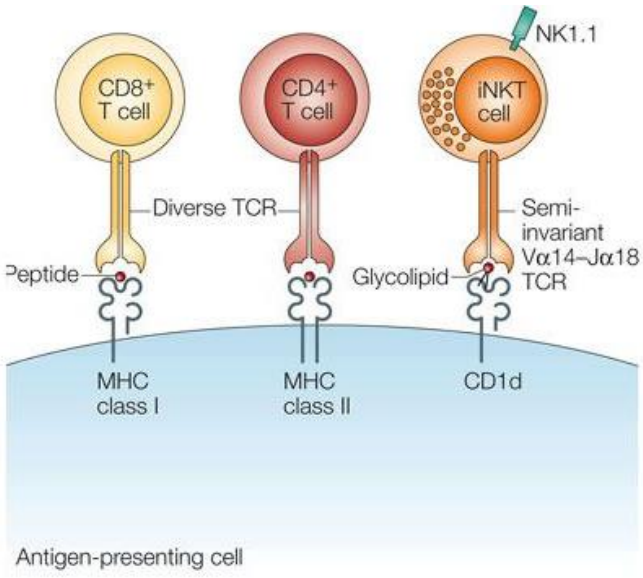

Figure 1.2 Conventional and non-conventional T cell subsets. Invariant natural killer T (iNKT) cells are a CD1d-restricted subset of T cells that express a semi-invariant T-cell receptor together with markers of NK cells. Adapted from L Van Kaer, Nature Reviews in Immunology, 2005 [50].

iNKT cells develop in the thymus following a differentiation pathway based on positive selection by thymocytes that conveys an antigen-experienced phenotype and a functional maturation status [49]. While some of them remain in the thymus as long-term residents exerting homeostatic functions [49], others egress from the thymus and reach peripheric lymphoid and non-lymphoid organs, thanks to their expression of chemokine and homing receptors [51]. Experiments with parabiotic mice showed that once in the periphery, most iNKT cells do not recirculate and become tissue-resident lymphocytes [52].

Murine iNKT cells have been well studied in spleen, intestine and liver where they represent 2%, 1% an 40% of the total T cell population, respectevely [19]. Less is known about iNKT cell distribution in humans, but they appear to be slightly less abundant [49]. Nonetheless, considering their high similar specificity and their prompt and potent response, a 1% population has the potential to greatly influence the outcome of the tissue immune response at mucosal sites.

As described in Section 1.5 for $CD4^+$ T cells, also iNKT cells differentiate into several subsets with distinct functional activities. The subset of NKT1 resembles the one of Th1 for T helper cells. They express Tbet transcription factor which drives the production of IFNγ and TNF and they are mainly localized in liver, spleen, lungs and intestine [49]. Lymph nodes have been shown to be enriched in NKT17 cells, producing IL-17 and expressing RORγt transcription factor. Differently from Th2 cells, NKT2 are not expressing GATA3 transcription factor so they are often tracked for their double negative expression of Tbet and RORγt, besides their secretion of IL-4 and IL-13 [49]. NKT2 are found in each organ at low frequencies under homeostatic conditions but can contribute to intestinal

inflammation in IBD patients [24]. More recently, the NKT10 subset has been described to exert regulatory functions through the production of the tolerogenic IL-10 cytokine [53]. NKT10 are mainly localized in the adipose tissue [54]. Interestingly, murine experiments of adoptive transfer with iNKT cells derived from different organs showed that the determining factor for iNKT cell phenotype and functional activity is the surrounding environment rather than an intrinsic cell lineage definition [51], suggesting a functional plasticity also for iNKT cells.

In addition to iNKT cells, there is another subset of CD1d restricted T cells, referred to as type 2 NKT cells, which have a more diverse T-cell receptor repertoire and do not recognize αGalCer. At least a subset of Type 2 NKT cells can be identified through sulfatide-loaded tetramers [22]

1.7 Gut microbiota

The intestinal tract represents the largest repertoire of microorganisms referred to as microbiota, a term that comprises a collection of bacteria, viruses and fungi [55]. The human body shelters 3.8×10^{13} bacterial cells, among which 10^{11} bacteria reside in the colon [56]. The number of bacterial cells is of the same order as the number of human cells [56]. As a result of this, the human host and the microorganisms inhabiting it are referred to as "superorganism" [55].

The host-microbiota interactions have co-evolved leading to the commensalism of many bacteria species. Indeed, the host takes advantage of many ecological services exerted by the microbiota, including the maintenance of epithelial integrity [57], the harvesting of

nutrients and the protection against pathogens, such as *Citrobacter rodentium* [58] and *Helicobacter hepaticus* [59]. One of the mechanisms through which the gut microbiota prevents pathogen infection is the so called “colonization resistance” [60]. This concept dates back in the 1960s and illustrates the role of indigenous microbiota in antagonizing pathogens through (i) competition for the same ecological niches and (ii) potentiation of the mucosal barrier function [61].

In the last decade the techniques used to study the microbiota have greatly improved due to the advent of culture-independent approaches that allow high throughput and low cost sequencing [62]. This started an enormous interest in this topic, as clearly illustrated by the 16 000 citations found in PubMed upon “gut microbiota” search, 70% of which dated after the year 2000 [63].

Combined data obtained by two international consortium projects, i.e. the European MetaHit (metagenomics of the human intestinal tract) and the American Human Microbiota Project, provided substantial information on human-associated microbial repertoire [64], [65] . They identified 2172 bacterial species, classified in 12 phyla, of which 93.5% belonged to *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* [55]. Most studies have utilized stool samples, however this provides an incomplete representation of the gut microbiota, since the composition of mucosa-associated microbes differs from the faecal one [66].

Moreover, it is becoming increasingly acknowledged that the sole description of microbial composition is of limited utility and a more detailed analysis of microbiota genome (microbiome) and metabolism is required. Indeed, the Human Microbiome Project

reconstructed the relative abundances of metabolic pathways in community metagenomes and coupled it with a metagenomic analysis of taxonomy [67]. Unlike microbial taxa, several pathways resulted to be ubiquitous among individuals, suggesting the existence of a high functional redundancy among different species [67].

The gut microbiota colonizes the gastrointestinal (GI) tract starting from the gestation period and then rapidly after birth. During the first years of life, the microbiota is constantly evolving in composition, increasing its diversity and typically resembling the gut microbiota of the mother [55]. In adulthood, the composition of the gut microbiota has been shown to be relatively stable. Adult individuals cluster in three main groups, called enterotypes, depending on the enrichment of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) [68].

Nonetheless, also the adult gut microbiota composition is subject to perturbations by life styles and events. For instance, a change in diet can dramatically impact on the microbiota. It has been shown that the microbiota is shaped by the availability of microbiota-accessible carbohydrates (MACs) that are found in dietary fibers. Administration of a low MAC diet to mice resulted in a drastic reduction in microbial diversity [69].

Moreover, important modifications in gut microbiota composition can also be caused by the use of antibiotics [70], especially in early life [71], and by the existence of defined pathologies in the host [60]. This reflects the strict relationship between gut microbiota and the host immune system.

1.8 Interactions between the microbiota and the adaptive immune system

Despite many intestinal bacteria developed a symbiotic relationship with the host, the proximity of the intestinal tissue with such a huge bacterial load leads to great health challenges. Thus, the mucosal immune system has evolved to restrain the microbiota while preserving the benefits deriving from host-microbiota association [72].

To do so, several molecular mechanisms are in place to allow bacterial recognition by the host. Even in the presence of an intact epithelial barrier, microbial products and metabolites can be sensed by epithelial and lamina propria residing cells. The host recognizes microbial-associated molecular patterns (MAMPs) through pattern recognition receptors (PRRs). The best studied PRRs are the Toll-like receptors (TLRs), a family of transmembrane receptors recognizing external components of microorganisms. TLRs have been shown to play a crucial role in the maintenance of intestinal homeostasis as well as in the protection from epithelial injuries [73]. NOD-like receptors (NLRs) are another well-known family of PRRs. They are cytoplasmic receptors that primarily sense bacterial MAMPs and they seem to be involved in Crohn's disease pathogenesis, since a mutation in the NOD2 gene has been linked to genetic predisposition for this disease [74].

A high frequency of T cells is found in mucosal tissues colonized by commensals, and this is especially true for the gastrointestinal tract. Indeed, many studies in which the gut microbiota was depleted using broad spectrum antibiotics or by maintaining mice colonies in germ free (GF) conditions, highlighted an impairment in T cell accumulation in the gut-associated lymphoid tissue (GALT) [58], [59], [75].

In recent years many efforts have been dedicated on the characterization of defined microbial strains or products directly associated to specific T cell subset expansion and activation (***Figure 1.3***). The Segmented Filamentous Bacteria (SFB) present in conventionally raised mice from Taconic Farms, but not from Jackson Laboratories, was shown to be associated to Th17 cells. Its mono-colonization of GF mice turned out to be sufficient to induce IL-17 secreting $CD4^+$ T helper cells [58]. The induction of Th17 cells by SFB resulted in protection from the infection of the intestinal pathogen *Citrobacter rodentium*. Following works showed that the adhesion of microbes to intestinal epithelial cells was critical for the induction of Th17 cells [76]. However, SFB or an equivalent family of adhesive bacteria failed to be identified in the human gut microbiota [77], posing impediments to the clinical translation of this finding.

Moreover, commensals have been shown to control also the induction of immune-modulatory responses. Remarkably, oral tolerance, the active suppression of immune responses against dietary antigens, is impaired in the absence of a healthy microbiota [78]. Consistently, GF mice colonization with a defined and benign mix of intestinal bacteria (Altered Schaedler Flora, ASF) specifically induced Treg expansion in the colonic lamina propria [75]. Furthermore, it has been described that a single bacterial metabolite (polysaccharide A, PSA) produced by *Bacteroides fragilis* could promote regulatory responses thus protecting mice from DSS-induced colitis [59]. This effect was achieved through the induction of IL-10 producing Treg cells. Moreover, also a mix of 46 *Clostridium* strains, particularly of clusters IV and XIV, promoted Treg expansion both in colon lamina propria and systemically [79]. These cells showed also a markedly suppressive phenotype, expressing high levels of IL-10 and CTLA-4, a key component of the immune checkpoint

[79]. The mechanism of Treg induction is still largely unknown, but the production of short chain fatty acids (SCFA) by *Clostridia* might be involved [80].

SCFA are a family of metabolites produced from the fermentation of undigested complex carbohydrates and can serve as important energy source for both gut microbiota and the host epithelial surface [81]. Many studies tackled the role of these metabolites in modulating frequencies and function of FoxP3^{+} Treg cells [82], [83]. Moreover it has been shown that SCFA might be also sensed by APCs, such as dendritic cells and monocytes, inducing a downregulation of the antigen presentation machinery (both MHCII and co-stimulatory molecules like CD80 and CD86), thus indirectly regulating T cell responses [84]. In addition, dendritic cell IL-12 production was inhibited in favor of IL-10 secretion upon SCFA stimulation, suggesting that they activate multiple immune pathways collectively aimed at the regulation of the inflammation [84]. The lack of SCFA has been linked to many human diseases, encompassing Type 2 diabetes and Inflammatory Bowel Disease [85]. The restoration of SCFA-producers in patients have been described as beneficial. To this purpose, they have recently suggested a dietary intervention on diabetic patients in order to promote the establishment of a core functional unit of SCFA-producing bacteria by modulating the availability of complex carbohydrates [86]. This approach resulted in a successful amelioration of patient clinical conditions and suggested a novel ecological approach for the manipulation of gut microbiota [86].

Non-conventional T cells, too, can interact with microbiota at mucosal surfaces. Recently, a skin commensal has been shown to be able to induce T cell homeostatic responses via non-classical MHC I molecules engagement [87].

Indeed, the laboratories of R.S. Blumberg and M. Kronenberg provided groundbreaking evidences revealing the existence of mutual mechanisms of regulation between the intestinal microbiota and iNKT cells [88], [89]. Although these cells respond to some commensal bacterial antigens, the presence of the gut microbiota is not strictly required for their development in the thymus. Indeed, they have been shown to be present in GF mice [71], [90]. Peripheral iNKT cells have been shown to be reduced in numbers and to have a less activated phenotype in GF mice [71], [90]. Interestingly, though, the same cells isolated in the intestinal lamina propria were shown to be increased [71]. This observation pointed to a negative shaping of iNKT cell repertoire by commensal bacteria, an evidence confirmed also by the use of short term oral antibiotics administration [91] and in mice with a restricted flora enriched in *Firmicutes* [92].

The gut microbiota has been shown to regulate iNKT cell functions through multiple pathways. Firstly, the absence of commensal bacteria increases the production of CXCL16 (the iNKT cell chemoattractant molecule) by intestinal epithelial cells through an epigenetic control of the *cxcl16* gene locus [71]. More recently, the commensal *Bacteroides fragilis* has been recognized as the source of an α-galactosylceramide antigen (Bf1717) which binds the CD1d but, unlike αGalCer, fails to activate iNKT cells, thus limiting CD1d-dependent iNKT cell proliferation [93]. Moreover, gut microbiota has been shown also to indirectly affect intestinal iNKT cell pool by modulating the expansion of other T cell subsets [92].

Importantly, the iNKT cell numbers in GF mice could be normalized by colonizing neonatal mice with a healthy microbiota, though this was not possible in young adult mice [71]. This suggests that iNKT cells are subject to an immunologic imprint by early microbial exposure

possibly aimed at preventing hyperreactive inflammatory responses towards the commensal microbiota in the intestine.

One possible interpretation of these observations, is that microbiota independent prenatal and neonatal iNKT cell development provides a first-line of defense at mucosal surfaces during early postnatal periods [88]. In this window of time, the host is at risk of intestinal infections because, on one hand, the unstable microbiota is not conferring a sufficient colonization resistance, and, on the other hand, the adaptive immune response is still immature and may not be able to confer protection against microbial pathogens. Later on during the host growth, iNKT cell activity at mucosal surfaces becomes less crucial because gut microbiota composition is less prone to modifications and the adaptive immune response has developed and is correctly in place. In fact, it has been proposed that in adults intestinal iNKT cell role in homeostasis becomes marginal, while instead they may importantly contribute to intestinal inflammation [88].

Interestingly, the gut microbiota- iNKT cells interaction is bidirectional. Interestingly, the gut microbiota- iNKT cells interaction is bidirectional. It has been recently shown that also NKT cells are impacting on microbial composition in the intestine [94]. Indeed, in CD1d deficient mice, bacterial colonization is favored due to an impairment in AMPs release by Paneth cells [94].

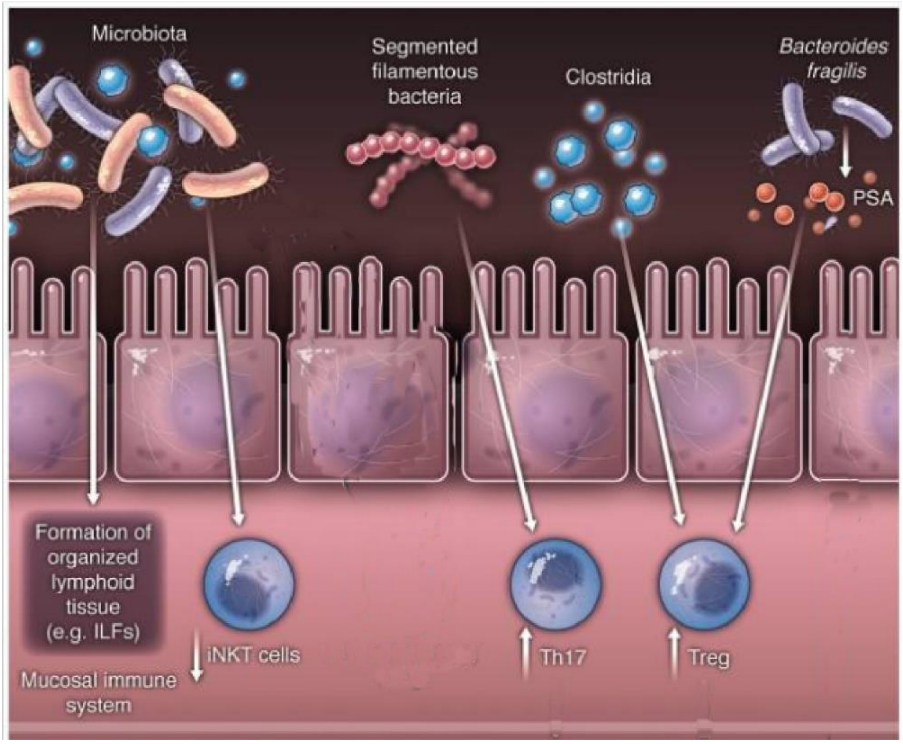

Figure 1.3 Microbiota shaping of intestinal T cell subsets. Some of the many ways that intestinal microbiota shape host immunity are depicted. These includes microbiota inhibition of mucosal iNKT cell proliferation, SFB induction of Th17 lineage and Clostridia and B.fragilis triggering of Treg. Adapted from LV Hooper, D Littman, AJ MacPherson, Science, 2012 [72].

1.9 Inflammatory Bowel Disease

As the largest reservoir of effector $CD4^+$ T cells and the site of the greatest secondary education of $CD4^+$ T cells, the bowel is at constant risk of developing immune related diseases such as Inflammatory Bowel Diseases (IBD). IBD, including Ulcerative Colitis (UC) and Crohn's Disease (CD), are a family of auto-immune chronic relapsing disorders characterized by pathologic inflammation of the gastrointestinal tract and epithelial injury [95]. While in UC patients the inflammation is restricted to the colon and has a continuous pattern starting from the rectum, CD has a preferential manifestation area in the terminal

ileum but can involve the whole digestive system and it is characterized by a discontinuous pattern of inflammation [96]. Moreover, CD inflammation is transmural, affecting all the layers of the intestinal wall, while UC patients have a superficial inflammation occurring in the mucosa and submucosa [97].

IBD occurs in genetically susceptible individuals and is considered a polygenic disorder, familial in the 5-10% of the population and sporadic for the remainder [95]. Studies on monozygotic twins demonstrated that there is phenotypic concordance in 50-75% of twins affected by CD, but only in 10-20% in those affected by UC, suggesting that heritability is less important in UC patients compared to CD ones [95]. The discordance in monozygotic twins and the occurrence of IBD in immigrants to high-prevalence Western countries, highlight the multifactorial nature of this family of disorders. However, the environmental factors involved in IBD pathogenesis are still poorly understood [95].

Genome wide association studies (GWAS) identified more than 240 genetic risk loci [98]. While some of them confirmed the role of already acknowledged immune-related pathways (IL-23, Th17, HLA/MHC), others previously unappreciated pathways such as autophagy and bacterial recognition (ATG16L1, NOD2) emerged from these analyses, underlying the importance of microbe-host interaction [99]. Interestingly, most genetic loci involved overlap between CD and UC [100].

1.9.1 Physical intestinal barriers in IBD

All the key components of the mucosal immune system that we described previously in this Chapter are markedly perturbed during IBD inflammation. Firstly, the epithelial barrier is dysfunctional, resulting in an increased intestinal permeability with the consequent translocation of luminal products, of both bacterial or dietary origin, into the lamina propria [96]. The main reason for epithelial barrier impairment is that tight junctions' proteins are differentially expressed in IBD patients, thus rendering the epithelial seal leaky [97], [101]. The expression of TJs proteins is known to be greatly modulated by pro-inflammatory cytokines that, as it will be discussed later, are drastically enriched in the lamina propria of IBD patients. Secondly, also the mucus layers are greatly altered during intestinal inflammation [7]. Patients with active disease display the mucus that is more penetrable to bacteria. UC patients have been found to have reduced goblet cell numbers in the colon as well as a thinner mucus layer due to an altered glycosylation profile [101]. Moreover, GWAS identified mutations in the MUC1 and MUC4 genes as more associated to CD patients [102], [103]. In addition, CD patients with the autophagy pathway mutated in ATG16L1 gene, have been shown to have abnormal Paneth cells with dysfunctionality in granule assembly and anti-microbial proteins secretion [8].

1.9.2 Adaptive immunity in IBD

Although an adaptive immune response is not strictly required for development of colitis in mice [104], it is undoubtedly a crucial player with the involvement of both bacterial-specific B and T cells. Originally, the pathogenesis of CD and UC was thought to be

characterized by a functional dichotomy of Th1/17 versus Th2 abnormal responses, respectively. Recent studies and randomized clinical trials (RCTs) have questioned this idea [105]–[107].

The discoveries that IL-17 expression is increased in both CD and UC patients [108] and that IL-17 secreting $CD4^+$ T helper cells, originating from $CD161^+$ $CD4^+$ precursors [109], are substantially infiltrating CD patient intestinal mucosa [110], have led to hypothesize that Th17 cells may actively contribute to CD pathogenesis [111]. This hypothesis has been challenged by the negative results of a clinical trial evaluating the efficacy of a monoclonal antibody against IL-17A in patients with luminal CD [106]. The recent understanding that Th17 cells do not represent a homogeneous entity but divide into at least two different subsets based on cytokine profile and pathogenicity, could provide an explanation for these apparently conflicting results. Classical Th17 cells, induced in presence of TGFβ and IL-6, have been found to possess regulatory activities, owing to the coproduction of IL-17 and IL-10 [39]. Alternative Th17 cells, on the opposite, are characterized by the concomitant production of IL-17 and inflammatory cytokines, including IFNγ, and have been found to mediate pathogenic activities in experimental models of inflammation [39]. Since then, different studies have corroborated the existence of a functional plasticity of Th17 cells towards the Th1 lineage, both in murine models and in human autoimmune diseases, including IBD [39]. At present, factors associated with pathogenicity of *in vivo*-differentiated Th17 cells in the gut are yet to be fully elucidated. In this context, we and others suggested that IFNγ produced by in vivo [35] and *in vitro*-differentiated[35], [112] Th17 cells might exert a direct pathogenic role in murine models of intestinal inflammation [35], [111] and in CD patients [35].

Experiments of *ex vivo* stimulation of lamina propria mononuclear cells (LPMCs) showed that, in contrast to CD, UC-derived cells expressed lower levels of IFNγ in favor of IL-5 [113]. This observation fueled the idea that the T helper cell lineage involved in the pathogenesis of UC could be the Th2 one. Moreover, experimental murine models of chemically induced colitis (oxazolone-induced colitis) [114], corroborated by some studies involving UC patients [115], implicated a role for $CD161^{+}$ IL-13-secreting NKT cells in the pathogenesis of UC. Interestingly, most of these CD1d reactive T cells were not iNKT, since they did not express the invariant Vα24-Ja18 TCR [115] and they were tracked by sulfatide-loaded tetramers [24]. However, the failure of a recent RCT aimed at the blockade of IL-13 production in UC patients questioned the prominent role of this cytokine in the pathogenesis of UC [107]. Moreover, experimental evidences on human UC-derived LPMCs [105] and mouse models of oxazolone colitis performed in different genetic backgrounds [116], revealed that the intestinal inflammation in UC patients was characterized by Th1 cytokine signature, as well.

1.9.3 Gut microbiota in IBD

One of the proposed mechanisms of IBD pathogenesis is an abnormal adaptive immune response against luminal microbiota [117], which results in the breakdown of the mucosal tolerance to enteric bacteria. Moreover, among all the IBD-associated risk factors, many are known to induce microbiota perturbations, i.e. mode of delivery (vaginal vs caesarean), milk feeding (breast vs artificial), antibiotics use, hygiene, diets [70]. Altogether, these

evidences suggest a strong association between intestinal inflammation and gut microbiota.

Indeed, in the past years, many studies have revealed an alteration of the microbiota composition and function in IBD patients, defined as dysbiosis [118]–[122] (***Figure 1.4***). In contrast to a healthy microbiota which shows a great plasticity and the capacity to adapt to short-term perturbations going back to a pre-disturbance state when possible, the IBD associated dysbiotic microbiota manifest a reduced resilience [99].

The IBD-associated dysbiosis presents different features [99], [123]. Firstly, IBD patients are characterized by a decrease in bacterial species α diversity, a measurement of the ecological biodiversity of the microbial community [118], [120] (***Figure 1.4***). In this setting, the colonization resistance, namely the ability to outcompete pathogens, is impaired [60]. Accordingly, it has been shown that repeated antibiotics use in CD patients amplifies the state of dysbiosis and that this results in an aggravation of the disease severity [120].

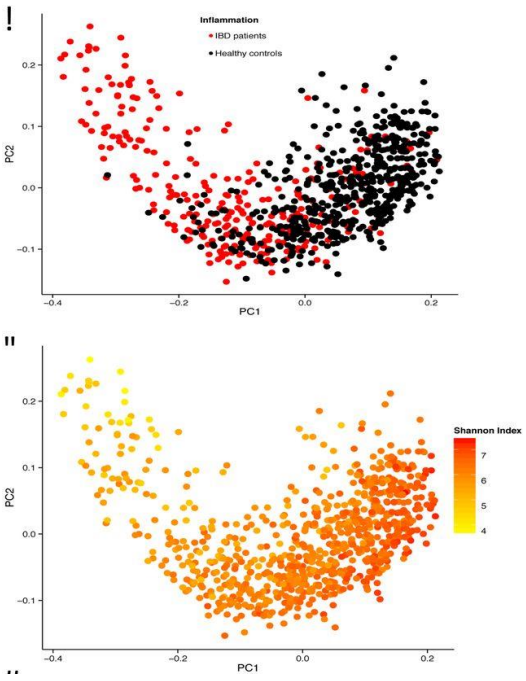

Figure 1.4 Gut microbiota dysbiosi in IBD patients. IBD patients experience a shift in overall gut microbiota composition compared to healthy patients (upper panel) and a decrease in microbial α diversity (lower panel), measured with Shannon index. Adapted from F Imhann et al.,Gut, 2016. [117]

Secondly, dysbiosis can also be intended as the expansion of pathobionts, namely symbiont species that are able to promote pathology if specific genetic or environmental conditions are altered in the host [124]. While pathobionts are found only in low frequency in healthy hosts, they can overgrow in dysbiosis and contribute to the disease in susceptible hosts.

In addition to pathobiont expansion, dysbiosis is often regarded as shifts in the overall microbial composition [99]. Since the advent of the sequencing technology for metagenomics analyses, many studies have addressed the microbiota composition of IBD patients, dealing with the intrinsic complexity created by the high variability among patients and with difficulties in using a proper healthy participant cohort [118]–[121]. A very elegant recent work tried to integrate the results of a considerably big case-control study with previous data [118]. The obtained consensus of overall microbial composition shift states that among IBD patients, CD ones experience a more marked dysbiosis compared to UC patients. Moreover, the phyla of *Bacteroidetes* and *Proteobacteria* were increased in both UC and CD patients, while *Firmicutes* was decreased only in UC. In contrast, *Actinobacteria* and *Tenericutes* were decreased in CD patients. Among the phylum *Bacteroidetes*, the order *Bacteroidales* was increased both in CD and UC compared to healthy controls, while the family *Rikenellaceae* and the genus *Bacteroides* were increased only in UC patients. Within the order *Clostridiales* of the *Firmicutes* phylum, CD patients experienced a decrease in families *Christensenellaceae*, *Clostridiaceae* and *Ruminococcaceae*. At lower taxonomic levels, also some species were observed to be significantly reduced in IBD patients, such as *Faecalibacterium prausnitzii* [125] and *Roseburia intestinalis* [119], [120].

Apart from bacterial changes in composition and diversity, the IBD dysbiosis is also associated to modifications of functional metabolic activities. In dysbiotic conditions, microbial pathways for oxidative stress tolerance, immune evasion, metabolite uptake, amino acid and carbohydrate synthesis are upregulated [119].

Despite the huge amount of observational results generated by the latest case-control studies, it remains still elusive whether the link between microbiota dysbiosis and inflammation is causative [126]. Nonetheless, gut microbiota similarities in twins both concordant and discordant for IBD have been shown in several studies [126]. Furthermore, in a healthy individual cohort, the IBD risk score (based on the presence of genetic mutations in IBD risk loci) has been associated with a decrease in *Roseburia spp* [118], similarly to what happens in IBD patients. Collectively, these evidences demonstrate that a genetic trigger can be responsible for the microbial alteration even in the absence of inflammation, suggesting that the establishment of dysbiosis could occur before intestinal inflammation [126]. A clearer evidence of gut microbiota direct contribution to the development of intestinal inflammation comes from experimental preclinical studies. The transplantation of disease-associated but not healthy microbiota can transmit the pathology in a CD mouse model of ileitis [127]. In the same way, mice deficient in CD1d expression bear a dysbiotic pro-inflammatory microbiota that can trigger a basal inflammation when transferred in healthy mice [128].

1.9.4 Therapeutic microbiota manipulation

Since an alteration of intestinal bacteria is associated with development of IBD, the modulation of gut microbiota has become an active area of research.

The use of antibiotics is the easiest way to impact on gut microbiota, thus, their use for IBD patients is of great interest. Antibiotics could act through a decrease in luminal bacterial load as well as through the selective targeting of pathobionts in favor of beneficial bacteria growth [70]. Nonetheless, as most of the commercially available antibiotics have a broad-spectrum activity, they often impact not only on harmful bacteria, but also on beneficial ones. To overcome this absence of specificity, a recent study on murine models of colitis utilized a tungsten-based treatment to target an inflammation-associated enzyme of the family *Enterobacteriaceae*, thus showing an amelioration of colitis due to antibiotic-independent precision editing of the gut microbiota [129]. Moreover, an important side effect of antibiotics treatment is the reduction in bacterial diversity, with the following impairment in colonization resistance microbiota properties [130]. For this reason, antibiotics treated IBD patients are at great risk of developing *Clostridium difficile* infection (CDI), the leading cause of infectious diarrhea in hospitalized patients [131]. Indeed, it has been demonstrated that the antibiotic driven microbiota depletion leads to a lack in primary bile acid processing in the colon. Since secondary bile acids can inhibit *C. difficile* growth by impairing the germination of its spores, their absence in antibiotic treated patients can promote *C. difficile* infection [70]. To avoid this risk, the clinical European and American guidelines (ECCO and ACOG respectively) do not recommend antibiotic treatment for IBD patients, despite their use resulted in a modest benefit when administered to active luminal CD patients and in prolonged maintenance of remission

[130]. However, antibiotic treatment remains highly recommended when the patient is at risk of infection as in case of abscesses and fistulas, or pouchitis [130].

In addition, another approach to modulate microbiota composition that has been proposed for IBD patients is the dietary intervention. Indeed, dietary composition has been associated with changes in the composition of gut microbial populations [132]. A western style diet (WSD) enriched in saturated fats and simple carbohydrates but depleted in fibers has recently been shown to induce intestinal barrier defects via the reduction in *Bifidobacteria* [133]. Conversely, a semi vegetarian diet (i.e. milk and eggs allowed, fish once a week and meat once every two weeks), resulted in enhanced maintenance of remission in CD patients [134]. Indeed, a diet enriched in resistant starch or supplemented with prebiotics, allows for complex carbohydrates arrival to the large intestine where they can ferment into SCFAs, an important energy source for both gut microbiota and the host epithelium. Nonetheless, two RCTs aimed at testing the use of prebiotics, namely food ingredients that promote beneficial bacteria growth, in CD patients failed in detecting any amelioration in the disease [135]. However, studies about dietary intervention and prebiotics use are greatly limited by the inability to include placebo controls and by challenges in collecting accurate information from the patients.

Probiotics are live organisms which provide a benefit to the host when administered in adequate amounts [136]. Many commensal bacteria have been tested *in vitro* and *in vivo* for their immunostimulatory functions or for their ability to protect the epithelial barrier. However, therapeutic use of such compounds requires caution, since it has been shown that in pathological conditions, such as colitis, they could amplify the inflammation [137].

There is emerging evidence that multi-strain probiotic mixes are more efficient than single-strain one and that their use in mild-to-moderate UC patients in combination with regular therapies is a valid approach to induce and maintain remission [138].

The concept that a multi-strain probiotic holds a higher efficacy in restoring a healthy microbiota leads to take into consideration a radical way to interfere with microbiota: the Faecal Microbiota Transplantation (FMT). The FMT is a relatively novel therapeutic approach that encompasses the application of faecal microbiota from healthy donors into the intestine of dysbiotic patients. FMT practice has been shown to be highly effective in treating recurrent *Clostridium difficile* infection patients [139]. The characterizing dysbiosis of IBD patients makes them eligible as well for testing this clinical approach. Currently, 231 RCTs investigating the safety and efficacy of FMT are registered and, among them, 38 are dealing with UC patients [140]. Nonetheless, so far, only three RCTs of phase II studies about FMT practice in UC patients have been completed and published [141]–[143]. The first study by Rossen et al. was carried out in The Netherlands and collected results from treatment of mild-to-moderate UC patients that were administered two doses of healthy FMT via nasoduodenal route of delivery. The Dutch team failed in detecting a significant improvement in attainment of remission (30% in FMT treated group, 20% in placebo group). Nonetheless patient responding to the treatment displayed an increased microbial diversity [141]. The second study held in Canada by Moayyedi et al. was conducted by treating patients with a total of 6 doses of FMT once a week supplied via enema. This time FMT treatment induced the achievement of the primary outcome of the study, namely the

remission of disease activity, in a percentage of patients statistically significantly higher compared to placebo group (24% vs 5%), suggesting that the route and frequency of administration play an important role [142]. Moreover, the placebo group of the Dutch study received an autologous FMT instead of a water enema, thus raising the doubt that this procedure could be considered as a true control placebo, since it likely results in a modification of the gut microbiota itself. The third study carried out by Paramsothy et al. in Australia introduced the use of multi-donor faecal material for the transplantation, with the aim to reduce the probability to receive ineffective donor stool [143]. Moreover, they prolonged the treatment and increased the frequency till 5 doses a week. They obtained a clinical remission in 27% of the treated patients versus the 8% of the water-placebo group ($p = 0.021$) and interestingly they showed that multi-donor preparations greatly increased the microbial diversity compared to individual donors [143]. Albeit the recent European Consensus Conference on FMT indicated the clinical practice of FMT only for CDI patients [131], the promising preliminary results on FMT efficacy in IBD patients recommend an accurate analysis on its application also for other pathologies.

Despite these encouraging results from the clinical practice, only poor mechanistic evidence is present about the actual function of FMT. Given the strict network of interactions between gut microbiota and the host immune system, it would not be surprising to observe a direct modulation of immune cells in response to microbial manipulation during intestinal inflammation. Indeed, in a murine model of intestinal inflammation, FMT has been shown to ameliorate colitis through a downregulation of pro-inflammatory cytokines and an increase in Treg cells [144].

2 Aims

The intestine has the challenge to preserve the integrity of the tissue while allowing pivotal exchanges with the external world [2]. To fulfill this task, the gut needs to maintain a balance between tolerance, required for food intake, and immunity, at risk of an overstimulation by the gut microbiota, including potential invading pathogens [3]. This delicate equilibrium is further demonstrated by the reciprocal functional shaping of the host mucosal immune system and of the gut microbiota throughout life [71], [145].

Here, we aim at investigating the functional interactions between the gut microbial community and the mucosal immune system during experimental and human intestinal inflammation and in the presence of microbial dysbiosis.

Since patients suffering from Inflammatory Bowel Diseases manifest abnormal T cell responses associated to the recognition of dysbiotic commensal bacteria [117], to gain a better understanding of the pathogenic mechanisms responsible of intestinal inflammation development, we will specifically focus on:

- Analyzing the effect of microbiota dysbiosis on intestinal T cell subsets activation;
- Phenotypically and functionally characterizing T cell subsets activation in mouse models of intestinal inflammation and in IBD patients;
- Unravelling the mechanism of action of therapeutic microbiota manipulation on the mucosal immune system during intestinal inflammation;
- Studying the role of microbiota-driven regulation on iNKT cell functions and activity.

3 Materials and Methods

3.1 Mice

C57BL/6 mice (Charles River, IT) and CXCR6 EGFP/+ mice of 8-10 weeks of age were housed at the European Institute of Oncology (IEO) animal facility in SPF conditions. Animal procedures were approved by the Italian Ministry of Health (Auth. 127/15, 27/13, 913/16, 415/17).

Experimental groups of mice receiving the different treatments were kept in separated cages. Littermates of same sex and age were randomly assigned to the different experimental groups.

CXCR6 EGFP/+ mice (B6.129P2-Cxcr6tm1Litt/J; IMSR_JAX: 005693) have C57BL/6 background and were purchased as GFP/GFP from JAX, USA, and then were bred to heterozigosity with C57BL/6 mice). At weaning mice are screened for the presence of the GFP and WT alleles by multiplexed PCR on genomic DNA using GoTaq G2 Hot Start Polymerase (Promega) and primers listed in ***Table 3.1***.

Primer	Allele	Sequence 5' --> 3'
oIMR0872	GFP	AAG TTC ATC TGC ACC ACC G
oIMR1416	MUT	TCC TTG AAG AAG ATG GTG CG
oIMR4077	WT Fw	TAG TGG CTG TGT TCC TGC TG
oIMR4078	WT Rev	GGC AGC CGA TAT CCT TCA TA

Table 3.1 Primer sequencing for CXCR6 EGFP/+ genotyping

The cycling protocol used is described in ***Table 3.2***.

Step #	Temp °C	Time	Note
1	94	3 min	-
2	94	30 sec	-
3	58	1 min	-
4	72	1 min	repeat steps 2-4 for 35 cycles
5	72	2 min	-
6	10	-	Hold

Table 3.2 Thermal cycler protocol used for CXCR6 EGFP/+ genotyping

The assay amplifies a 173 bp band for the wild type allele and a 340 bp band for the GFP allele as shown in ***Figure 3.1***.

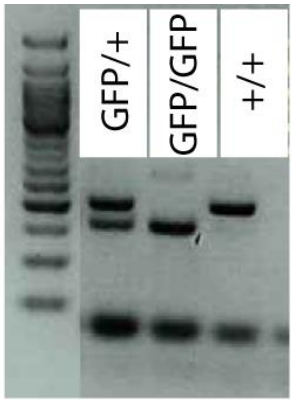

Figure 3.1 CXCR6 EGFP mice genotyping assay

3.2 Murine experimental colitis models

For the induction of DSS-induced acute and chronic colitis, mice were given 2% (w/v) dextran sodium sulphate (DSS, MW 40 kD; TdB Consultancy) in their drinking water for 7 days followed by 2 days of recovery or for 3 cycles of 7 days of DSS followed by 14 days of water recovery, respectively. The weight curve was determined by weighing mice daily in

the acute models or twice a week for the chronic one. At sacrifice colons were collected, their length was measured and divided in portions to be fixed in 10% formalin for histological analyses, snap-frozen for RNA extraction and for lamina propria mononuclear cells (LPMC) immunophenotyping. Additionally, the luminal content was collected and immediately frozen.

3.3 In vivo gut microbiota depletion

To eliminate the whole gut microbiota, mice were administered with a whole spectrum antibiotic cocktail of neomycin (1g/L), ampicillin (1g/L), vancomycin (0.5 g/L) and metronidazole (1g/L) in their drinking water for at least 2 weeks.

3.4 In vivo faecal microbiota transplantation

The faecal microbiota transplantation (FMT) was performed through oral gavage of mucus (1st day) and faecal (2nd and 3rd days) preparations from healthy donor mice (normobiotic), DSS-treated mice (dysbiotic) or antibiotic treated mice (***Figure 3.2***). This protocol facilitates the engraftment of the mucus-associated bacteria that are also considered to be the ones that more likely interact with the host immune system. Mucus was scraped from the colons of the donor mice, diluted in PBS and administered to recipients at 1:1 ratio. Faeces were collected, diluted in PBS (50 mg/ml) and administered to recipients by oral gavage (10 mg/mouse). To deplete distinct taxa of bacteria donor mice were treated with metronidazole (1g/L), vancomycin (1 g/L) or streptomycin (2g/L).

For in vivo IL10R blockade, mice were injected intraperitoneally with 250 µg InVivoMAb anti-mouse IL10R (BioXCell, clone 1B1.3A) daily for 4 days starting from one day before FMT treatment.

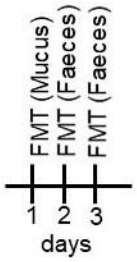

Figure 3.2 Representative scheme of Faecal Microbiota Transplantation

3.5 Microbiota identification by 16S rRNA gene- amplification, -sequencing and data analysis

The intestinal mucus was scraped from the colon, collected in TES buffer (50 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM EDTA) and stored at -80°C. The faecal content was collected directly from the specimen and stored at at -80°C. The bacterial DNA was extracted with G NOME DNA isolation kit (MP Biomedicals) following the manufacturer's instructions. The analyses of 16S rRNA amplification, sequencing and data analyses was performed by GenProBio s.r.l. (Parma, Italy). Partial 16S rRNA gene sequences were amplified using primer pair Probio_Uni and / Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence [146] 16S rRNA gene sequencing was performed using a MiSeq (Illumina) at the DNA sequencing facility of GenProbio srl (www.genprobio.com) according to the protocol previously reported. Following sequencing, the obtained individual sequence reads were

filtered by the Illumina software to remove low quality and polyclonal sequences. All Illumina quality-approved, trimmed and filtered data were exported as .fastq files. The .fastq files were processed using a custom script based on the QIIME software suite. Paired-end reads pairs were assembled to reconstruct the complete Probio_Uni / Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score >20 while sequences with homopolymers >7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 99 % sequence homology using uclust and OTUs with less than 10 sequences were filtered. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the SILVA database. Biodiversity of the samples (alpha-diversity) were calculated with Chao1 and Shannon indexes. Similarities between samples (beta-diversity) were calculated by unweighted uniFrac. The range of similarities is calculated between the values 0 and 1.

3.6 Metabolomic analyses by metabolite extraction, derivatization, identification with mass spectrometry and data analyses

Metabolome extraction, purification and derivatization was carried out by Theoreo s.r.l. (Montecorvino Pugliano [SA], Italy) by means of the MetaboPrep kit according to the manufacturer's instruction.

Two µL samples of the derivatized solution were injected into the GC-MS system (GC-2010 Plus gas chromatograph coupled to a 2010 Plus single quadrupole mass spectrometer; Shimadzu Corp., Kyoto, Japan). Chromatographic separation was achieved with a 30 m 0.25 mm CP-Sil 8 CB fused silica capillary GC column with 1.00 µm film thickness from Agilent (Agilent, J&W Scientific, Folsom, CA, USA), with helium as carrier gas. The initial oven temperature of 100 °C was maintained for 1 min and then raised by 4 °C/min to 320 °C with a further 4 min of hold time. The gas flow was set to obtain a constant linear velocity of 39 cm/s and the split flow was set at 1:5. The mass spectrometer was operated in electron impact (70 eV) in full scan mode in the interval of 35-600 m/z with a scan velocity of 3333 amu/sec and a solvent cut time of 4.5 minutes. The complete GC program duration was 60 minutes. Untargeted metabolites were identified by comparing the mass spectrum of each peak with the NIST library collection (NIST, Gaithersburg, MD, USA). To identify metabolites identity, the linear index difference max tolerance was set at 10, while the minimum matching for the NIST library search was set at 85%. The chromatographic data for PLS-DA analysis were tabulated with one sample per row and one variable (metabolite) per column. According to MSI level 1 standard [147], the VIP putative metabolites identity was confirmed by means of an independent analytical standard analysis. The normalization procedures consisted of data transformation and scaling. Data transformation was made by generalized log transformation and data scaling by autoscaling (mean-centered and divided by standard deviation of each variable). Partial least square discriminant analysis (PLS-DA) [148] was performed on Internal Standard peak area [149] normalized chromatogram using R (Foundation for Statistical Computing, Vienna, Austria). Mean centering and unit variance scaling was applied for all analyses. Classes separation was

archived by PLS-DA, which is a supervised method that uses multivariate regression techniques to extract, via linear combinations of original variables (X), the information that can predict class membership (Y). PLS regression was performed using the plsr function included in the R pls package [150]. Classification and cross-validation was performed using the corresponding wrapper function included in the caret package [151]. A permutation test was performed to assess the significance of class discrimination. In each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross validation for the model based on the original class assignment. Variable Importance in Projection (VIP) scores were calculated for each component. A VIP is a weighted sum of squares of the PLS loadings, taking into account the amount of explained Y-variation in each dimension.

3.7 Histological analysis

The histological analyses were performed in collaboration with the Pathology Unit of IRCCS Policlinico Hospital Milan. Tissue processing was performed with a LEICA PELORIS processor before paraffin embedding. Murine samples were embedded using an automated system (SAKURA Tissue-Tek). After Hematoxylin and Eosin staining, snapshots of histology were taken using an Aperio CS2 microscope with a scanning resolution of 50,000 pixels per inch (0.5 µm per pixel with 10x objective and 2.5 µm per pixel when scanning at 4x). Scoring of disease activity was performed according to the criteria described in ***Table 3.3*** [91] (Appendix I).

	Criterion	Definition	Score
Inflammatory cell infiltrate	Severity (leukocyte density of lamina propria area infiltrated)	No infiltrate	0
		Minimal acute (<10%)	0.25
		Mild chronic (10-25%, scattered neutrophils)	0.5
		Moderate chronic (26-50%)	0.75
		Marked (>51%, dense infiltrate)	1
	Extent (expansion of leukocyte infiltration)	Mucosal	0.5
		Mucosal and submucosal	0.75
Epithelial changes	Hyperplasia (increase in epithelial cell numbers in longitudinal crypts, visible as crypt elongation)	No hyperplasia	0
		Minimal (<25%)	0.25
		Mild (26-35%)	0.5
		Moderate (36-50%, mitoses in the upper third of the crypt epithelium)	0.75
		Marked (>51%, mitoses in crypt epithelium distant from crypt base)	1
	Goblet cell loss (relative to baseline goblet cell numbers per crypt)	No loss	0
		Minimal (<25%)	0.25
		Mild (26-35%)	0.5
		Moderate (36-50%)	0.75
		Marked (>51%)	1
Mucosal architecture	Ulceration (epithelial defect reaching beyond muscolaris mucosae)	No ulcers	0
		Ulcers	0.25
	Granulation tissue (connective tissue new capillaries, surrounded by infiltrating cells, hypertrophic areas)	No granulation tissue	0
		Granulation tissue	0.25
	Mucosal thickness and crypt depth	No thickening	0
		Thickening	0.5
	Glandular rarefaction	No rarefaction	0
		Rarefaction	0.5
	Dysplasia	No dysplasia	0
		Dysplasia	0.5
		MAX SCORE	**6**

Table 3.3 Scoring scheme for histological evaluation of intestinal inflammation

3.8 Quantitative reverse transcription PCR (RT-qPCR) of tissue mRNA

Colonic tissues were homogenized in 500 µl TRIzol (Invitrogen) and then RNA extraction was performed adding 100 µl of chloroform, precipitating the aqueous phase with 300 µl of 70% ethanol and purifying RNA with Quick-RNA MiniPrep (ZymoResearch) according to manufacturer's specifications. cDNAs were generated from 1µg of total RNA with EasyScript Plus™ cDNA Synthesis kit (abm). Gene expression levels were evaluated by qPCR on 10ng cDNA using BrightGreen 2X qPCR Mastermix (abm) and normalizing to the housekeeping gene (*Rpl32* for mouse and *Gapdh* for human) expression. The primer sequences are collected in ***Table 3.1***.

Primer	Species	Forward	Reverse	bp	Vendor
CXCL16	Mouse	AGCGCAAAGAGTGTGGA	GGTTGGGTGTGCTCT	193	SIGMA
CXCR6	Mouse	CCTTTTTGGGCCTATGCA	ATGCCTCGAAGAGTT	71	SIGMA
MCP-1	Mouse	CAAGATGATCCCAATGA	GGTTCCGATCCAGGT	161	SIGMA
CXCL10	Mouse	CGCTGCAACTGCCATCCA	CCGGATTCAGACATC	148	SIGMA
TNF	Mouse	TCTTCTCATTCCTGCTTG	CACTTGGTGGTTTGCT	200	SIGMA
IL6	Mouse	CTCTGGGAAATCGTGGA	GCAAGTGCATCATCG	77	SIGMA
IL12	Mouse	CCTGCTGAAGACCACAG	AGCTCCCTCTTGTTGT	200	SIGMA
Muc1	Mouse	TACCCTACCTACCACACTCACG	CTGCTACTGCCATTACCTGC	95	SIGMA
Muc2	Mouse	GTGTGGGACCTGACAATGTG	TTGCCACCAGAACATTTCTTT	124	SIGMA
Muc3	Mouse	CTTCCAGCCTTCCCTAAACC	TCCACAGATCCATGCAAAAC	119	SIGMA
Muc4	Mouse	GAGAGTTCCCTGGCTGTGTC	GGACATGGGTGTCTGTGTTG	101	SIGMA
CXCL16	Human	AGCGCAAAGAGTGTGGA	GGTTGGGTGTGCTCT	193	SIGMA
CXCR6	Human	CCTTTTTGGGCCTATGCA	ATGCCTCGAAGAGTT	71	SIGMA
MCP-1	Human	CAAGATGATCCCAATGA	GGTTCCGATCCAGGT	161	SIGMA
CXCL10	Human	CGCTGCAACTGCCATCCA	CCGGATTCAGACATC	148	SIGMA
TNF	Human	TCTTCTCATTCCTGCTTG	CACTTGGTGGTTTGCT	200	SIGMA
IL6	Human	CTCTGGGAAATCGTGGA	GCAAGTGCATCATCG	77	SIGMA
Primer		**Cat no.**			**Vendor**
IL17a	Mouse	QT00103278			Qiagen
IFNg	Mouse	QT01038821			Qiagen
IL10	Mouse	QT00106169			Qiagen
IL23	Mouse	QT01663613			Qiagen
IL22	Mouse	QT00128324			Qiagen

Rpl32	Mouse	QT00131992	Qiagen
Tjp1	Mouse	QT00493899	Qiagen
Camp1	Mouse	QT00241003	Qiagen
S100A8	Mouse	QT00250264	Qiagen
DEFb3	Mouse	QT00265517	Qiagen
IL1b	Mouse	QT010483555	Qiagen
IL17	Human	QT00009233	Qiagen
IFNg	Human	QT00000525	Qiagen
Gapdh	Human	QT00079247	Qiagen

Table 3.4 qPCR primers

3.9 Tissue ELISA of murine IL-10

Colonic tissues were homogenized in 300 µl RIPA Buffer (Cell Signaling Technology) supplemented with Phosphatase inhibitors (Sigma) and Protease inhibitors (Complete Ultra tablets, Roche). The samples were then incubated at 4°C for 30 minutes under slow rotation and then centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was quantified at the NanoDrop with Bradford Assay (BioRad). mIIL-10 was measured on 6.25 µg of lysate using the ELISA assay (Purified anti-mouse IL-10 and Biotin anti-mouse IL10, Biolegend) performed following manufacturer's instructions.

3.10 Faecal Bacteria Plating

One faecal pellet from each mouse was smashed, filtered with 100 µm nylon cell strainer and resuspended in 1 mL of sterile PBS. 200 µL were then plated on Chocolate II Agar plates (BD) and grown at 37°C under aerobic conditions overnight or in anaerobic conditions for 48 hours.

3.11 Murine Cell isolation

For lamina propria mononuclear cell (LPMC) isolation, the colons were cut open, washed in PBS and then incubated in RPMI supplemented with 1 mM EDTA at 37°C for 30 minutes to detach the epithelial layer. The tissue was mechanically dissociated with GentleMACS™ (Miltenyi) and then serially smashed and filtered on a metal strainer first, and then with 100 µm and 70 µm nylon cell strainers. The LPMC were then counted and stained. In some experiments after isolation, cells were re-stimulated *ex-vivo* for 3 hours with 0.1µM PMA (Sigma), 1µg/ml Ionomycin (Sigma-Aldrich) and 10µg/ml Brefeldin A (Sigma).

3.12 Flow cytometry

Human and murine cells were stained with combinations of directly conjugated antibodies as specified in ***Table 3.5***, all sourced from BD, eBioscience, Biolegend or Tonbo.

Marker	Reactivity to	Clone	Vendor
mCD1d:PBS57 Tet	Mouse		Gift from NIH Tet facility
CD45.2	Mouse	104	Biolegend
CD3	Mouse	17A2	BD
CD8α	Mouse	53-6.7	eBioscience
CD4	Mouse	GK1.5	BD
CD11c	Mouse	HL3	BD
CD19	Mouse	1D3	BD
CD11b	Mouse	M1/70	BD
F4/80	Mouse	BM8	Biolegend
Ly6g	Mouse	1A8	Biolegend
Ly6c	Mouse	AL-21	eBioscience
MHC-II	Mouse	M5/114.15.2	eBioscience
Ki67	Mouse	16A8	Biolegend
CD69	Mouse	H1.2F3	BD

Ter119	Mouse	TER-119	BD
TCR α/β	Mouse	H57.597	BD
TCR γ/δ	Mouse	UC713B5	Biolegend
CD90	Mouse	53-2.1	BD
CD117	Mouse	2B8	BD
CD127	Mouse	A7R34	Biolegend
Nkp46	Mouse	29A1.4	BD
RorγT	Mouse	AFKJS9	eBioscience
ST-2	Mouse	DIH9	Biolegend
CD25	Mouse	PC61	BD
FoxP3	Mouse	FJK-16s	eBioscience
IFNγ	Mouse	XMG1.2	BD
IL10	Mouse	JE65-16E3	BD
IL22	Mouse	Poly5164	Biolegend
IL17A	Mouse	TC11-18H10.1	Biolegend
IL13	Mouse	eBio13A	eBioscience
TNF	Mouse	MP6-XT22	Biolegend
Zombie Dye			Biolegend
Zo-1	Mouse	ZO1-1A12	Invitrogen
hCD1d:PBS57 Tet	Human		Gift from NIH Tet facility
CD3	Human	OKT3	Tonbo
CD4	Human	RPA-T4	Tonbo
CD8α	Human	OKT8	Tonbo
CD25	Human	BC96	BD
CD11c	Human	B-ly6	BD
CD19	Human	HIB19	BD
IL17A	Human	BL168	BD
IFNγ	Human	B27	BD
TNF	Human	Mab11	R&D Systems
IL13	Human	142928	Biolegend
Anti-CD1d	Human	CD1d42	BD
Anti-IFNγ	Human	NIB42	eBioscience
Anti-TNF	Human	Mab1	eBioscience
Anti-IL17	Human	eBio64CAP17	eBioscience
Anti-IL13	Human	JES10-5A8	Biolegend

Table 3.5 Antibodies and FACS reagents

iNKT cells were identified by CXCR6 EGFP expression or by m/hCD1d:PBS57 Tetramer (NIH Tetramer core facility) staining. Prior staining, the Fcγ-receptors were blocked with anti-

CD16/32 mAb (10μg/ml). Murine and human tetramers were then incubated at 4°C for 30 minutes with fresh cells at the concentration of, respectively, 0.1 μl/ $3x10^6$ cells for murine cells or 0.2 μl/ $3x10^6$ cells for human ones. The gating strategy to identify murine and human iNKT cells are described respectively in ***Figure 3.3*** and ***Figure 3.4***. Intracellular cytokines were detected after stimulation of cells for 3 hours with 0.1μM PMA (Sigma), 1μg/ml Ionomycin (Sigma-Aldrich) and 10μg/ml Brefeldin A (Sigma). Cells were fixed and permeabilized with Cytofix/Cytoperm (BD) before the addition of the antibodies detecting the cytokine released. Intranuclear transcription factors were detected after fixation and permealisation with Foxp3/Transcription Factor Staining Buffer Set (eBioscience™). Multiplexing analysis of cytokines in supernatants collected after *in vitro* stimulation was performed with a Cytokine Bead Array (CBA, BD), according to manufacturer's protocol. Samples were analyzed by FACSCanto or FACSCelesta flow cytometer (BD), gated to exclude non-viable cells on the basis of light scatter. Data were analyzed using FlowJo software (BD).

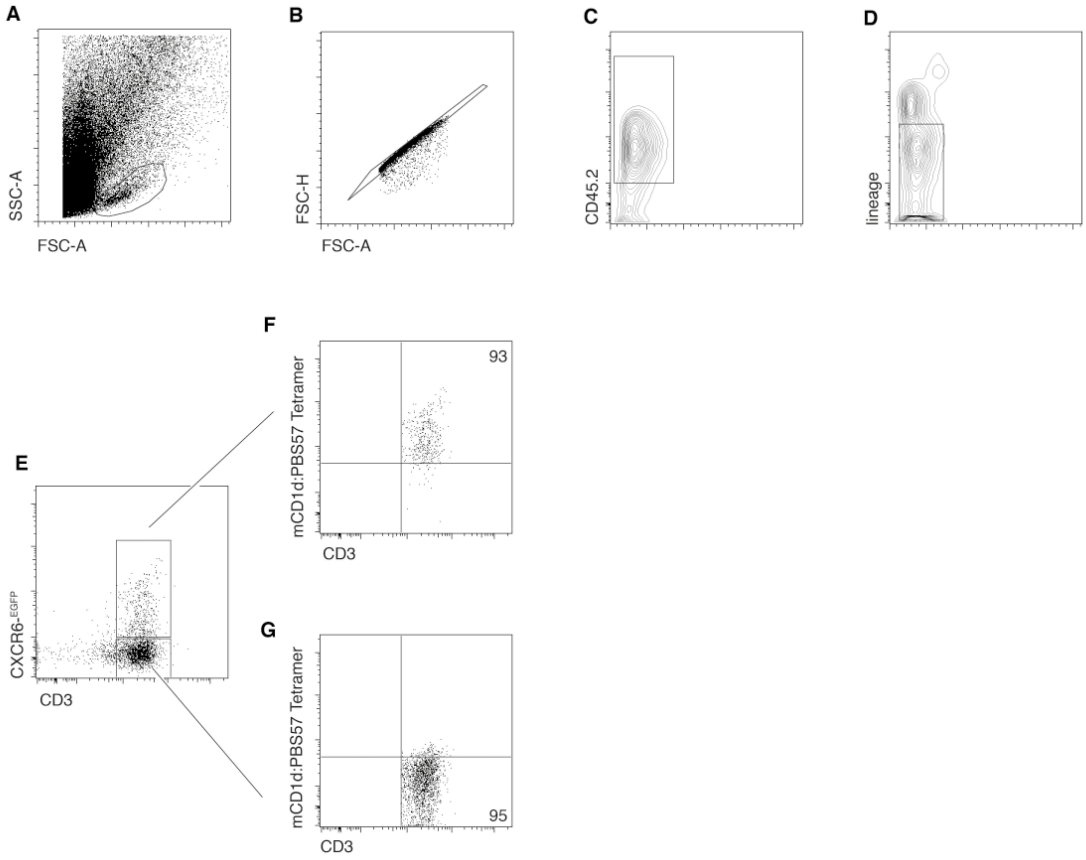

Figure 3.3 Gating strategy for tracking murine iNKT cells (A) Forward and Side Scatter of colonic LPMC gate indicates living lymphocytes (B) Doublets-excluding gate (C) Epithelial cell-excluding gate based on CD45.2 expression. (D) Lineage (CD19, CD11c, CD11b)-excluding gate. (E) CD3 expression and $CXCR6^{EGFP}$ (F) mCD1d:PBS57 Tet staining on gated $CD3^{+}$ $EGFP^{+}$ cells confirms that 93% of $EGFP^{+}$ cells are iNKT cells. (G) mCD1d:PBS57 Tet staining on gated $CD3^{+}EGFP^{-}$ cells.

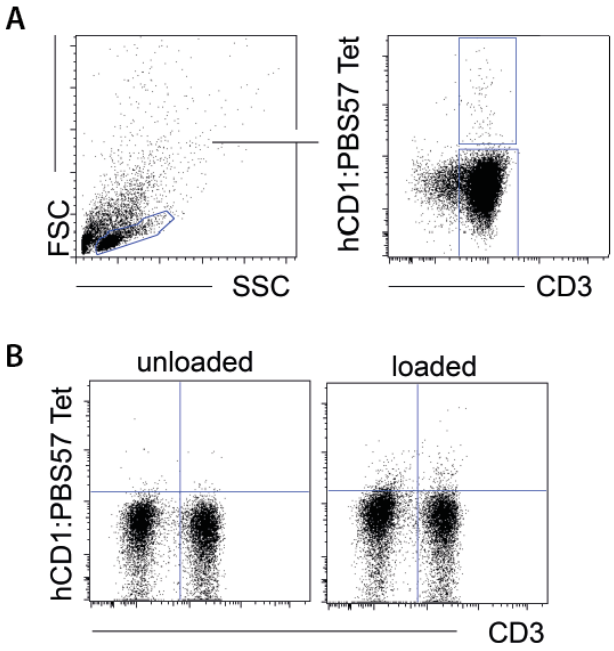

Figure 3.4 Gating strategy for tracking human iNKT cells (A) From left, Forward and Side Scatter to identify lymphocytes and CD3/hCD1d:PBS57 tetramer expression. (B) Dot plots indicate iNKT cells staining with unloaded (left panels) or PBS57-loaded hCD1d Tetramer.

3.13 In vitro murine intestinal cell activation assay

Colonic lamina propria and mesenteric lymph node leukocytes were collected from untreated mice. 2×10^6 cells were plated and exposed to 0.05 mg of bacteria (wet weight) derived from faeces of untreated, DSS-treated or FMT-treated mice at the time of sacrifice. Gentamycin (50 µg/ml) and a cocktail of antibiotics (P/S) were added after 2 hours of incubation. The cells were left in culture for 96hrs. In some experiments, anti-MHCII blocking antibody (clone M5/114.15.2, TONBO) was added at a final concentration of 10 µg/ml. At the end of the experiment cells were analysed by flow cytometry. Their viability was checked with Zombie Yellow™ Fixable Viability Kit (Biolegend).

3.14 Human Subjects

Buffy-coated blood (HD, n=15, IBD n=5) and intestinal specimens of UC patients (n=16), CD patients (n=24), and patients undergoing intestinal surgical resection for pathologies unrelated to IBD, including diverticular disease and intestinal tumors (n=27) were obtained from the IRCCS Policlinico Ospedale Maggiore, Milan, Italy. The clinical characteristics and concomitant therapies of IBD patients are summarized in ***Table 3.6***. The Institutional Review Board approved the study (permission ref. no. EA1/107/10) and informed consent was obtained from the subjects involved in the study.

Clinical parameter	**Healthy controls n=27**	**Ulcerative Colitis n=16**	**Crohn's disease n=24**
Male/Female, (n)	14 / 13	9 / 7	12 / 12
Age at enrolment, (mean ± SD, yr)	69.5 (± 12.3)	40.75 (± 13.3)	39.61 (± 7.4)
Disease duration, (mean ± SD, yr)	-	8,8 (± 4.8)	10.5 (± 6.2)
Smoking status, (yes/no/ex)	-	2/13/1	3/19/2
Crohn's disease, (n)			
L1 ileal	-	-	10
L2 colonic	-	-	3
L3 ileocolonic	-	-	11
L4 upper	-	-	0
B1 (no strict/no penetr)	-	-	6
B2 (stricturing)	-	-	13
B3 (penetrating)	-	-	5
Ulcerative Colitis, n			
E1 proctitis	-	1	-
E2 left-sided	-	11	-
E3 pancolitis	-	4	-
Concomitant therapy at enrolment			
No therapy, (n)	-	7	15
Antibiotics, (n)	-	0	1
Mesalamine, (n)	-	6	3

Thiopurines, (n)	-	-	5
Corticosteroids, (n)	-	3	2
Anti-TNF, (n)	-	-	2

Table 3.6 Patients description

3.15 Human primary cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich). Human LPMC were isolated as previously described [111]. Firstly, the tissue was resected, and the mucosa separated from the submucosa with forceps and surgical scissors. The intestinal mucosa was freed of mucus with a pretreatment of 15 minutes at RT with dithiothreitol (DTT, 0.1 mmol/L, Sigma) and then washed with 3 cycles of 5 minutes with HBSS supplemented with Penicillin/Streptomycin (Gibco, Life Technology) and Gentamycin (Roche). To detach the epithelial layer the mucosa was then minced with scalpels and incubated in EDTA (1 mmol/L, Sigma) at RT (3 cycles of 50 minutes). After three washes with HBSS supplemented with antibiotics the mucosa was incubated with collagenase D to digest the extracellular matrix (400 U/ml, Worthington Biochemical Corporation, Lakewood, NJ) o/n at 37°C and 5% CO_2. The cell suspension was then filtered with 100 µm nylon strainer and centrifuged at 1930 rpm for 10 minutes at RT. The cell suspension was then separated with a Percoll (Sigma) gradient (100%, 60%, 40% and 30%) at 1930 rpm for 30 minutes at RT. Lamina Propria Mononuclear Cells (LPMC) were then collected between the 60% and 40% layers, washed twice with PBS supplemented with antibiotics and then cultured in complete RPMI 1640 medium.

3.16 Intestinal iNKT cell lines and clone generation

The population of $CD45^+CD3^+$ CD1d:$PBS57Tet^+$ cells was sorted with FACSAria (BD) from total LPMC or PBMC. For the generation of human iNKT cell lines, sorted iNKT cells were expanded *in vitro* for two weeks in the presence of irradiated peripheral blood feeders ($5x10^5$/mL), hIL2 (100 U/ml, Proleukin) and PHA (1 µg/ml, Remel™).

iNKT cell clones were generated via cloning by limiting dilution according to the protocol described in [152]**.** Briefly, the sorted iNKT cells were resuspended at three serial dilution in order to plate them at 5 cells/well, 1 cell/well and 0.2 cell/well in 96 well plates. The first dilution resulted in a positive control as the majority of the wells showed growing cells. The plating efficiency was estimated by counting the number of negative wells and only the cells grown from the dilutions with a negative growth rate lower than 37% were considered as clones [152].

Both iNKT cell lines and clones were then re-stimulated with irradiated feeder cells (5x105/mL), PHA (1 µg/ml, Remel™) and hIL-2 (100 U/ml, Proleukin) every 21 days.

3.17 Human iNKT-cell in vitro stimulation

Polycolonal iNKT cell stimulation was performed with 0.1 µM PMA and 1 µg/ml ionomycin (Sigma-Aldrich). Neutralizing antibodies to CD1d (BD) were used at the concentration of 10 µg/ml.

For antigen specific stimulation, $5x10^4$ antigen presenting cells (monocytes derived Dendritic cells or $CD45^+HLA\text{-}DR^+$ intestinal LPMC) were plated in each well at 1:1 ratio with human iNKT cells. Sonicated αGalCer was used at 40 ng/mL.

For bacteria stimulation assays, purified bacterial strains (*AIEC* LF82 and *S.Thyphymurium*) underwent cycles of heat inactivation and freezing/thawing before being serially diluted starting from $5x10^6$ CFU/well (100:1 bacteria:APC) to $5x10^5$/well (1:1 bacteria:APC). The isolates of patient mucosa-associated microbiota were normalized according to their protein content (Pierce BCA Protein Assay kit, Thermo Fisher). After 36 hours T cell activation was estimated by measuring cytokine released in culture supernatants by ELISA assays, Cytometric Bead Array (BD) or intracellular staining.

3.18 Measurement of trans-epithelial electrical resistance (TEER)

The intestinal epithelial cell line Caco-2 was sourced from the American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 20% FCS, 2MM L-glutamine, 1mM sodium pyruvate, 0.1 mM non-essential amino acids (NEAA) and penicillin/streptomycin. The cells were split three times a week. For TEER measurement the cells at passage 10-30 were plated at $15x10^3$ cells/well on polyester permeable Transwell-clear inserts (6.5-mm diameter, 0.4-mm pore size, Corning®) and grown for 5–7 days, until ΔTEER >300 Ω*cm2 (Millicell-ERS Volt-Ohm Meter Millipore, Bedford, MA).

The supernatants collected from intestinal $CD4^+$ T cell clones, stimulated for 3 hours with PMA/ionomycin in Caco-2 medium, in the presence or absence of neutralizing Ab (anti-human -IFNγ, -IL-17A, -TNF (eBioscience), -IL13 (Biolegend) at concentration of 20 μg/ml

for anti-IFNγ and 10 μg/ml for the others) were applied in the lower transwell chamber.

Measurements were carried out at the beginning of the assay, after 2 hours, after 16 hours and then every 2 hours until 26 hours after stimulation. The ohmic resistance of a blank (culture insert without cells) was measured in parallel. To obtain the sample resistance, the blank value was subtracted from the total resistance of the sample. The final unit area resistance (Ω*cm2) was calculated by multiplying the sample resistance by the effective area of the membrane. For comparison among treatments with different clones, TEER was normalized to the supernatant of each unstimulated clone.

3.19 Fluorescence in situ hybridization (FISH)

Formalin fixed paraffin embedded tissues are sectioned 5 μm thickness. The probes (EUB1, EUB2, EUB3) used are designed to specifically target different regions of the 16S rRNA. All the probes are manufactured by SIGMA and labelled with Alexa488. Probes are applied to slides at a concentration of 5 ng/μl in prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris pH 7.4, 0.01% SDS). Slides are incubated over night at 50°C in a humid chamber and washed at 50°C in pre-warmed washing buffer (0.9 M NaCl, 20 mM Tris pH 7.4). The slides are counterstained with DAPI. Confocal images are acquired through HCX PL APO 40X (NA 1.25) oil immersion objective. The probes sequences are collected in Sup Tab.4.

3.20 Statistics

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). Statistical significance was calculated using a Mann Whitney test for comparison within two groups or Kruskal-Wallis test with Dunn's multiple comparison correction within more than two groups. $P < 0.05$ (*), $P < 0.01$ (**) $P < 0.001$ (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

4 Results I

Several evidences suggest that functional activation of immune cell populations in the lamina propria might be secondary to the recognition of an altered (dysbiotic) microbiota [153]. Nonetheless, it is currently unclear if any type of intestinal dysbiosis might be equally capable to trigger mucosal immune cells activation.

Indeed, the disruption of the intestinal barrier and the following translocation of luminal bacteria and metabolites in the lamina propria are two key events in the aetiology of intestinal inflammation and colitis [154],[95].

To study the mechanisms leading to the activation of immune cell populations in the intestinal mucosa in the presence of a microbial altered composition, we adopted two different models of dysbiosis induction, i.e. by Dextran Sodium Sulphate (DSS) or by broad-spectrum antibiotics administration.

Antibiotic-induced dysbiosis was obtained by treating for two weeks mice with broad-spectrum antibiotics (vancomycin, metronidazole, ampicillin, neomycin) in their drinking water, while DSS-induced dysbiosis was generated by administering DSS for one week, according to the acute colitis model [155].

Both experimental settings are characterized by a relevant alteration in the microbiota composition, nonetheless they show peculiar differences, including the presence or absence of inflammation, of epithelial damage and of bacterial translocation.

At the end of the treatments, colonic immune cells were analyzed, with a specific focus on conventional $CD4^+$ T cells and unconventional iNKT cell phenotype and functions.

Since iNKT cells are scarcely present in mucosa-associated lymphoid tissues [20], we took advantage of the CXCR6 EGFP/+ reporter mice to faithfully track them in the intestine. As reported in ***Figure 3.3***, mCD1d:PBS57 Tet staining on gated $CD3^+$ $CXCR6^+$ cells confirms that 93% of $CXCR6EGFP^+$ cells are iNKT cells.

4.1 DSS-induced dysbiosis

4.1.1 DSS induces intestinal inflammation and $CD4^+$ and iNKT cells colonic accumulation

DSS is a sulfated polysaccaride that is directly causing colonic epithelial toxicity and intestinal barrier dysfunction [156].

DSS administration induced a strong intestinal inflammatory response, characterized by weight loss (***Figure 4.1A***), colon shortening (***Figure 4.1B***) and by histological signs of colonic inflammation (***Figure 4.1C-D***). These colonic tissues are characterized by epithelial changes including epithelial hyperplasia visible as crypt elongation and goblet cell loss, tissue architecture modifications such as ulcers and mucosal thickening, and by a marked immune cell infiltration [157], (Appendix I and ***Figure 4.1D***). The extent of inflammation is also well documented by the expression of a broad array of inflammatory genes such as *tnf, il17a, ifng, il23* and *il12* ***Figure 4.1D***) and chemokines associated to iNKT cell recruitment (***Figure 4.1F***).

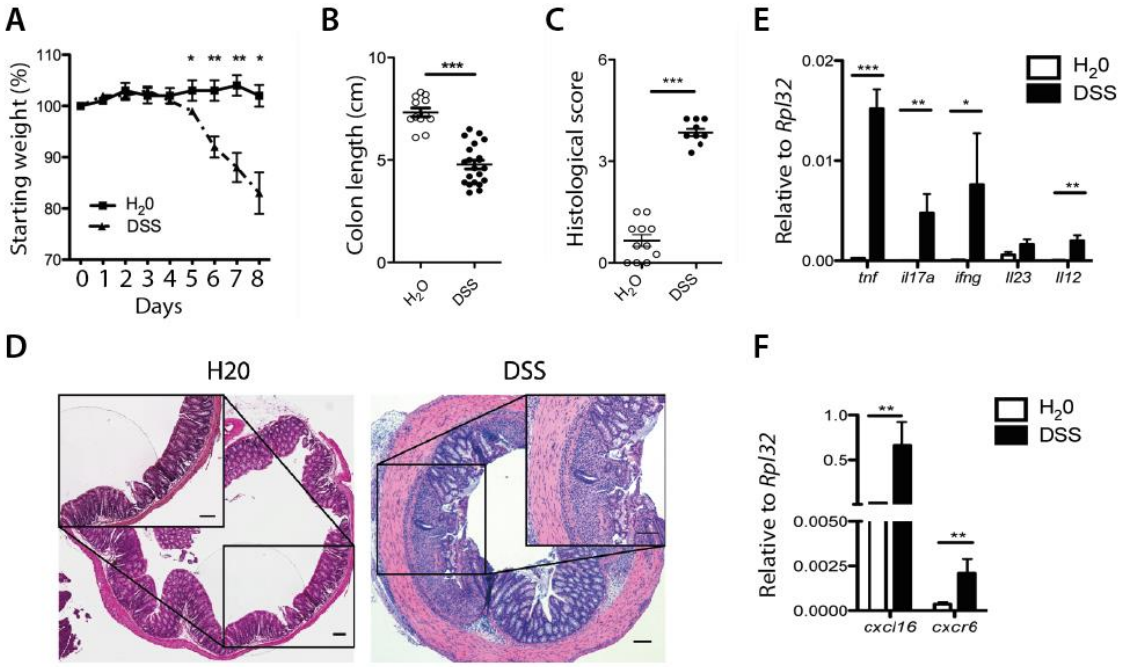

Figure 4.1 DSS induces acute intestinal inflammation. Acute experimental colitis was induced in CXCR6-EGFP/+ mice by DSS administration in drinking water. (A) Weight loss, (B) colon length and (C) histological score were compared in mice treated with DSS (black box) or water (white box). (D) Colonic expression levels of tnf, il17a, ifng, il23, il12, il10, Il22, il6, mcp-1, cxcl10 in untreated (white bars) and DSS-treated (black bars) mice by qPCR. (E) Colonic expression levels of cxcl16 and cxcr6 in untreated (white bars) and DSS-treated (black bars) mice by qPCR. *Statistical significance was calculated using a Mann Whitney test for comparison within two groups. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Albeit DSS-induced intestinal inflammation model is known to be principally driven by the recruitment of innate immune cells, i.e. neutrophils and macrophages [156], we hypothesized that the translocation of luminal antigens into the lamina propria could also trigger the accumulation of T lymphocytes. We performed flow cytometric analysis of colonic lamina propria mononuclear cells (LPMC) comparing DSS-treated (DSS) and untreated (H2O) mice. DSS-induced inflammation was associated with the accumulation of both $CD4^+$ T cells and $CXCR6^+$ iNKT cells (93% of which were also mCD1d:PBS57 Tetramer+,

Figure 3.3) in the colonic mucosa (***Figure 4.2A-B***). This corresponded to an increased colonic expression of both CXCR6 and CXCL16 (CXCR6 ligand, Figure 4.1***D***), thus confirming previously published data [71].

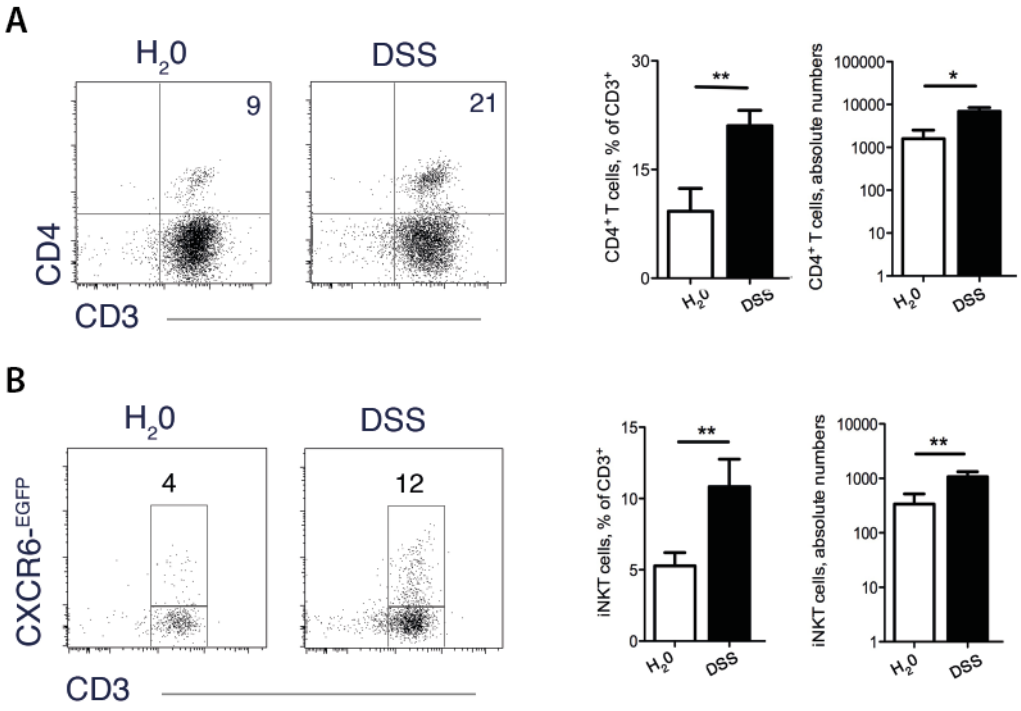

Figure 4.2 T cells infiltrate the inflamed colonic mucosa. CD4⁺ T cells (A, CD45⁺lin⁻CD3⁺EGFP⁻CD4⁺) and iNKT cells (B, CD45⁺lin⁻CD3⁺ EGFP⁺) representative dot plots, frequency among CD3+ T cells and absolute numbers in colon of DSS-treated (black box) or controls (white box). Statistical significance was calculated using a Mann Whitney test for comparison within two groups. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.

Having observed that CD4⁺ T and iNKT cells were associated with DSS- induced intestinal inflammation, we analyzed their ability to exert pro- or anti- inflammatory functions. We performed intracellular cytokine stainings of colonic LPMC. Interestingly, upon DSS treatment the production of the tolerogenic IL-10, the main player of the regulation and

resolution of the inflammation, was shut down both in CD4⁺ T and iNKT cells (***Figure 4.3***). During intestinal inflammation, the colonic CD4⁺ T helper cells were producing higher levels of IFNγ and IL-17 cytokines compared to untreated controls (***Figure 4.3A***). Collectively, in DSS-induced acute intestinal inflammation the T cell population displays a Th1/Th17 cytokine profile, consistently to what described in Inflammatory Bowel Disease (IBD) patients [96].

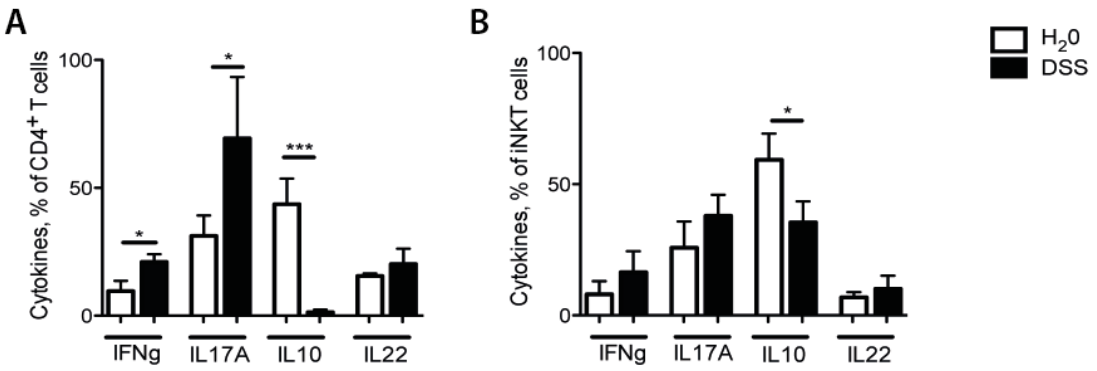

Figure 4.3 iNKT and CD4⁺ T cells exhibit a pro-inflammatory profile during acute intestinal inflammation in CXCR6 ^{EGFP} mice. Intracellular stainings of cytokine production by CD4⁺ T cells (A) and iNKT cells (B) in DSS-treated and untreated mice. Statistical significance was calculated using a Mann Whitney test for comparison within two groups. P < 0.05 (*), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

Taken together, these data suggest that CXCR6 EGFP/+ reporter mice are susceptible to DSS-induced colitis and can be used as a valuable tool to track and study intestinal T cell subset, including the rare population of iNKT cells.

4.1.2 Gut microbiota composition is altered during DSS-induced colitis

Since during DSS-induced colitis the epithelial barrier is disrupted, and microbial components have been indicated as possible triggers of immune activation in the gut [153], we considered important to evaluate if changes in the colonic bacterial population might have occurred upon DSS treatment. To do so, faecal samples of DSS-treated and untreated mice were collected and subjected to microbiome profiling using 16S rRNA gene sequencing on the Illumina MiSeq platform. An unweighted UniFrac-based comparison of the microbiota was performed. Principle component analysis (PCoA) differentiated colitic mice from healthy controls (***Figure 4.4A***). Similarly, the microbiota isolated from DSS-treated mice showed a lower α-diversity when compared to untreated mice microbiota, as reflected by the Chao1 and Shannon indexes (***Figure 4.4B***). A more detailed phylogenetic analysis of the taxonomic composition of the microbiome was carried out (***Figure 4.4C***). Interestingly, we found many taxa, already known to be altered in IBD patients, to be modified also during DSS treatment. For instance, at the phylum level, *Actinobacteria* and *Firmicutes* were reduced upon inflammation in favour of an enrichment of *Bacteroidetes* (***Figure 4.4C-D***) [118]. At the family level instead, we observed a significant reduction of *Lachnospiraceae* and *Christensenellaceae* (***Figure 4.4D***) [120], while the genus *Desulfovibrio* was strongly enriched in DSS-treated mice (***Figure 4.4D***), similarly to what reported in patients [158].

This alteration of the healthy microbial composition, referred to as dysbiosis, is characterizing DSS-treated mice as well as IBD patients [120], [159].

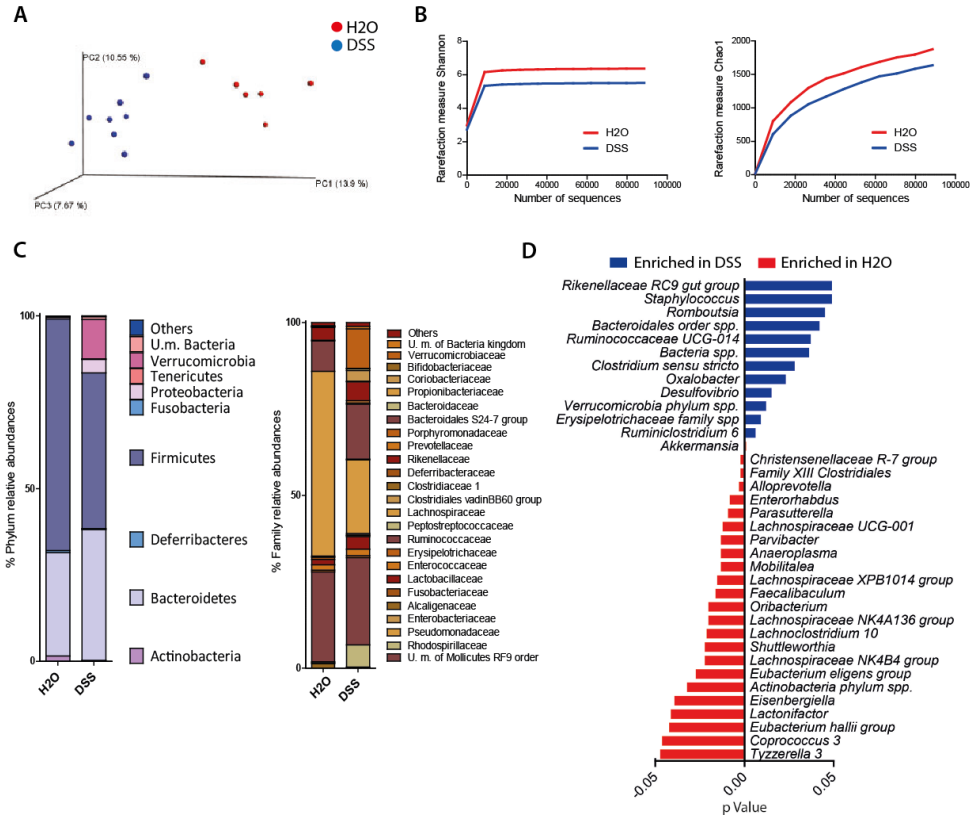

Figure 4.4 DSS treated mice experience gut microbiota dysbiosis *(A) Microbiome clustering based on unweighted Principal Coordinate Analysis (PCoA) UniFrac metrics of faecal gut microbiota derived from DSS treated (blue dots) and untreated (red dots) mice. (B) Rarefaction curves showing microbial microbial richness and evenness on the Shannon index (left panel) and richness based on the Chao1 index (right panel). (C) Bar plots of the taxonomic composition showing relative abundances >1% of bacterial phyla (left panel) and families (right panel). (D) P value of the comparison between the relative abundances of different taxa between DSS treated and untreated mice. Blue bars, taxa enriched in DSS treated mice, red bars, taxa enriched in untreated controls. Statistical significance was calculated with One Way Anova test with LSD post-hoc test.*

4.2 Antibiotic-induced dysbiosis

To alter the gut microbiota composition by mean of antibiotics administration, CXCR6 EGFP/+ mice were treated for two weeks with a broad- spectrum antibiotic cocktail (ABX,

vancomycin, metronidazole, ampicillin, neomycin), targeting both aerobic and anaerobic bacteria. Subsequently, mice were either maintained in ABX treatment (ABX, left scheme) or reconstituted by oral gavage with mucosa-associated and faecal bacteria (FMT; ***Figure 4.5A***, middle scheme). The complete depletion of bacteria was confirmed by qPCR analyses and CFU plating.

To evaluate whether the characteristics of the microbiota utilized to reconstitute the gastrointestinal tract upon ABX treatment might impact, mucus and feces derived from healthy mice (nFMT, normobiotic Faecal Microbiota Transplantation) or from mice with intestinal dysbiosis (dFMT, dysbiotic FMT) were engrafted to ABX-treated mice. Dysbiotic microbiota was obtained from mice with acute DSS-induced colitis. To address the presence of inflammation these groups were compared to DSS-treated mice as positive control (***Figure 4.5A***, right scheme).

The histological evaluation of colonic tissues revealed that neither antibiotic treatment nor recolonization with normobiotic or dysbiotic microbiota induced macroscopic changes in the colonic tissue architecture (***Figure 4.5B***). Next, we performed qPCR analyses on tissue derived mRNA to address the expression of il-6, il-17A, tnf, mcp-1 and il-12. We observed that all the above-mentioned microbiota-manipulating treatments failed to upregulate inflammatory genes (***Figure 4.5D***).

Thus, we can conclude that microbiota depletion through short-term oral antibiotics treatment or its reconstitution with either normobiotic or dysbiotic microbiota do not induce a macroscopic gut inflammation.

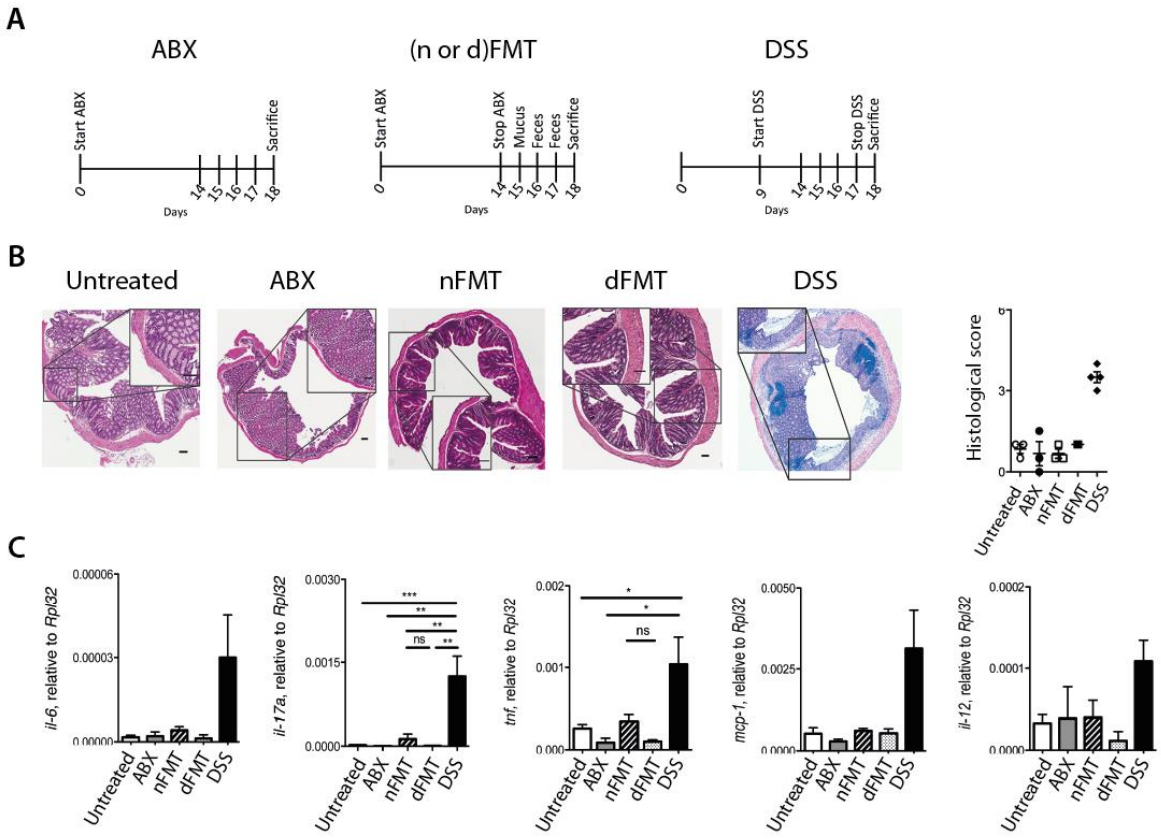

Figure 4.5 Antibiotic treatment does not alter mucosal architecture under homeostatic conditions. (*A) Schematic representation of the treatments. (B) H&E staining and cumulative histological score on colon specimens of untreated (open circles), antibiotic treated (ABX, closed circles), transplanted with eubiotic (open squares) or dysbiotic FMT (closed squares) and of DSS-treated mice (closed diamonds). Scalebar 100um. (C) Colonic expression levels of Il6, il17a, tnf, mcp-1, il-12 in untreated (white bars), ABX-treated (gray bars), reconstituted with eubiotic (striped bars) or dysbiotic (dotted bars) FMT or in DSS-treated mice (black bars). Statistical significance was calculated with Kruskal wallis test with Dunn's multiple comparison correction. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Though no macroscopic signs of inflammation were present in mice treated with antibiotics, we wondered if a modulation of the immune response at the cellular level might have occurred. We performed flow cytometric analyses on freshly isolated colonic

LPMCs of mice treated with a cocktail of whole-spectrum antibiotics or reconstituted with normobiotic or dysbiotic microbiota, as described above. The microbiota depletion was sufficient to induce a significant expansion of iNKT cells in the colon of adult mice, both in terms of frequency and absolute numbers (***Figure 4.6A-C***, upper panels). Importantly, reconstitution of the gut microbiota with a normobiotic FMT restored iNKT cell frequency, a phenomenon that was not observed upon microbial reconstitution with the microbiota derived from DSS- treated mice (***Figure 4.6B***). Conversely, $CD4^+$ T cells accumulation in the colon was unaffected by antibiotic treatment or by microbiota recolonization, regardless its origin (***Figure 4.6A-C***, lower panels).

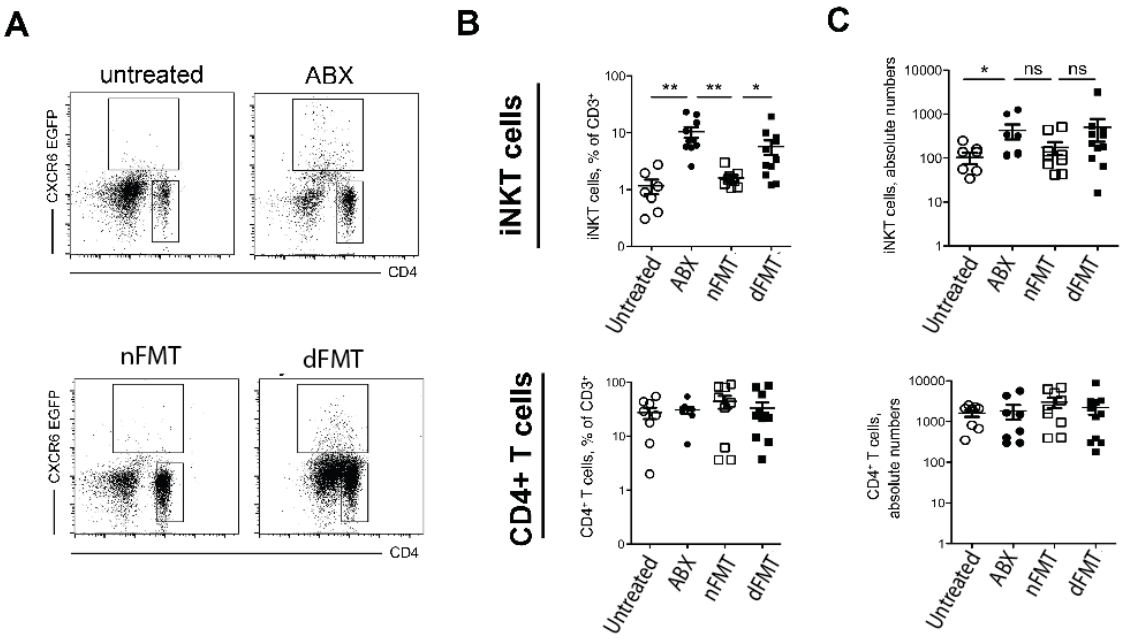

Figure 4.6 Antibiotic treatment influences colonic iNKT cell frequency (A) Representative dot plots, (B) cumulative frequency and (C) absolute numbers of iNKT cells (upper panels) and CD4+ T cells (lower panels) in untreated mice (open circles), ABX-treated mice (closed circles), mice reconstituted with normobiotic FMT (open squares) or with microbiota from DSS-treated mice (dysbiotic FMT, closed squares). Statistical significance was calculated with Kruskal wallis test with Dunn's multiple comparison correction. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.

To further characterize the mucosal T cell population upon microbiota manipulation, we analyzed the expression of specific activation markers and intracellular cytokines. We found that CD69, a surface molecule associated to T cells functional activation, in both iNKT and $CD4^+$ T cells isolated from ABX-treated mice did not significantly differ from that of cells isolated from mice reconstituted with a normobiotic microbiota (*Figure 4.7**A***). Conversely, re-colonization of ABX-treated mice with a dysbiotic microbiota triggered an upregulation of CD69 expression by iNKT cells. Despite no great difference in terms of cytokine profile was found upon ABX treatment and reconstitution with a normobiotic microbiota, IFNγ secretion was enhanced upon dysbiotic FMT in both iNKT cells and $CD4^+$ colonic T cells (***Figure 4.7B***).

Collectively, these results suggest that expansion of colonic iNKT cells and $CD4^+$ T cells is differentially modulated upon the manipulation of the intestinal microbiota in the absence of intestinal inflammation. Moreover, the microbiota composition influences both the accumulation and the functional activation of colonic iNKT cells and $CD4^+$ T.

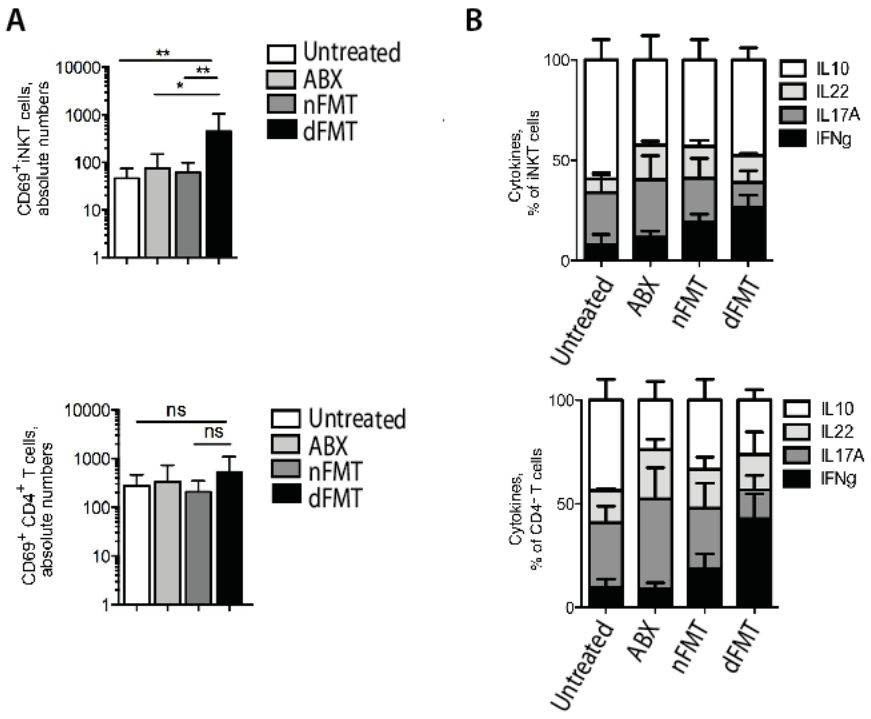

Figure 4.7 Antibiotic treatment influences colonic iNKT cell frequency *(A) Absolute numbers of CD69⁺ cells among iNKT cells (upper panels) and CD4+ T cells (lower panels) in untreated mice (white bars), ABX-treated mice (light gray bars), mice reconstituted with normobiotic FMT (dark gray bars) or with microbiota from DSS-treated mice (dysbiotic FMT, black bars) (B) Cytokine production by iNKT cells (upper panel) and CD4⁺ T cells (lower panel) in untreated, ABX-treated, reconstituted with normobiotic or with dysbiotic FMT. Statistical significance was calculated with Kruskal Wallis test with Dunn's multiple comparison correction. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.*

4.3 Therapeutic modulation of gut microbiota during intestinal inflammation

Our previous results demonstrate that the mucosal immune response, and especially T cells, become fully activated in the presence of a dysbiotic microflora associated to intestinal inflammation and that, even in the absence of inflammation, specific subsets of T cells can be functionally affected by the alteration of the gut microbiota composition. Starting from these observations, we asked whether gut microbiota manipulation through faecal microbiota transplantation (FMT) could impact on T cells functions and whether this could have a therapeutic relevance for the treatment of intestinal inflammation.

4.3.1 FMT ameliorates DSS-induced acute colitis

To evaluate the functional effects of therapeutic FMT, mucus and faeces derived from normobiotic untreated donor mice were collected and transplanted by oral gavage to DSS-treated colitic recipient mice for 3 consecutive days, according to the scheme described in ***Figure 4.8A***. When sacrificed in the acute phase of DSS-induced inflammation, FMT-treated mice showed reduced signs of intestinal inflammation, as indicated by a reduction in weight loss (***Figure 4.8B***) and increased colon length (***Figure 4.8C***). These effects were associated to an increase in variability in the histological score of mice treated with FMT compared to the ones treated with DSS only (***Figure 4.8D***). Some mice experienced a global amelioration

of the intestinal inflammation, measurable with all the histological parameters used to score the tissue (***Figure 4.8E***).

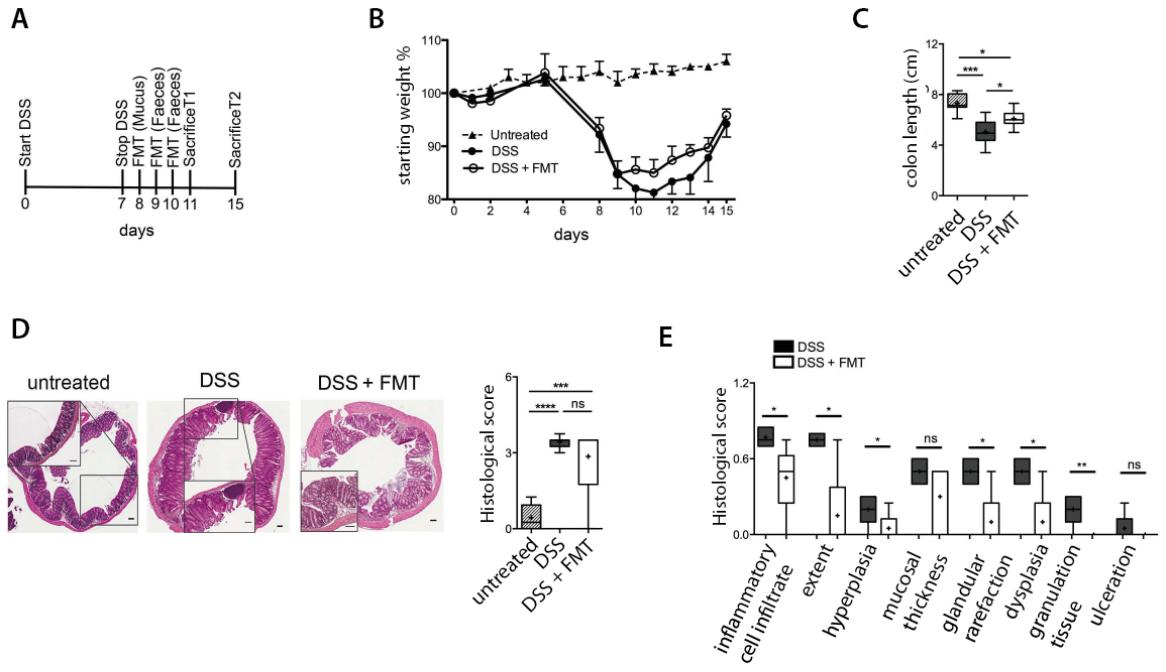

Figure 4.8 Therapeutic FMT ameliorates DSS-induced experimental colitis (A) Schematic representation of FMT treatment during acute DSS experimental colitis. (B) Weight loss and colon length (C) of untreated (striped boxes), colitic (black boxes), or colitic mice treated with FMT (white boxes). (D) H&E staining (scalebar 100μm) and cumulative histological score of colon specimens obtained from DSS-treated and FMT-treated mice; (E) Detailed histological evaluation of mice with decreased histological score (white boxes, n=5) compared to DSS-treated mice (black boxes, n=5). Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

Since DSS-induced colitis is a self-resolving disease experimental model [155], to address if FMT beneficial effects were limited to a specific timeframe, we sacrificed mice also at a later timepoint. Colon length measurement fifteen days after the start of the treatment

indicated that the acute inflammation was resolved in the DSS-treated group (***Figure 4.9A***). Nonetheless, histological evaluation demonstrated that the intestinal tissue of DSS-treated mice remained injured also at later timepoints. Conversely, FMT treatment of colitic mice resulted in mucosal protection in half of the mice sacrificed at day 15 (***Figure 4.9B***).

These results suggest that FMT might sustain cellular processes involved in the resolution of the intestinal inflammation in DSS-treated mice.

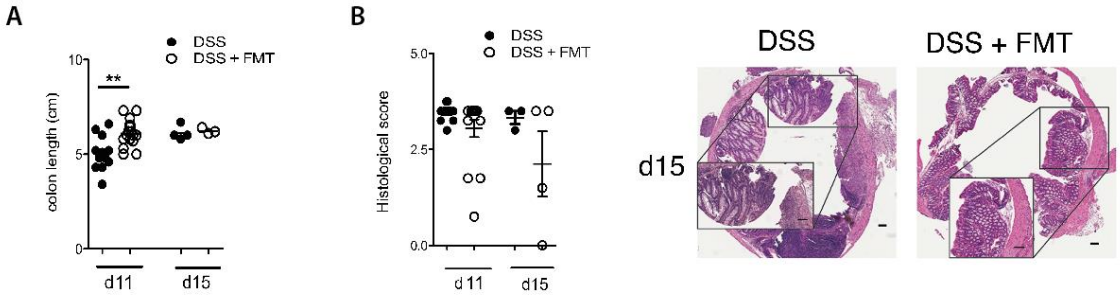

*Figure 4.9 FMT contributes to accelerate the resolution of intestinal inflammation. (A) Colon length and (B) histological evaluation of DSS treated (black dots) and DSS + FMT treated (open dots) of mice sacrificed 11 or 15 days after the beginning of the treatment. Statistical significance was calculated with Mann-Whitney test. P < 0.05 (*), P < 0.01 (** were regarded as statistically significant. Outliers were detected with Grubb's test.*

In agreement with previously published reports [144], FMT reduced the expression of the pro-inflammatory il1β (***Figure 4.10A***) in the colonic mucosa. To evaluate whether FMT might exert protective effects on gut barrier functions, the colonic expression of antimicrobial peptides and mucins was also tested (***Figure 4.10C***). The colonic expression of Camp and s100A8, two antimicrobial peptides playing anti-inflammatory roles during acute intestinal inflammation [160] were upregulated upon FMT administration. Similarly, a tendency toward up-regulation of Muc1 and Muc4, two mucins exerting anti-

inflammatory functions in response to pathogens [102], and a downregulation of Muc3 were observed by FMT treatment in acute DSS-colitis.

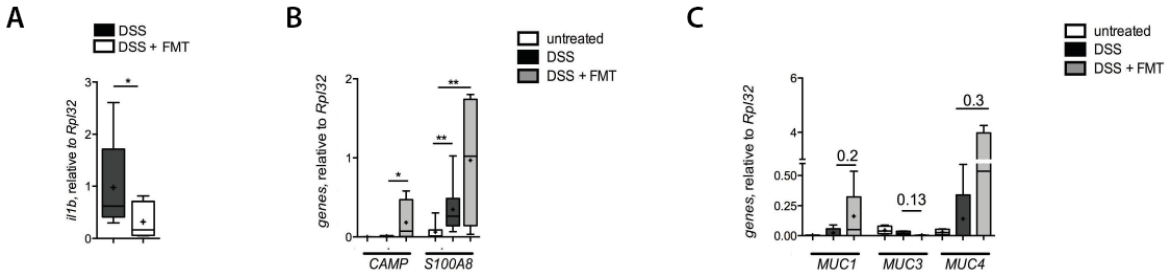

Figure 4.10 FMT modulates the colonic expression of pro-inflammatory genes, anti-microbial peptides and mucins. (A) Colonic expression levels of il1β in colitic (black boxes) and FMT-treated (white bars) mice. (B) Colonic expression levels of camp, S100A8 (C) and muc1, muc3, muc4 in untreated (white boxes), DSS treated (black boxes) or DSS + FMT treated (gray boxes) mice. Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

4.3.2 FMT treatment induces variations in gut microbiota composition and metabolic activity in colitic mice

Having observed that mice experiencing DSS-induced acute colitis are characterized by a state of microbial dysbiosis, we hypothesized that FMT beneficial effect could correlate with a restoration of a healthy microbiota composition. Thus, we evaluated variations in the microbial communities in colitic mice treated or not with FMT. Faecal samples of DSS and DSS + FMT mice were collected at day 11 post colitis induction and subjected to microbiome profiling using 16S rRNA gene sequencing on the Illumina MiSeq platform. An unweighted UniFrac-based comparison of the microbiota isolated from untreated, DSS and

DSS + FMT-treated recipient mice was performed. As shown in ***Figure 4.11A***, principle component analysis (PCoA) differentiated untreated mice from the experimental groups (DSS and DSS + FMT). The intestinal microbiota of DSS-treated mice receiving or not a FMT did not macroscopically differ at sacrifice, possibly due to similar relative abundances of the top 10 most abundant species among DSS and DSS + FMT-derived samples (***Figure 4.11C***). Similarly, the microbiota isolated from DSS + FMT treated mice did not show an increase in α-diversity when compared to colitic mice derived microbiota, as reflected by the Chao1 and Shannon indexes (***Figure 4.11B***).

Nonetheless, a detailed phylogenetic analysis of the taxonomic composition of the microbiome of colitic mice treated or not with FMT showed that the reduced inflammatory conditions observed upon FMT administration were associated to variations in the abundance of specific taxa, including an increase in *Firmicutes* and a reduction of *Verrucomicrobia* (***Figure 4.11D***). Significant changes towards restoration of normobiosis were detected among the less abundant families belonging to the *Firmicutes* phylum in the DSS + FMT–derived microbiota. For instance, *Clostridiaceae*, which were expanded in colitic mice, were reduced upon FMT and returned to levels comparable to those observed in untreated mice (***Figure 4.11E***). On the other hand, FMT-derived samples showed significant increases of beneficial commensals, also used in probiotics preparations [161], including *Lactobacillaceae* and *Streptococcus sp*., and of the SCFA-producing taxa *Erysipelotrichaceae*, *Odoribacter* and *Olsenella* (***Figure 4.11E-F***), taxa usually reduced in IBD patients [118]–[120].

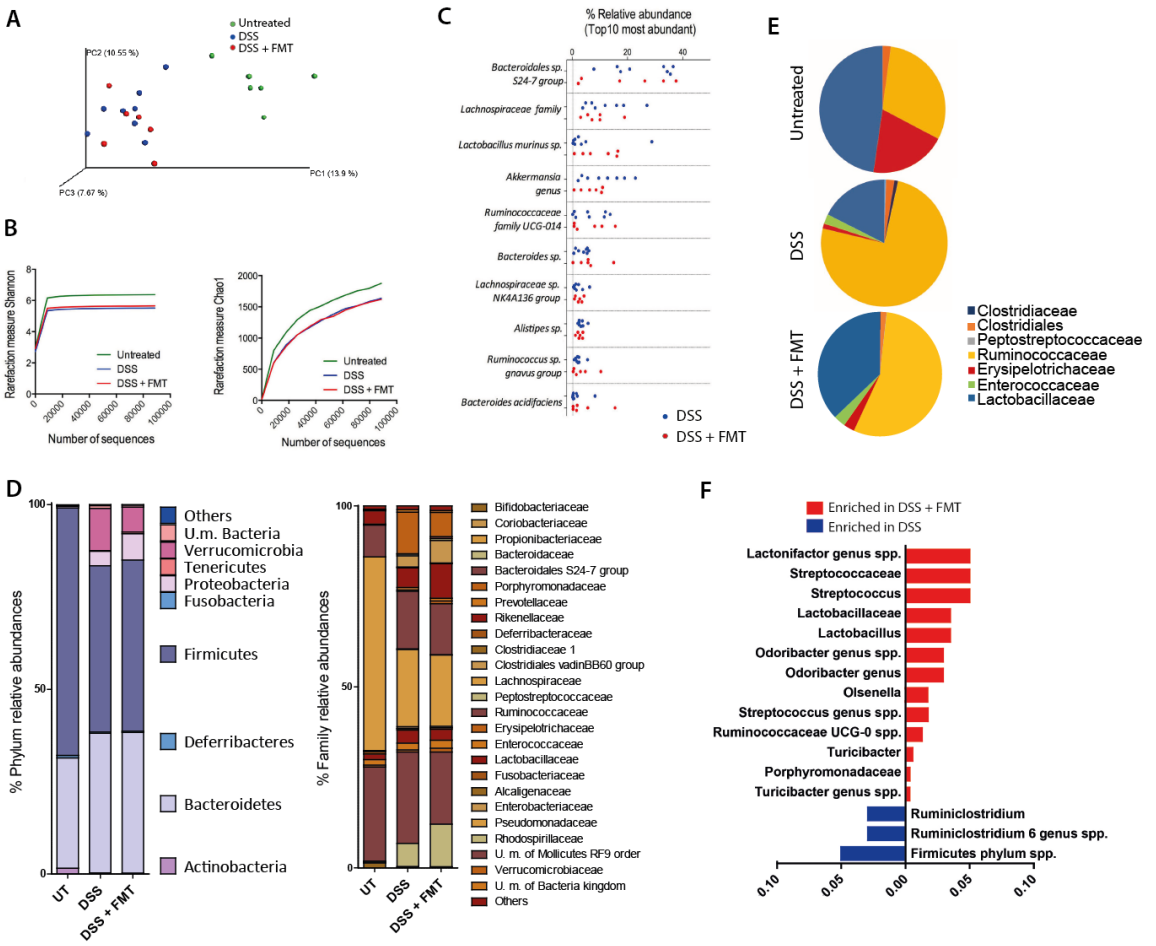

Figure 4.11 Gut microbiota analysis upon FMT treatment in colitic mice *(A) Microbiome clustering based on unweighted Principal Coordinate Analysis (PCoA) UniFrac metrics of faecal gut microbiota derived from DSS treated (blue dots), DSS+FMT treated (orange dots) and untreated (red dots) mice. (B) Rarefaction curves microbial richness and evenness on the Shannon index (left panel) and showing microbial richness based on the Chao1 index (right panel) (C) Relative abundance of the top 10 most abundant OTUs in DSS (blue) and DSS + FMT (red) treated mice. (D) Bar plots of the taxonomic composition showing relative abundances >1% of bacterial phyla (d, left panel) and families (d, right panel). (E) Pie charts showing the relative abundance of the less abundant families belonging to the Firmicutes phylum. (F) P value of the comparison of the relative abundances of different taxa between DSS (blue) and DSS + FMT (red) treated mice. Statistical significance was assessed through One way ANOVA with LSD post-hoc test * p<0.05*

In addition to an alteration of the gut microbiota composition, IBD patients have been reported to be characterized also by a perturbation in bacterial metabolites [162]. To unravel if this was the case also in DSS- and DSS + FMT- treated mice, we studied faecal bacterial metabolites with a metabolomic approach. As already observed in human IBD patients [162], DSS-treated mice showed increased faecal content of complex sugars including lactose and maltose, a possible consequence of defective intestinal absorption, which were normalized upon FMT-treatment. Similarly, glutamic acid, a metabolite altered in IBD patients [163], decreased upon FMT while gluconic acid and dihydroxiacetone, involved in natural detoxification activities [164], increased upon FMT ***(Figure 4.12***).

Altogether, these findings suggest that therapeutic FMT exert beneficial effects during acute intestinal inflammation and that this may arise from the reshuffling of the microbiota communities towards restoration of functional normobiosis.

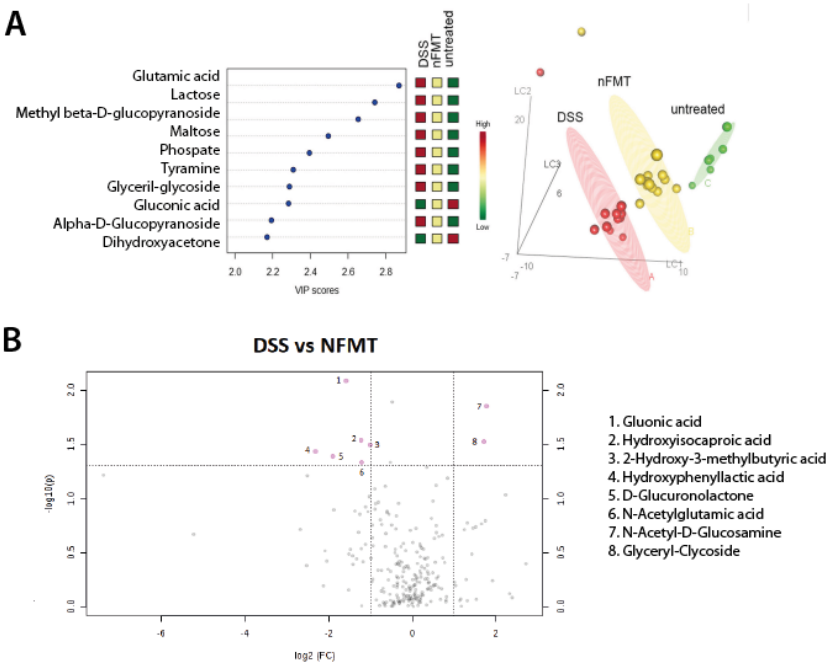

Figure 4.12 FMT modulates gut microbiota metabolism *(A) Partial Least square-discrimination analysis (PLSD-DA) and heat map of metabolites that have contributed most to class separation and (B) volcano plot on metabolomics data of faecal samples of untreated, DSS and DSS + FMT treated mice.*

4.3.3 The transplant of dysbiotic microbiota does not ameliorate intestinal inflammation

Our previous data indicated that the composition of the microbiota transplanted was essential to modulate the mucosal immune response under steady state conditions (***Figure 4.6***). Thus, we obtained donor mucus and faecal samples from normobiotic or dysbiotic mice, *i.e* from healthy mice left untreated or treated for 7 days with DSS, and we performed FMT in colitic mice as described in ***Figure 4.8***. As expected, an amelioration of the intestinal inflammation was reported in mice receiving a normobiotic FMT but not in those receiving a dysbiotic FMT, as demonstrated by an increase in colon length (***Figure 4.13A***) and a

decrease, albeit not significant, in the expression levels of colonic pro-inflammatory *il1b* and *tnf* (***Figure 4.13B***).

Consistently, the microbial composition of recipient mice that were transplanted with a dysbiotic FMT was also more similar to that of DSS-treated mice, enriched in pathobionts such as *E.Coli/Shigella* (***Figure 4.13C***) while the microbiota of mice receiving a normobiotic FMT were more similar to that of untreated mice, enriched in protective SCFA-producing bacteria (***Figure 4.13D***). Consistently, metabolomic analysis of the faeces of recipient mice treated with normobiotic FMT revealed the upregulation of metabolic pathways associated with the resolution of the inflammation (***Figure 4.13E***), *i.e.* metabolites associated to scavenging of free radicals (d-glucunolactone) and metals (gluconic acid) [163], to the control of ROS production and neutrophils activity (hydroxiphenillactic acid) and chemotaxis (LPA) [164] , and to SCFA production (Valeric acid) [81].

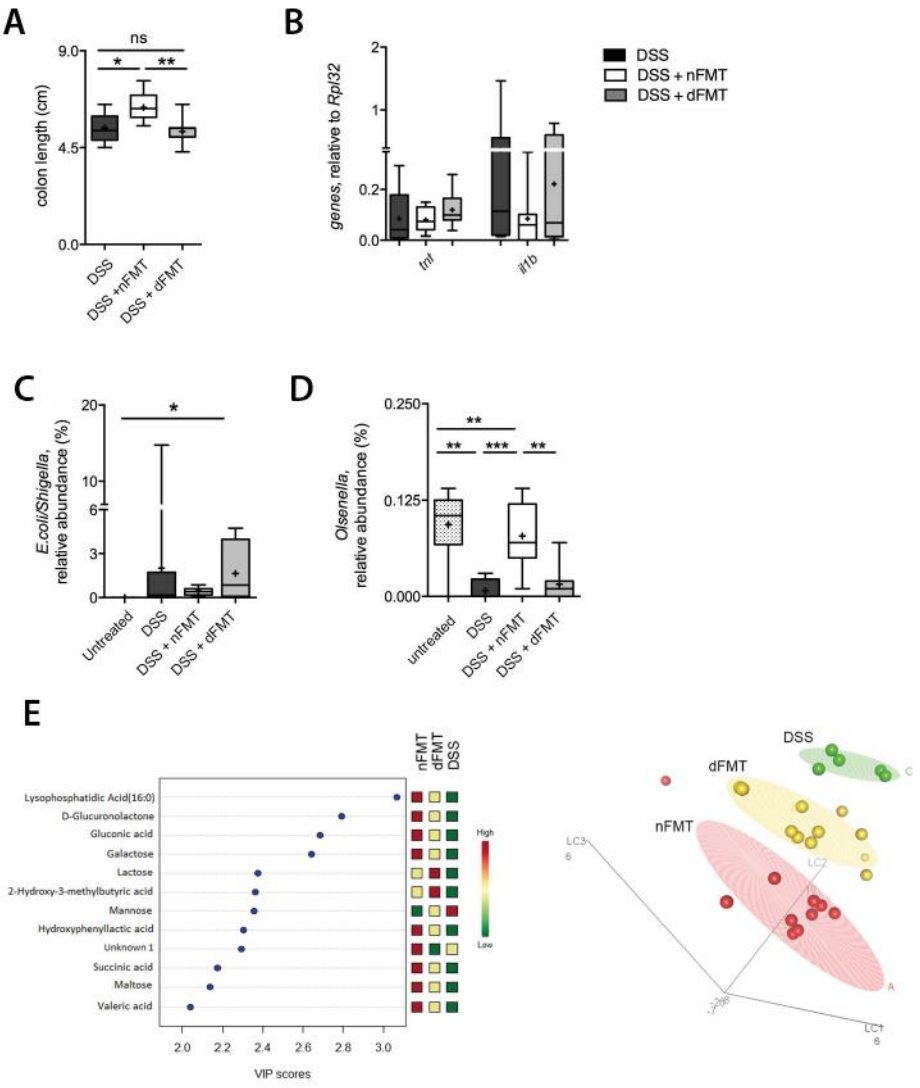

Figure 4.13 Transplant of normobiotic or dysbiotic microbiota. (A) Colon length of DSS (black boxes), DSS + nFMT (white boxes) or DSS + dFMT (gray boxes) treated mice. (B) Colonic expression of tnf and il1b in DSS (black boxes), DSS + nFMT (white boxes) or DSS + dFMT (gray boxes) treated mice. (C) Relative abundance of E.Coli/Shigella and (D) Olsenella in untreated (dotted white boxes), DSS treated (black boxes), DSS + nFMT (white boxes) and DSS + dFMT (gray boxes) mice.(E) Partial Least square-discrimination analysis (PLSD-DA) on metabolomics data (right panel), heat map and cluster plot of metabolites that have contributed most to class separation (left panel) of faecal samples of DSS, DSS + nFMT and DSS + dFMT treated mice. Statistical significance was calculated with Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

Relevant differences between the two types of donors used for FMT experiments were confirmed by metagenomic analyses on the transplanted faecal material (***Figure 4.13***). As previously shown in ***Figure 4.4***, also in this experimental setting the microbiota of dysbiotic mice was characterized by a contraction of *Bacteroidales S24-7*, *Lachnospiraceae* and *Bifidobacteriaceae* and an expansion of *Enterobacteriaceae* and *Bacteriaceae* as compared to that of normobiotic mice (***Figure 4.14A***). The dysbiotic microbiota transplanted displayed also a lower microbial richness and evenness, as indicated by the Shannon and Chao1 α-diversity measurement (***Figure 4.14B***).

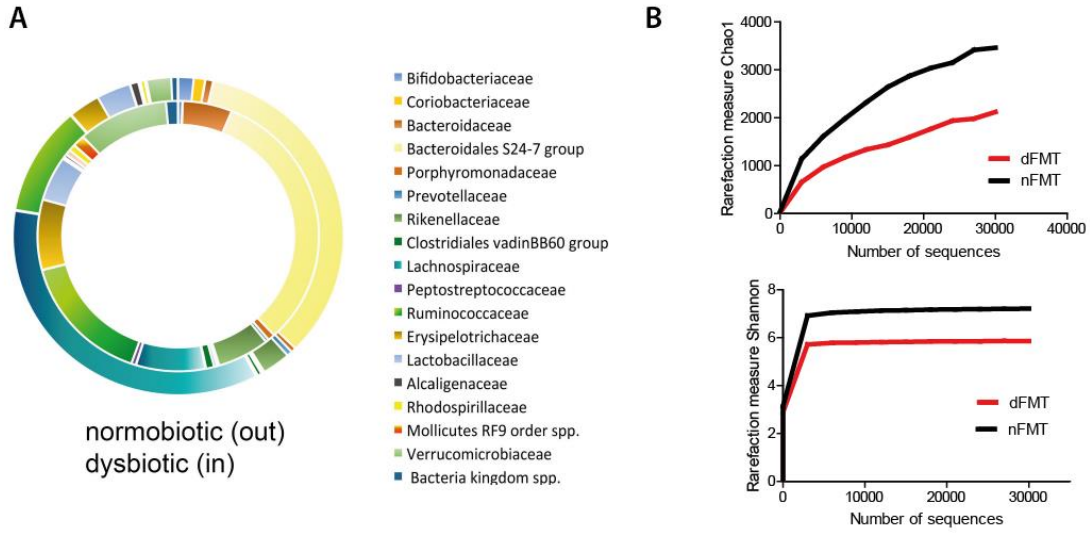

Figure 4.14 Differences between normobiotic and dysbiotic microbiota donors. (A) Comparison of relative abundancies of different taxa between normobiotic (outer chart) and dysbiotic (inner chart) faecal microbiota. (B) Rarefaction curves showing microbial richness based on the Chao1 index (right panel) and microbial richness and evenness on the Shannon index (left panel).

Collectively, these results suggest that the normobiotic nature of the donor microbiota is a key prerequisite for the restoration of a healthy microbial composition and the resolution of the inflammation in the recipient.

4.3.4 The beneficial effect of FMT is independent on the housing origin of the donor mice

Next, we evaluated if normobiotic donors of different origins might be equally capable to control intestinal inflammation when transplanted into colitic mice. Mucus and faecal samples were isolated from age and sex matched C57Bl/6 mice obtained from different sources, *i.e.* from two commercial animal vendors (Charles River srl and Envigo srl) and from in-house bred C57Bl/6 colony (IEO animal facility). Interestingly, the FMT performed with the microbiota isolated from the different normobiotic donors was equally capable to control intestinal inflammation, as shown by similar colon length (***Figure 4.15A***), histological score (***Figure 4.15B***), and expression of colonic pro-inflammatory genes such as *tnf* and *il1b* (***Figure 4.15C***).

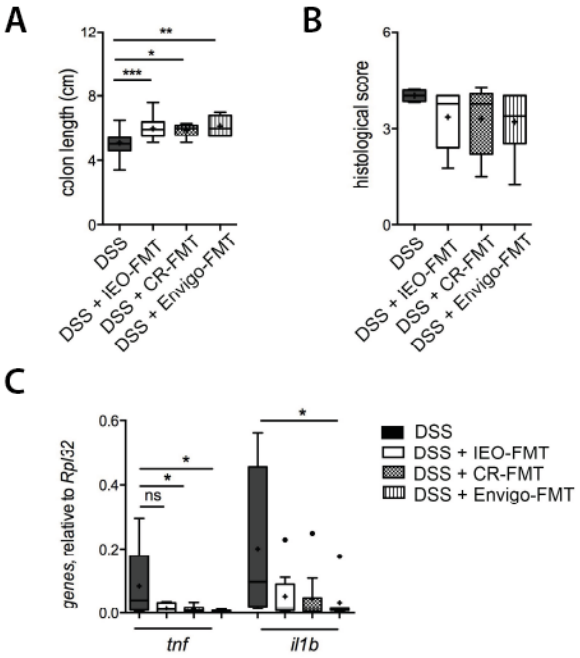

Figure 4.15 Transplant of microbiota from donors of different housing origins. (A) Colon length and (B) cumulative histological score of DSS (black boxes), DSS + IEO-FMT (white boxes), DSS + CR-FMT (dotted boxes) and DSS + Envigo-FMT (striped boxes) treated mice. (C) Colonic expression of tnf and il1b in DSS (black boxes), DSS + IEO-FMT (white boxes), DSS + CR-FMT (dotted boxes) and DSS + Envigo-FMT (striped boxes) treated mice. Statistical significance was calculated with Kruskal Wallis *test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

4.3.5 FMT beneficial effect in intestinal inflammation relies on the transplant of normobiotic microbial ecologies

As previously observed [58], [165], [166], donor mice sharing the same genetic background, sex and age, but raised in different animal facilities harbored a genetically similar, though not identical, microbiota (***Figure 4.16A***) capable to perform overlapping metabolic activities when transplanted into a colitic recipient mouse (***Figure 4.16B***).

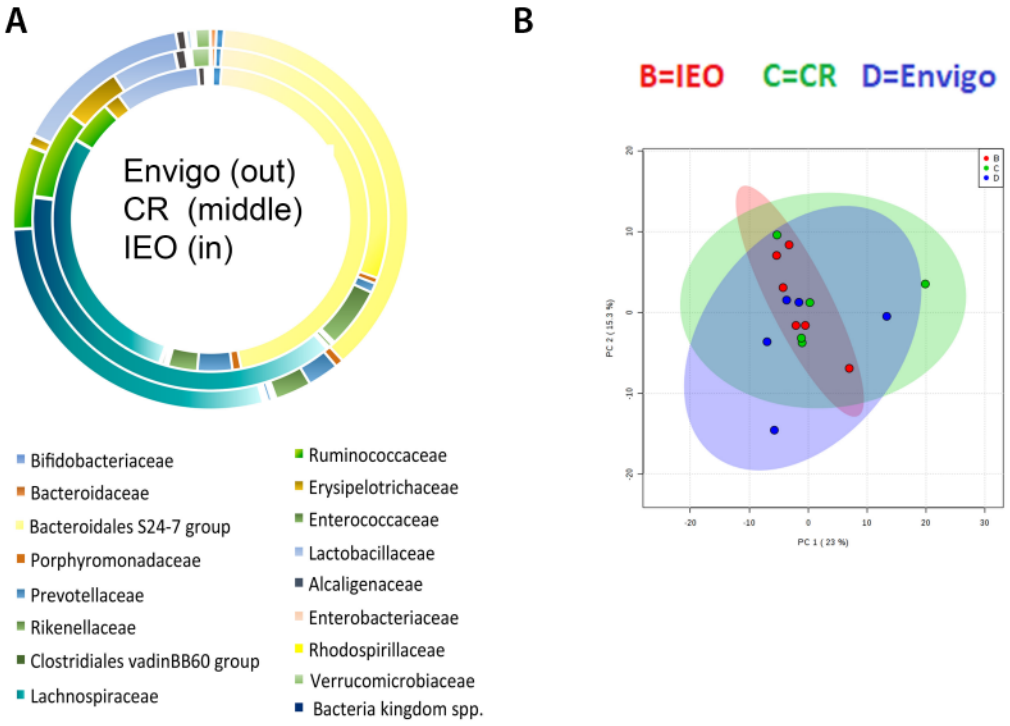

Figure 4.16 Differences between microbiota donors of different housing origins. (A) Comparison of relative abundancies of different taxa between Envigo- (outer chart), Charles River- (CR) (middle chart) or IEO-derived faecal microbiota. (B) PCA showing the partial least square-discrimination analysis (PLSD-DA) on metabolomics data between Envigo-, Charles River- (CR) or IEO-derived faecal microbiota.

In accordance to very recent data [86], 90 to 93% of the total taxa relative abundance of untreated normobiotic mice donors of different housing origins, was composed by a similar core microbial ecology of *Bacteroidales S24-7* (30-45% among groups), *Lachnospiraceae* (28-38%), *Lactobacillaceae* (6-15%), *Ruminococcaceae* (5-8%), *Rikenellaceae* (3-6%), *Bifidobacteriaceae* (1-2%) and *Erysipelotricaceae* (1-5%), that in dysbiotic mice accounted only for a total 75% (***Figure 4.17***). These taxa have been functionally associated to homeostatic metabolic activities, including SCFA production leading to Treg cells

differentiation and IL-10-production [82], [83]. On the contrary, the taxa expanded in dysbiotic mice accounting for a 25% of the total microbial ecology, which were either completely absent or present in extremely low abundancies in normobiotic mice, were mostly *Enterobacteriaceae*, *Bactaeriaceae*, *Rhodospirillaceae*, *Streptococcaceae,* (***Figure 4.17***) i.e. taxa that have been shown to be increased both in colitic mice and IBD patients [118], [167].

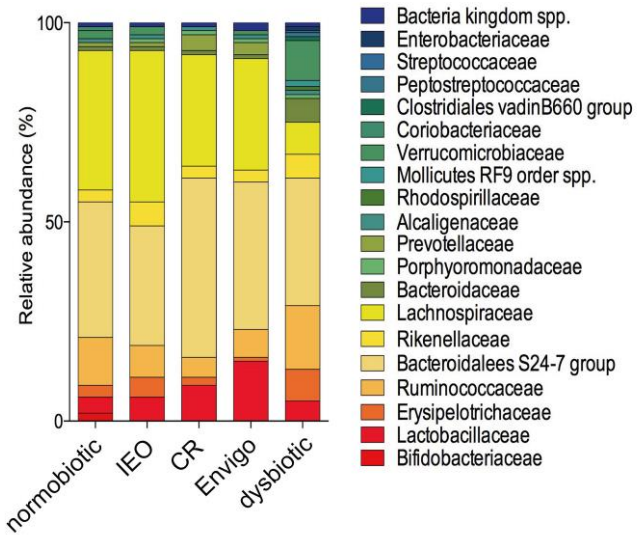

Figure 4.17 Variations of microbial ecologies among microbiota donors. Comparison of relative abundancies of different family between normobiotic-, IEO-, CR- Envigo- and dysbiotic-derived faecal microbiota.

Taken together, these data indicate that only a normobiotic healthy microbiota, composed by bacteria that are contributing to beneficial ecological functions, can ameliorate the intestinal inflammation.

4.4 Modulation of the colonic immune response by therapeutic FMT

Since the host immune system and the gut microbiota shape each other throughout life [71], we next asked if the gut microbiota reshuffle induced by therapeutic FMT administration in colitic mice could trigger variations in the frequencies and in the functional activities of the immune cell colonic infiltrate.

4.4.1 Therapeutic FMT influences T cell abundance and proliferation

The presence of specific bacterial strains in the gut has been linked to the differentiation and expansion of conventional [58] and unconventional [93] T lymphocytes. An increase of iNKT cell frequency, but not absolute numbers, was observed in DSS + FMT- treated mice after FMT, while $CD4^+$ T cells were decreased both in frequency and numbers (***Figure 4.18 A***). This observation might find an explanation in their reduced proliferative capacity (***Figure 4.18B***). Given that iNKT cells isolated from FMT-treated and untreated mice exhibited similar Ki67 expression (***Figure 4.18B***), the increase of iNKT cell percentage on total $CD3^+$ lymphocytes was likely a consequence of the overall reduction of $CD4^+$ T cells rather than of their expansion.

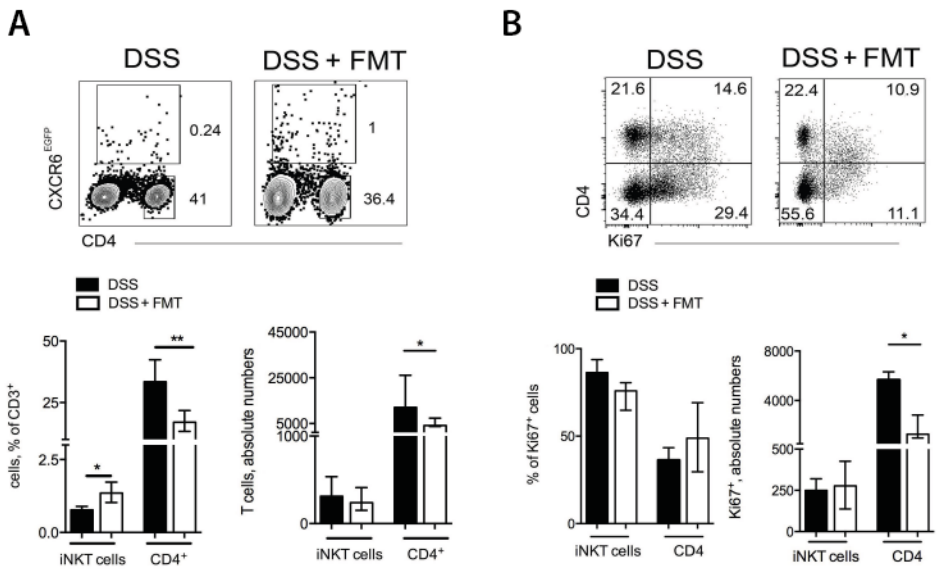

*Figure 4.18 Modulation of T cell populations upon FMT. (A) Representative dot plots (upper panels), frequencies and absolute numbers (lower panels) of colonic CD4+ T cells and iNKT cells in DSS-treated (black bars) and FMT-treated (white bars) mice 11 days after starting DSS administration (B) Representative dot plots, (upper panels), frequencies and absolute numbers (lower panels) of Ki67-expressing colonic CD4+ T cells and iNKT cells in DSS-treated (black bars) and FMT-treated (white bars) mice. Statistical significance was calculated with Mann-Whitney test. P < 0.05 (*), P < 0.01 (** were regarded as statistically significant. Outliers were detected with Grubb's test.*

4.4.2 Therapeutic FMT influences the myeloid cell compartment and its antigen presentation potential

Antigen presenting cells (APC) and immune cells of myeloid origin infiltrating the inflamed lamina propria have been shown to contribute to sustain and propagate intestinal inflammation in IBD patients [95] and in DSS-induced acute colitis model [156]. Thus, we analysed by flow cytometry the myeloid cell compartment (***Figure 4.19***). Following therapeutic FMT, a reduction in F4/80$^+$ macrophages and CD11b$^+$Ly6G$^+$ neutrophils frequency (***Figure 4.19A***) and partially in absolute numbers (***Figure 4.19B***) was observed.

In contrast, CD19^{+}B cells and CD11c^{+}dendritic cell abundances were mostly unaffected (***Figure 4.19A-B***).

Moreover, several evidences suggest that mucosal T-helper cells activation during intestinal inflammation depends on antigenic stimulation [95]. Upon FMT treatment, both the number of colonic MHC-II-expressing professional APC, including dendritic cells and macrophages (***Figure 4.19C***), and MHC-II expression levels on APC (***Figure 4.19D***) were strongly reduced.

These observations lead us to hypothesise that FMT might directly act on the antigen presenting capacity of professional APC.

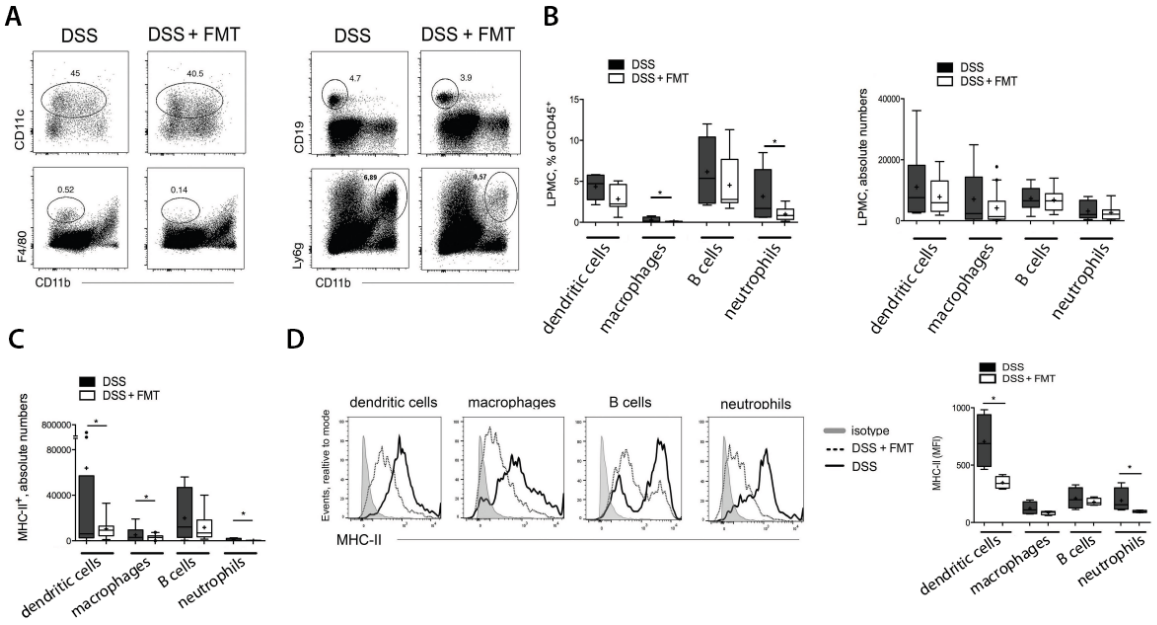

Figure 4.19 Modulation of antigen presenting cells upon FMT. (A) Representative dot plots and frequency of dendritic cells (CD45.2^{+}CD3^{-}CD11c^{+}), macrophages (CD45.2^{+}CD3^{-}F4/80^{+}CD11b^{-}), B cells (CD45.2^{+}CD3^{-}CD19^{+}), neutrophils (CD45.2^{+}CD3^{-}Ly6g^{+}CD11b^{+}) in DSS-treated (left panels) and FMT-treated (right panels) mice 11 days after starting DSS administration. (B) Frequency and absolute numbers of colonic dendritic cells, macrophages, B cells, neutrophils in DSS-treated (black boxes) and DSS+FMT-treated (white boxes). (C) Absolute numbers of MHC-II expressing colonic dendritic cells,

macrophages, B cells and neutrophils in DSS (white boxes) and DSS+FMT treated (black boxes) mice. (D) Representative histograms of MHC-II expression and cumulative Mean fluorescence intensity (MFI) on colonic dendritic cells, macrophages, B cells, neutrophils in DSS (black boxes) and DSS+FMT (white boxes) treated mice. Statistical significance was calculated with Mann-Whitney test. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.

Collectively, these data indicate that therapeutic FMT exerts a specific effect on intestinal infiltrating immune cell population frequency and phenotype.

4.5 Bacterial antigen presentation on T cell activation during FMT treatment

We next evaluated if FMT administration might influence specific immune cells functional activities directly correlated to bacterial antigens presentation.

The faeces of untreated, DSS-treated and FMT-treated mice were collected and used to stimulate *in-vitro* intestinal lamina propria mononuclear cells (LPMC) freshly isolated from healthy mice. The infection was stopped after 2 hours of stimulation by adding Gentamycin, Penicillin and Streptomycin antibiotics. The cells were left in culture for 96 hours and their phenotype was assessed by flow cytometry (***Figure 4.20***).

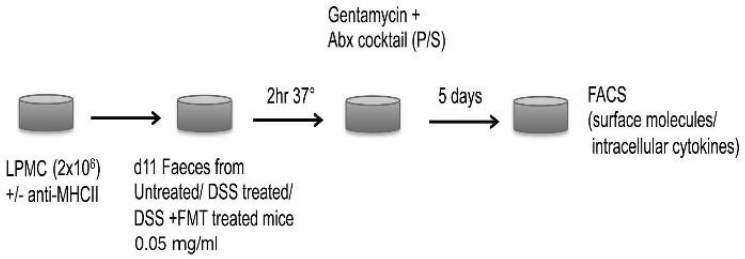

Figure 4.20 In-vitro bacterial antigen presentation assay *(A) Schematic representation of in vitro stimulation of mononuclear cells with faeces derived from untreated, DSS treated and DSS + FMT treated mice*

This *in vitro* assay perfectly recapitulated what observed *in vivo.* MHC-II levels on dendritic cells, macrophages and monocytes were down-regulated upon exposure *in vitro* to normal (untreated) or FMT-derived microbiota, as compared to the ones exposed to DSS-derived faeces (***Figure 4.21A***).

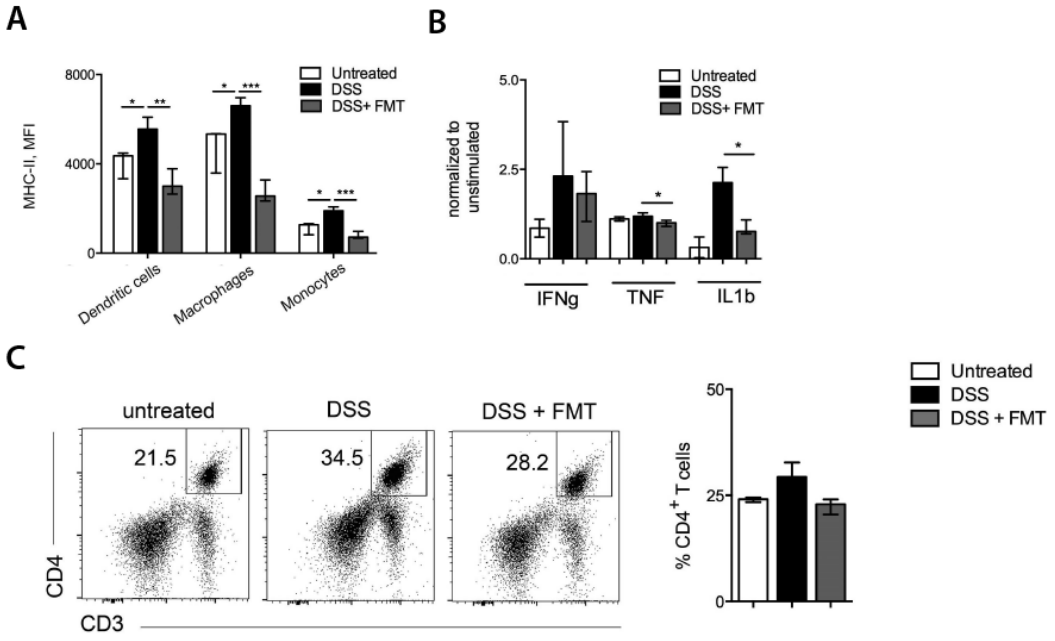

Figure 4.21 The antigen presentation assay recapitulates in vivo FMT effect *(A) Mean fluorescence intensity (MFI) of MHC-II on dendritic cells, macrophages, monocytes, neutrophils exposed in vitro to faeces isolated from untreated (white bars), DSS (black bars) and DSS + FMT (grey bars) treated mice. (B) IFNγ, TNF and IL1b cytokines levels in supernatants of LPMC in vitro exposed to faeces isolated from untreated (white bars), DSS (black bars) and DSS +F MT (grey bars) treated mice. Cytokines levels were measured with Cytokine Bead Array (BD) and normalised to unstimulated spontaneous secretion by LPMC. Statistical significance was calculated with Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Cytokines produced by mucosal immune cells play a pivotal role in the initiation and propagation of intestinal inflammation, as in the maintenance of homeostasis [96]. To evaluate the cytokine milieu generated upon exposure to FMT-derived microbiota, we analysed the supernatants of stimulated intestinal LPMC. Interestingly, LPMC exposed to FMT-derived microbiota showed low levels of pro-inflammatory cytokines, such as TNF,

IL1β and IFNγ, which were instead increased in the supernatants of LPMC exposed to DSS-derived microbiota (***Figure 4.21B***).

Moreover, $CD4^+$ T cell expansion occurred only after stimulation with DSS-derived, but not with untreated- or FMT- derived microbiota (***Figure 4.21C***). Likewise, DSS-derived microbiota also induced a strong upregulation of the CD69 activation marker on intestinal $CD4^+$ T cells, while untreated or FMT-derived microbiota failed to do so (***Figure 4.22A***).

Interestingly, when an MHCII blocking antibody was added to the medium, the expression of CD69 on T cells was not affected, suggesting that in this *in vitro* setting upregulation of CD69 was not antigen dependent (***Figure 4.22A***). Conversely, T cell cytokine production seemed to be mainly dependent on antigen presentation, as both IFNγ and IL-10 secretion were significantly reduced in presence of MHC-II blockade (***Figure 4.22B-C***). More importantly, only FMT-derived microbiota increased IL-10 secretion by $CD4^+$ T cells (***Figure 4.22B-C***).

Altogether these data confirm a crucial role for bacterial antigen presentation in the tolerogenic skewing of innate and adaptive colonic immune populations upon FMT treatment.

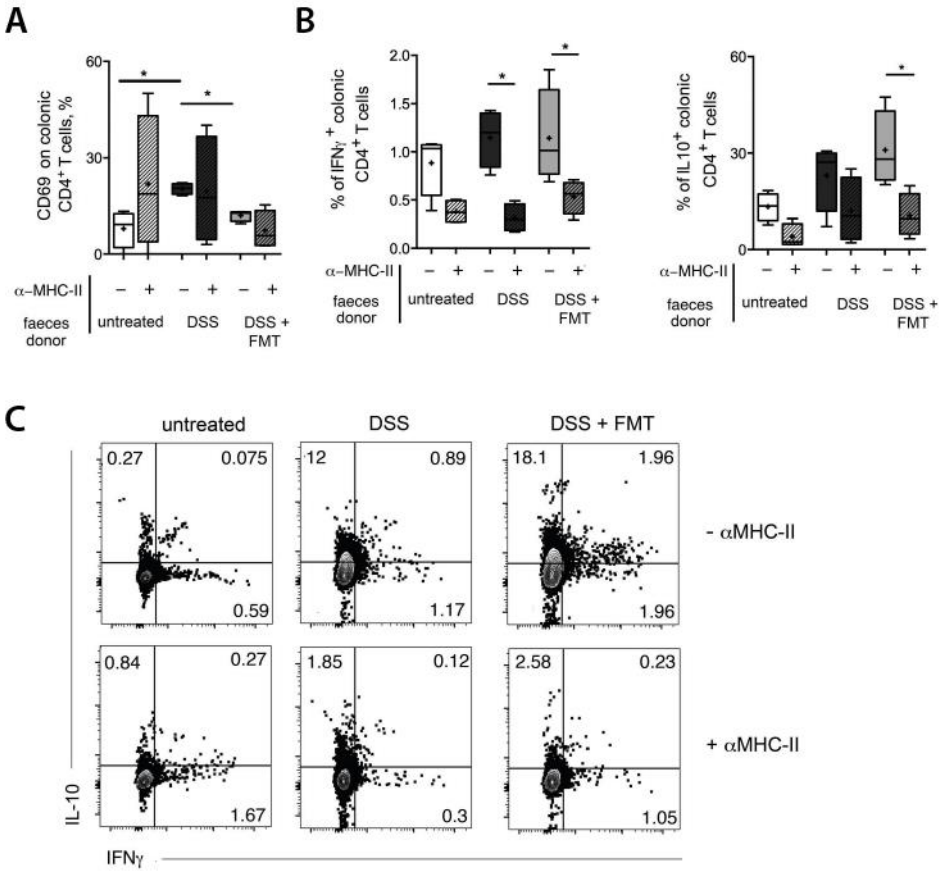

Figure 4.22 MHC-II-dependent presentation of FMT- derived bacterial antigens is required for CD4+ T cells cytokine profile skewing. (A) CD69 expression on intestinal CD4+ T cells exposed in vitro to faeces isolated from untreated (white boxes), DSS (black boxes) and DSS + FMT (grey boxes) treated mice in the presence (striped bars) or absence (filled bars) of blocking MHC-II antibodies. (B) Frequencies and representative dot plots of IFNγ and IL-10 secreting intestinal CD4+ T cells exposed in vitro to faeces isolated from untreated (white bars), DSS (black bars) and DSS + FMT (grey bars) treated mice in the presence (striped bars) or absence (filled bars) of blocking anti-MHCII antibodies. Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

4.6 The role of FMT-driven IL-10 secretion

The production of the tolerogenic IL-10 is one of the key mechanisms for the maintenance of mucosal homeostasis and for the regulation and resolution of intestinal inflammation [39], [40]. Our *in vitro* experiments indicated that IL-10 production by intestinal immune cells might be critically involved in the tolerogenic mechanisms triggered by therapeutic FMT during experimental colitis (***Figure 4.22B***). We thus wondered if IL-10 production might be responsible for the observed therapeutic effects of FMT *in vivo*.

4.6.1 Colonic IL-10 production is increased upon FMT treatment

Colonic tissue lysates of DSS + FMT treated mice showed higher amounts of IL-10 protein level (**Figure 4.23A**). With a flow cytometric approach we sought for possible IL-10 producers among the mucosal immune cell infiltrate and we observed increased frequencies of IL-10-producing APC ($CD45.2^+CD3^-Lin^+$, ***Figure 4.17B***) as well as $CD4^+$ T and iNKT cells in the colons of FMT-treated mice as compared to DSS-treated mice (***Figure 4.23C-D***).

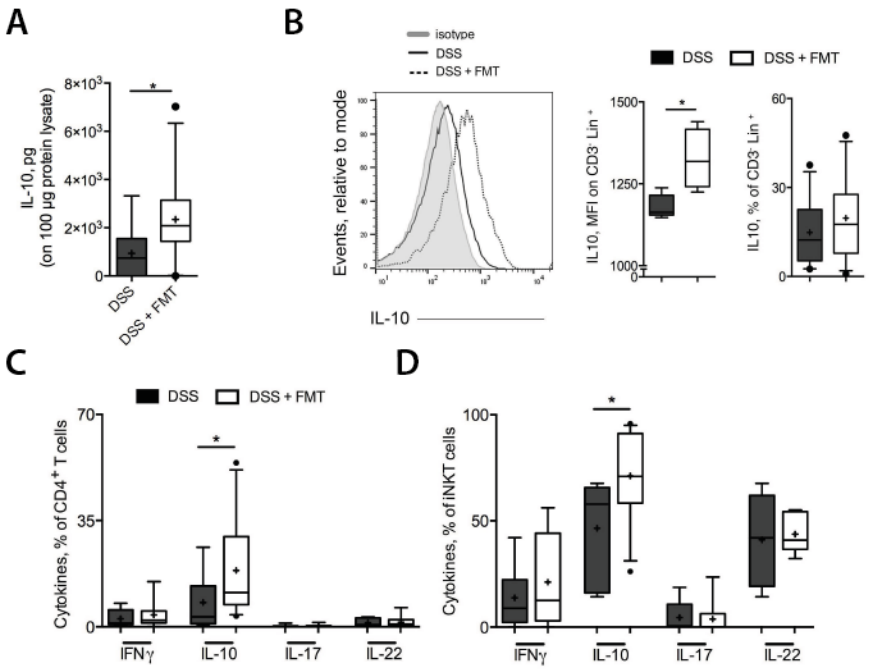

Figure 4.23 IL-10 production increases upon FMT (A) Colonic IL-10 protein level in DSS- (black boxes) and DSS + FMT- (white boxes) treated mice. (B) Representative histograms of IL-10 expression and cumulative Mean fluorescence intensity (MFI) (left panel) and frequency (right panel) of IL-10-producing colonic professional antigen presenting cells (CD45⁺ CD3⁻ MHC-II⁺) in DSS (black boxes) and DSS + FMT (white boxes) treated mice. Summary of cytokines secreted by (C) colonic CD4⁺ T cells and (D) iNKT cells in DSS-treated (black boxes) and FMT-treated (white boxes) mice 11 days after starting DSS administration. Statistical significance was calculated with Mann-Whitney test for comparison between two groups. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

4.6.2 IL-10 critically contributes to FMT-driven beneficial activity

To test the specific contribution of IL-10 on the anti-inflammatory properties of FMT, DSS-treated mice were intraperitoneally injected with IL-10 receptor (IL-10R) blocking antibody administered concomitantly to FMT treatment , as shown in ***Figure 4.24A***. Inhibition of the tolerogenic functions of IL-10 on IL-10R expressing cells, such as antigen presenting cells

(APC), T cells and epithelial cells [168], hampered FMT protective activity as shown by reduced colon length (***Figure 4.24B***), increased weight loss (***Figure 4.24C***), and higher colonic expression of *tnf, ifnγ* and *il1ß* (***Figure 4.24D***). These effects were not observed when IL-10R was blocked in colitic mice without a concomitant FMT administration, suggesting a direct contribution of microbiota induced IL-10 in mediating FMT-driven control of inflammation.

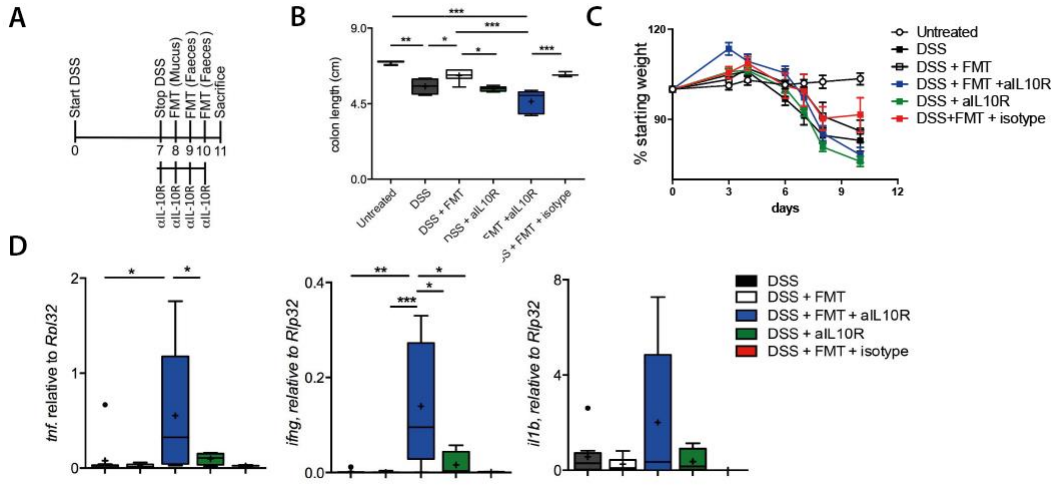

Figure 4.24 In vivo blockade of IL-10 pathway impairs FMT effects. *(A) Schematic representation of αIL-10R blocking antibody administration in colitic mice during FMT treatment. (B) Colon length and (B) weight loss of untreated mice (white dotted boxes and open circles) or receiving DSS (black boxes and black symbols), or DSS + FMT (white boxes and white symbols) or DSS + αIL-10R (green boxes and green symbols), or DSS + FMT + αIL-10R (blue boxes and blue symbols) or mice receiving DSS + FMT + αIL-10R isotype antibody (red boxes and red symbols). (h) Colonic expression of tnf, ifnγ and il1ß in DSS treated mice (black boxes), DSS + FMT treated (white boxes), DSS + αIL-10R (blue boxes), DSS + FMT + αIL-10R treated (green boxes) and DSS + FMT + αIL-10R isotype antibody treated mice (red boxes). Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

As expected, IL-10R blockade reverted the inhibition of $CD4^+$ T cell proliferation occurring upon FMT, making it comparable to the levels of DSS- and DSS + aIL10R- treated mice (***Figure 4.25A***). iNKT cell proliferation was not affected by IL-10R blockade (***Figure 4.25B***). Importantly, blocking of IL-10R also resulted in inhibition of IL-10 production by T and iNKT cells, possibly through a feedback regulatory loop (***Figure 4.25C***).

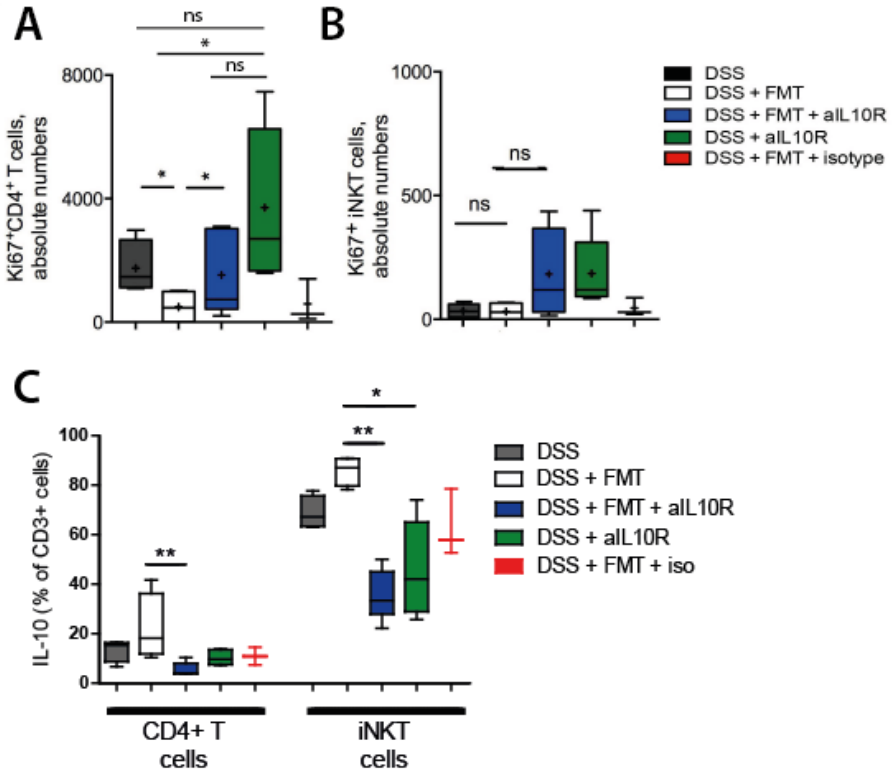

Figure 4.25 In vivo blockade of IL-10 pathway impairs FMT effects on T cells (A) Absolute numbers of KI67 expressing colonic $CD4^+$ T cells and (B) iNKT cells (right panels) in DSS treated mice (black boxes), DSS + FMT treated (white boxes), DSS + αIL-10R (blue boxes), DSS + FMT + αIL-10R treated (grey boxes) and DSS + FMT + αIL-10R isotype antibody treated mice (green boxes) and DSS + FMT + αIL-10R isotype antibody (red boxes). Statistical significance was calculated with Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

4.6.3 Selective elimination of protective bacterial communities impairs FMT-induced IL-10 production and its beneficial effect

Further, we addressed the specific contribution of different gut microbiota communities to the induction of protective immune-mediated functions exerted by FMT. To do so, as shown in ***Figure 4.26***, therapeutic FMT was performed with donor mucus and faeces isolated from mice pre-treated for two weeks with different antibiotics, targeting either Gram-positive organisms (vancomycin), Gram- negative bacteria (streptomycin), strict anaerobes (metronidazole) or having a broad-spectrum bacterial depletion capability (ABX, cocktail of vancomycin, metronidazole and streptomycin) [169].

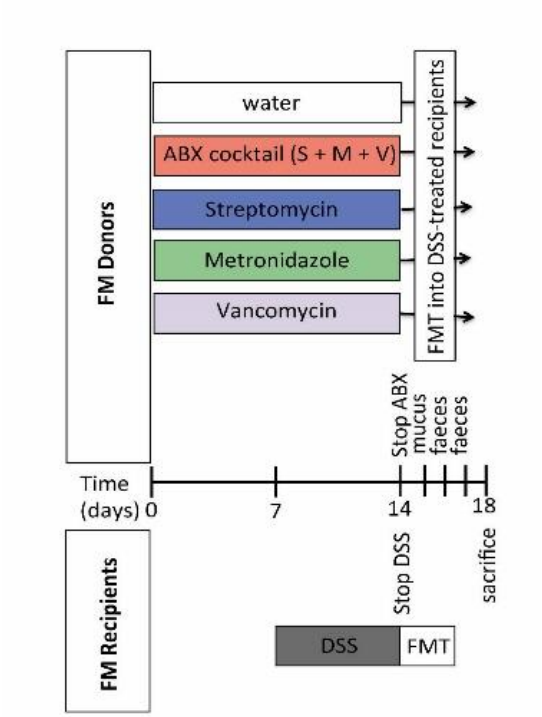

Figure 4.26 FMT of antibiotics treated donors. Schematic representation of the experiment.

The taxonomic composition of the different donor microbiota was analysed by 16S rRNA profiling before administration to colitic mice. As expected, the α-diversity of gut

microbiota in antibiotics-treated mice was lower as compared to those derived from normobiotic samples. The microbiota of mice treated with just one antibiotic displayed an intermediate α-diversity (*Figure 4.27**A***). This evidence was also confirmed by plating of the faecal material in aerobic and anaerobic conditions (*Figure 4.27**B***). As a proof of concept, the microbial population of broad spectrum antibiotic-treated mice was completely depleted while, the single use of the three abovementioned antibiotics resulted in a selection of the live bacteria thus shaping the overall population (*Figure 4.27**B***).

The detailed phylogenetic analysis of the taxonomic composition highlighted a relevant dysbiosis in the antibiotic- treated donor samples as compared to the untreated normobiotic ones (*Figure 4.27**C***). In particular, metronidazole treatment favoured the presence of *Lactobacillaceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae* (*Figure 4.27**D***), families belonging to the protective normobiotic microbial ecologies [86] previously described in Figure 4.17. On the contrary, pathobionts such as, *Burkholderiales*, *Listeriaceae* and *Gastranaerophilales* significantly emerged in Vancomycin-treated samples at the expenses of the abovementioned protective families (*Figure 4.27**D***). Streptomycin-treatment, instead, favoured a limited presence of *Erysipelotricaceae* and *Ruminococcaceae* while not expanding pathobionts (*Figure 4.27**D***), but rather allowing the survival of families whose function could be protective or detrimental according to the context [118], [170].

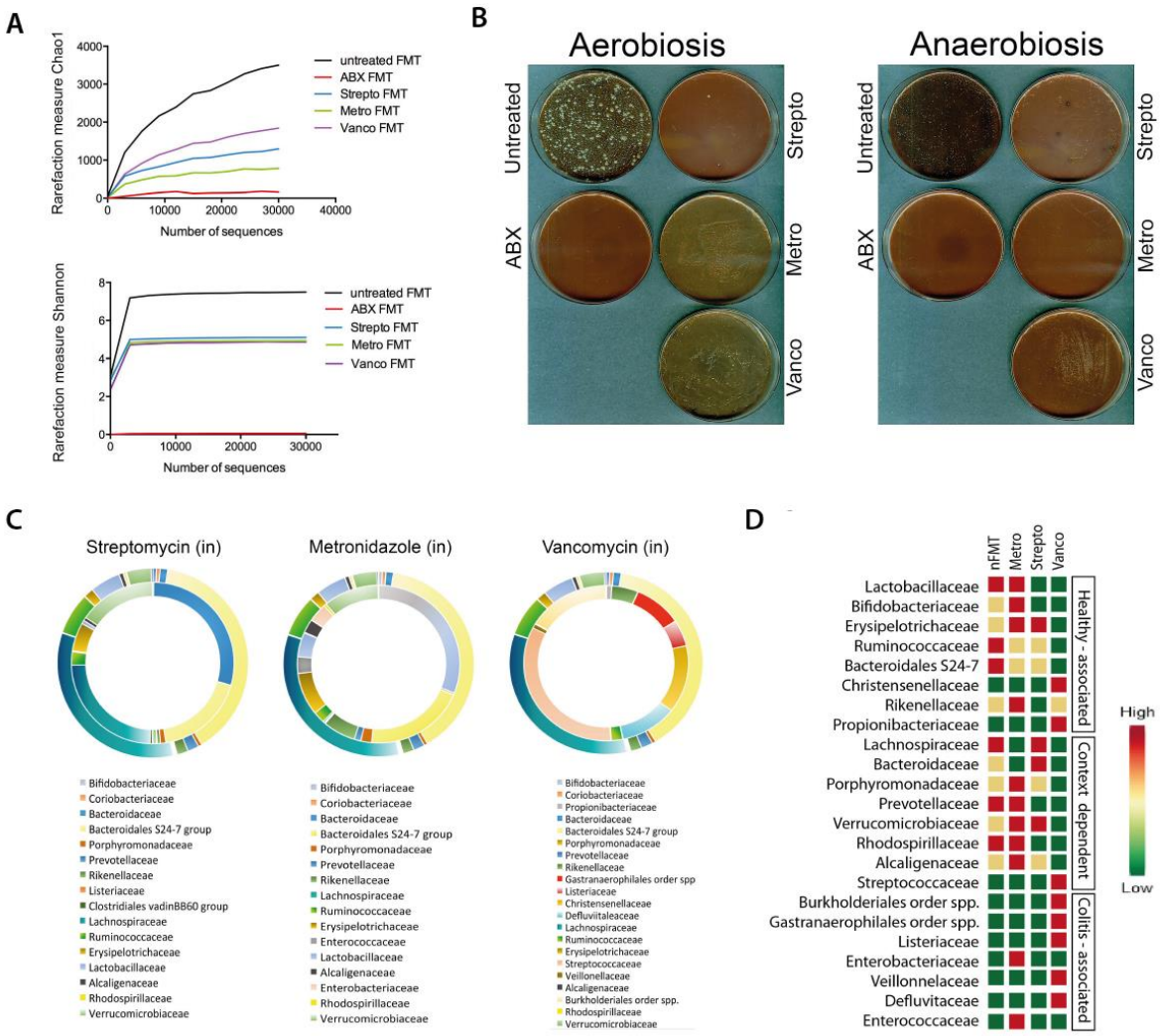

Figure 4.27 Antibiotics treated microbiota donors. (A) Rarefaction curves showing microbial richness based on the Chao1 index (top panel) and microbial richness and evenness on the Shannon index (bottom panel) (B) Plating of faecal material derived from untreated, treated with the antibiotic cocktail (ABX) or with streptomycin (Strepto), Metronidazole (Metro) or Vancomycin (Vanco) in aerobiosis (left) and anaerobiosis (right). (C) Comparison of relative abundancies of different families between faecal microbiota obtained from normobiotic (outer chart) and Streptomycin-treated mice (inner chart, left panel), or Metronidazole-treated (inner chart, middle panel) or Vancomycin-treated mice (inner chart, right panel). (e) Heat map comparing the expression levels of the different taxa between faecal microbiota obtained from normobiotic, Streptomycin-, Metronidazole- or Vancomycin-treated mice.

As expected, among all the experimental groups shown in ***Figure 4.26*** only the FMT performed with metronidazole-treated donor microbiota remained fully capable to control intestinal inflammation. Indeed, colon length of mice receiving metronidazole treated microbiota was higher compared to that of DSS treated mice (***Figure 4.28A***). Consistently, only in this group the colonic expression of the pro-inflammatory il1b was significantly decreased (***Figure 4.28B***).

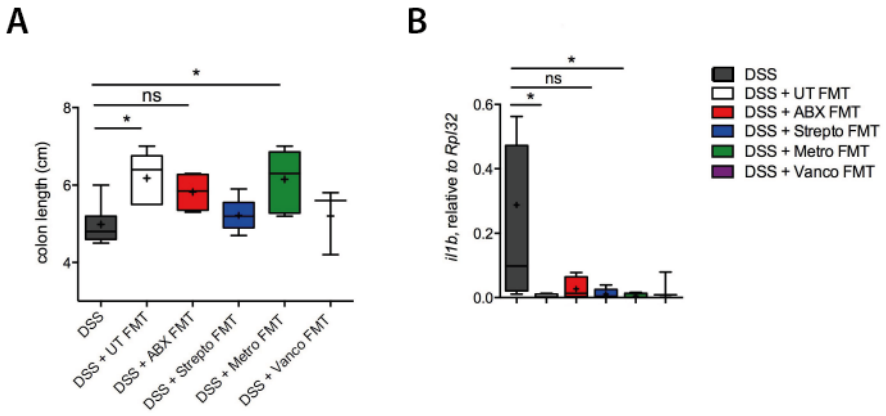

Figure 4.28 Alteration of the healthy microbial ecology by antibiotic treatment selectively abolishes FMT beneficial effects. (A) Colon length and (B) il1β colonic expression in DSS-treated (black boxes), DSS + Untreated FMT (white boxes), DSS + ABX FMT (red boxes), DSS + Streptomycin FMT (blue boxes), DSS + Metronidazole FMT (green boxes) and Vancomycin (Violet boxes)-treated mice). Statistical significance was calculated with Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.

The FMT driven inhibition of $CD4^+$ T cell accumulation in the colon lamina propria was impaired in mice receiving FMT from antibiotics-treated donors (***Figure 4.29A***). Interestingly, intestinal iNKT cells expansion was triggered upon transplant of microbiota from whole-spectrum antibiotics treated donors (***Figure 4.29B***), in line with our previous

results during homeostatic conditions (***Figure 4.6***) and with published evidences about germ free mice [71].

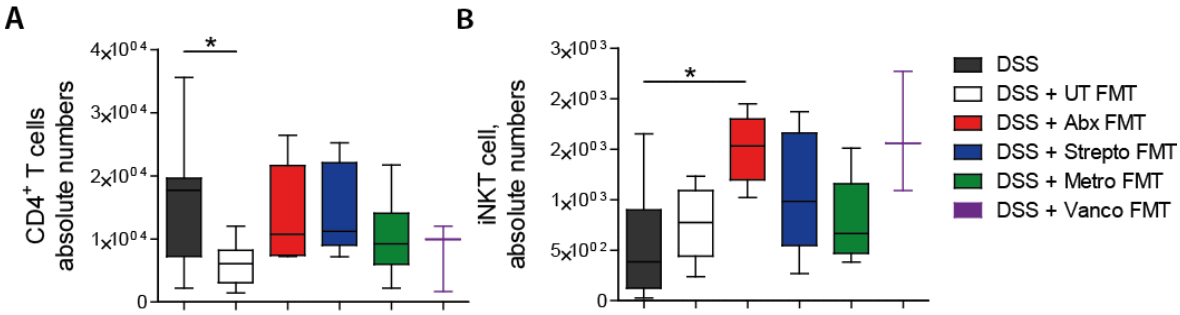

Figure 4.29 Alteration of the healthy microbial ecology by antibiotic treatment selectively impairs FMT effects on T cells. Absolute numbers of colonic (A) CD4+ T cells and (B) iNKT cells cells isolated from DSS-treated (black boxes), DSS + untreated FMT (white boxes), DSS + ABX FMT (red boxes), DSS + Streptomycin FMT (blue boxes), DSS + Metronidazole FMT (green boxes) and DSS + Vancomycin FMT (Violet boxes)-treated mice. Statistical significance was calculated with Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.

IL-10 production by the intestinal immune infiltrate (CD45.2$^+$ cells) remained high in mice receiving metronidazole- treated FMT (***Figure 4.30***), enriched in *Lactobacillaceae* and B*ifidobacteriaceae* which are known to promote IL-10 production [161]. IL-10 production was maintained in dendritic cells and B cells, but also neutrophils and macrophages showed higher IL-10 secretion, though not statistically significant, compared to mice treated with only DSS or receiving ABX, streptomycin or vancomycin treated FMT (***Figure 4.30***). Conversely, IL-10 production by CD4$^+$ T cells was impaired in all the antibiotic treated experimental groups (***Figure 4.30***).

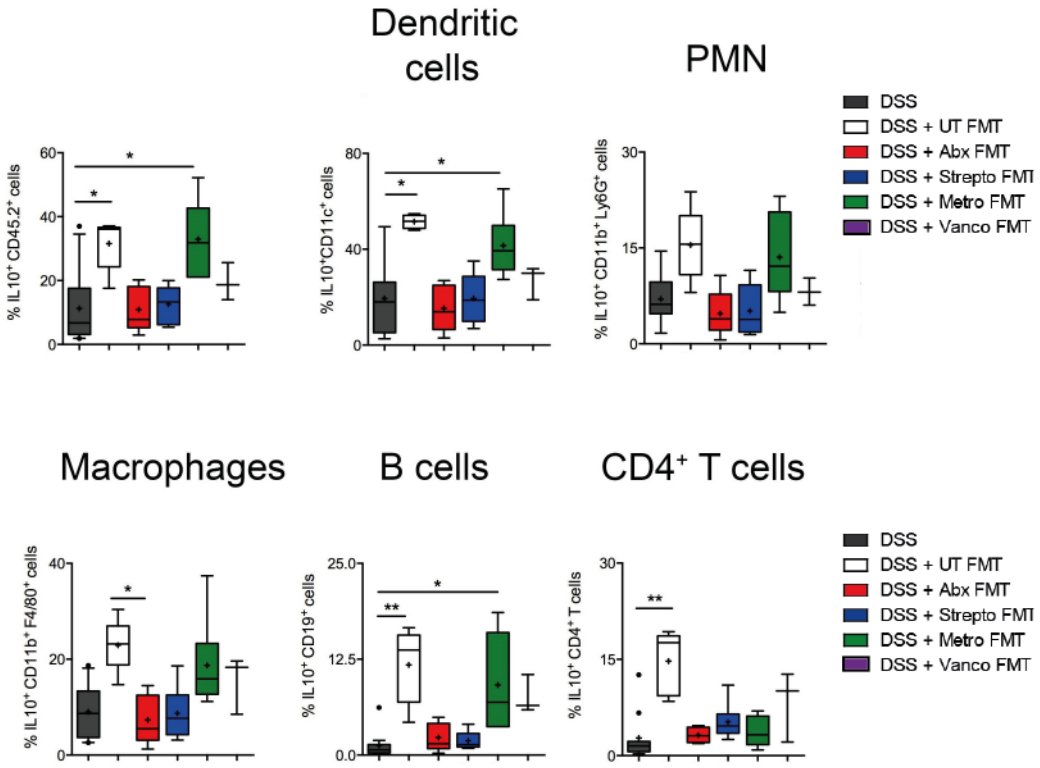

Figure 4.30 Alteration of the healthy microbial ecology by antibiotic treatment selectively impairs FMT induced IL-10 production. Frequencies IL-10 secreting colonic total CD45+ immune cell populations or gated dendritic cells (CD11c+), neutrophils (Cd11b+ Ly6g+), Macrophages (Cd11b+F4/80+), B cells (CD19+) and CD4+ T cells isolated from DSS-treated (black boxes), DSS + untreated FMT (white boxes), DSS + ABX FMT (red boxes), DSS + Streptomycin FMT (blue boxes), DSS + Metronidazole FMT (green boxes) and DSS + Vancomycin FMT (Violet boxes)-treated mice. Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.

These results suggest that FMT anti-inflammatory activity is differentially acting on APC and T cells and that the therapeutic effect driven by FMT treatment might be relying primarily on IL-10 produced by intestinal B lymphocytes and myeloid derived infiltrating cells.

It is well acknowledged that the murine models only partially resemble human pathologies. Nevertheless, keeping in mind this caveat, our data clearly show that the manipulation of the gut microbiota is associated with a general amelioration of the inflammatory status through a modulation of the mucosal immune response.

5 Discussion I

The acknowledgement of the association between microbiota dysbiosis and the pathogenesis of several immune-related diseases triggered an increasing interest about the idea of using microbiota-derived information for diagnostic and therapeutic purposes. The clinical complexity of multifactorial diseases, such as IBD, renders the identification of a disease-associated dysbiotic profile relatively difficult. Patients diagnosed with the same diseases (e.g. Crohn's disease or Ulcerative colitis) often manifest wide ranges of pathological conditions. This high interindividual variability among patients requires an individualized approach to undoubtedly link microbiota structure and function and disease phenotype [123]. The effort to solve this issue could nonetheless prove effective in the detection of early microbial biomarkers of disease. For instance, it has been recently reported that a cohort of Parkinson disease patients displayed constipation symptoms and dysbiotic shift in gut microbiota composition years before the development of motor dysfunction [171], promisingly suggesting the use of microbiota-based screening tests to help the early detection of disease development among individuals at risk. The same concept has been proposed also for Crohn's Disease patients. In this context a microbial signature associated to the early onset of the pathology was identified after the analyses of a large cohort of paediatric treatment-naïve patients [120]. Similarly, the decrease in some protective bacterial species in healthy individuals correlated to a higher IBD genetic risk score[118]. The diagnostic value of microbial biomarkers could also additionally lie in the stratification of patients belonging to similar pathologies, such as UC and CD [121], or in the prediction of patient's responses to defined therapies [172]–[174].

Once suggested a causality link between gut microbiota dysbiosis and the onset of intestinal pathologies, it is therefore not surprising that great effort has been posed to offer therapeutic approaches aimed at manipulating the gut microbiota status. Among the different proposed approaches, antibiotic administration has been taken into consideration also in the management of IBD patients, showing positive effects on the amelioration of intestinal inflammation in several clinical trials involving CD patients, especially in defined pathologic conditions (i.e, postoperative recurrence, perianal fistulae, pouchitis [130], [175]. We also showed that metronidazole treatment could contribute to the amelioration of colonic inflammation by selecting protective bacteria such as *Lactobacillaceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae (Figure 4.27).* However, we and others [176] also demonstrated that antibiotics not only modulate gut microbiota composition, but also mucosal immune cell activity and frequency (Section 4.2). Their administration to patients should therefore take into account antibiotic immunostimulatory ability, a condition that in patients with autoimmune disorders could easily become a double-edged sword. On top of the abovementioned conflicting observations, antibiotic administration to IBD patients has been linked to a worsening of their dysbiotic state [120] and to an increased risk in developing *Clostridium difficile* infection, due to a reduction in colonization resistance [70]. As a consequence of these and other considerations concerning the long-term administration of antibiotics to IBD patients, ECCO guidelines concluded not to recommend their use [177], [178] with the notable exceptions, as mentioned before, of postoperative recurrence, perianal fistulae and pouchitis in CD patients and before hospitalization for UC patients.

On the contrary, other approaches aimed at the manipulation of the gut microbiota quickly became increasingly utilised in the clinical practice. For instance, FMT is becoming the first-line therapy in antibiotic resistant recurrent CDI [131]. However, its therapeutic application to other gastrointestinal diseases is at the very beginning and data on its mechanism of action during intestinal inflammation are still scarce. While it is known that restoration of normobiosis correlates with clinical remission in successful trials involving UC patients [141]–[143], it is still unclear whether FMT might have an effect on the immune system. To date, results obtained in BALB/c mice suggest that FMT can induce $CD4^{+}CD25^{+}$ regulatory T cells and reduce colonic expression of Il1β and ifnγ [144].

In our study, we showed that the manipulation of the gut microbiota by FMT induces a general amelioration of the inflammatory status in colitic animals (Sections 4.3). Moreover, analyses of the gut microbiota in our model showed that 3 days of therapeutic FMT are sufficient to introduce modifications in the dysbiotic microbiota, with a relevant change in the relative proportions of *Firmicutes* (***Figure 4.11***). Interestingly, we found an alteration of the levels of *Erysipelotrichaceae* and *Lactobacillaceae* in DSS-treated mice, similarly to what happens in IBD patients [118], [120], and these taxa abundances were restored upon therapeutically successful FMT (***Figure 4.11***).

It is now acknowledged that the gut microbiota composition is heavily influenced by age, gender, genotype, diet and environmental factors [55]. In this context, animal models are valuable tools to study complex biological phenomena, such as those occurring during intestinal inflammatory processes, while controlling confounding variables. It also emerged from several recent studies that different research institutions or commercial vendors

might harbour variations in the microbiological environment leading to differences in microbiota composition [58], [166]. Here, we showed that the microbiota derived from three different sources were equally capable to control intestinal inflammation in our experimental model (*Figure 4.15*), and this ability was dependent on the presence of a core microbial ecology composed by *Bacteroidales S24-7*, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae*. As recently shown also in metabolic diseases [86], this core ecology consists of bacteria from different phyla but sharing similar metabolic functions (i.e: SCFA production, pH control, free radicals scavenging) and capable to create environmental conditions sufficient to inhibit the growth of pathobionts while supporting optimal host health. We also demonstrated that a healthy gut environment, shaped by the presence of a healthy functional microbial ecosystem, is also fundamental to instruct the immune system towards homeostasis (***Figure 4.13***).

Furthermore, our results provided a strong evidence about the close relationship between gut microbiota and the mucosal immune system. We observed that different types of microbial dysbiosis, i.e. inflammation-induced and antibiotic-driven, are impacting on T and iNKT cell phenotype and functions (**Figure 4.3** and ***Figure 4.6***). Moreover, for the first time we proved that the gut microbiome modifications upon FMT treatment exert a profound impact on both the adaptive and innate mucosal immune system and that this correlates with the reduction of the intestinal inflammation (Section 4.4). FMT beneficial anti-inflammatory effects support changes in immune cell frequencies, reduction of colonic ifnγ and il1β, the increase in antimicrobial peptides and mucins, and the decrease of bacterial antigen presentation by APC. Most importantly, a normobiotic FMT induces the skewing of

innate and adaptive immune cells toward a tolerogenic IL-10 secreting cytokine profile that, altogether, concur to restore intestinal homeostasis (***Figure 5.1***).

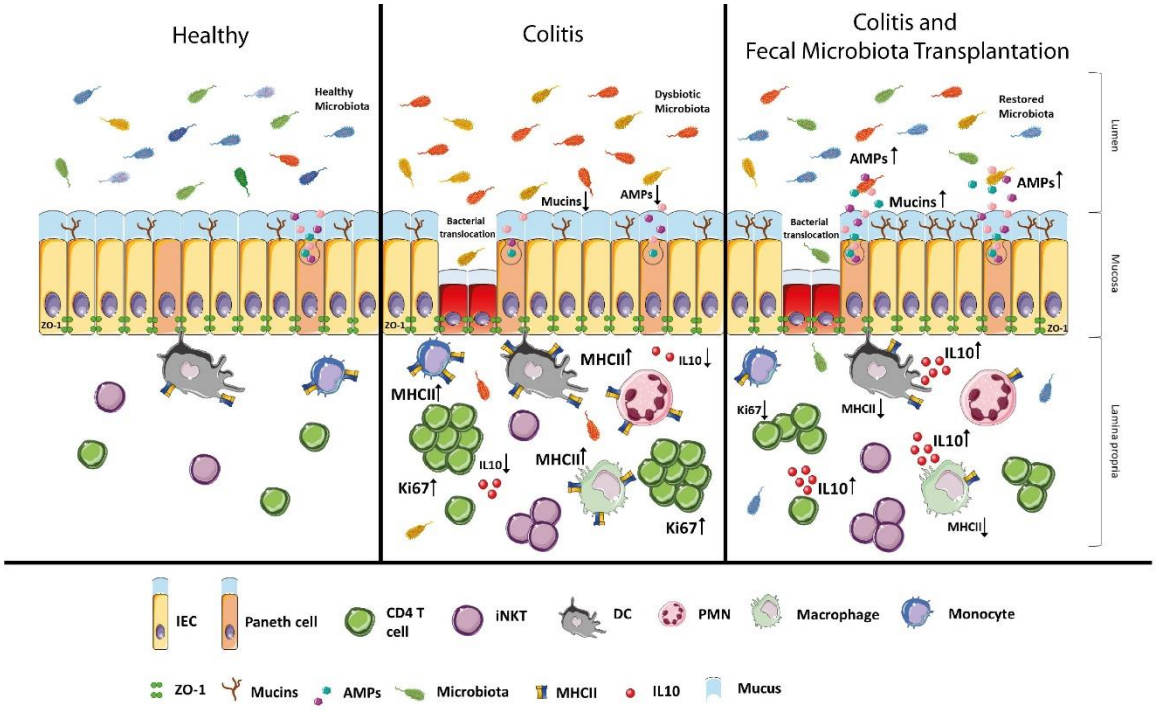

Figure 5.1 Schematic representation of mucosal immune response regulation by FMT during intestinal inflammation.

Among the different tolerogenic mechanisms developed to maintain intestinal immune homeostasis [179], we found that IL10 production was one of the most potent anti-inflammatory pathway induced by FMT administration in colitic mice. Our results are in line with the notion that IL-10-deficient mice and patients with genetic defects in the IL-10/IL-10R pathway develop intestinal inflammation following the onset of a dysbiotic state [180]. Several studies report that a probiotic preparation (i.e. #VSL3) composed by a cocktail of

eight strains of *Lactobacilli* and *Bifidobacteria* is effective in controlling intestinal inflammation through the IL-10-mediated restoration of intestinal homeostasis [161].

The resolution of intestinal inflammation has been often linked to the functional plasticity of $CD4^+$ T cells from pro-inflammatory to tolerogenic IL-10-producing subtypes [53], [181]. Upon therapeutic FMT, both $CD4^+$ and iNKT cells produced IL-10 (***Figure 4.23***). Importantly, since IL-10 also negatively affects the proliferative capacity of cells, the overall increased availability of colonic IL-10 might be directly responsible for the reduced proliferative capacity of T cells (***Figure 4.18***) together with the decreased antigen presentation potential of APC, as shown by their reduced levels of MHC-II (***Figure 4.19***). This, in turn, might contribute to the reduction of the presentation of bacterial antigens to $CD4^+$T cells, known to occur in IBD patients [117]. Importantly, colonic HLA-DR expression levels discriminate between healthy, quiescent and active IBD patients, confirming a prominent role of MHCII-dependent antigen presentation in IBD immunopathology [182].

In conclusion, we demonstrated that the restoration of normobiosis could be the first hint to simultaneously trigger several immune pathways leading to tolerogenic functions of innate and adaptive immune cells that altogether contribute to the resolution of the inflammatory processes. These findings represent an important contribution toward the elucidation of the complex interplay between the immune system and the gut microbial ecosystem and are instrumental for a better understanding of the immune events occurring during therapeutic FMT in humans.

However, further pivotal aspects concerning FMT mechanisms of action still need to be addressed to optimize its translation into the clinics. The relatively low fraction of UC patients responding to FMT treatment in the completed RCT (25-30% [141]–[143]), strictly associated to an increase in microbiota α-diversity, underlines the presence of a high interindividual variability in the response to the treatment. This finding is likely a direct consequence of the multifactorial nature of IBD, but it also suggests that an improved knowledge about the mechanisms of bacterial engraftment could give useful insights for the best selection and clinical management of those patients. Moreover, the transplant of multi-donor faecal samples resulted in a higher FMT endoscopic and clinical success by reducing the probability to receive ineffective donor stools [143]. Interestingly, this observation provides the rationale for more accurate studies of the best donor/recipient matching to increase the success rate of FMT. In addition, the combination of FMT with other gut microbiota manipulation approaches could increase the engraftment rate by favouring the colonization of specific beneficial strains. For instance, as mentioned before we showed that metronidazole treatment is selecting protective SCFA-producing and IL10-inducing bacteria such as *Lactobacillaceae*, *Bifidobacteriaceae* and *Erysipelotrichaceae* (***Figure 4.27***). This, together with other interventions such as diet, could provide a favourable environment for the engraftment of the microbiota transplant.

Intriguingly, induction of a shift in the microbiota composition could have positive effects on the host also in other clinical contexts, such as treatment of cancer patients [123]. Several studies have shown that the gut microbiome may influence antitumor responses by shaping both adaptive and innate immunity and that this relationship could be exploited to enhance anticancer therapy efficacy [183], [184]. More recent reports showed a

stratification of melanoma patients on the basis of their microbiome. The ones displaying high α-diversity and abundance of certain taxa demonstrated a better response to checkpoint inhibitor immunotherapy [174], [185].

These evidences, together with our demonstration of the existence of a close crosstalk between the immune responses and the gut microbiota, could thus pave the path to the clinical use of a combination of cancer immunotherapy and the gut microbiota manipulation.

6 Results II

6.1 Characterization of intestinal T lymphocytes in Inflammatory Bowel Diseases

Our data indicate that experimental manipulation of the gut microbiota composition during intestinal inflammation, as well as in the presence of antibiotic-induced dysbiosis, results in an activation of both conventional and non-conventional T cell subsets.

Since current hypotheses suggest that an abnormal T cell responses against a dysbiotic intestinal microbiota in genetically predisposed individuals [153] might be linked to Inflammatory Bowel Disease (IBD) pathogenesis, we aimed at evaluating if and which phenotypical and functional variations might have occurred in human iNKT and $CD4^+$ T cells in a cohort of human IBD patients.

To phenotypically and functionally characterize T lymphocytes in the intestinal mucosa of IBD patients, lamina propria mononuclear cells (LPMCs) were obtained from individuals affected by Ulcerative Colitis (UC, n=16), Crohn's Disease (CD, n=24) or non-inflammatory IBD-unrelated intestinal pathologies referred to as healthy donors (HD, n=27) (Figure *6.1*). Patients were enrolled from March 2014 to July 2018 at the IRCCS Policlinico Ospedale Maggiore, Milan, Italy. The clinical characteristics and concomitant therapies of IBD patients are summarized in ***Table 3.6***.

6.1.1 CD4$^+$ T and iNKT cells infiltrate the lamina propria of IBD patients

Human iNKT cells were identified by hCD1d:PBS57 Tetramer recognition, whose specificity was confirmed by unloaded CD1d tetramer staining (***Figure 3.4***).

The whole population of CD3$^+$ tetramer^{neg} conventional T cells did not manifest frequency differences between IBD patients and uninflamed donors (Figure *6.1**A**-**B***). Similarly, iNKT cell frequency was slightly increased in the LPMCs of CD patients compared to UC patients and healthy donors, albeit not significantly (Figure 6.1***A,C***). As already known for other tissues [19], also intestinal iNKT cells display a high frequency variability, spanning from the 0.1% to the 10% of the total CD3$^+$ T lymphocytes (Figure *6.1**A**-**B***). iNKT cells were slightly more abundant in the intestinal lamina propria than in the peripheral blood (*Figure 6.1**B***), in accordance with the notion that most iNKT cells do not circulate but localize preferentially within tissues [186].

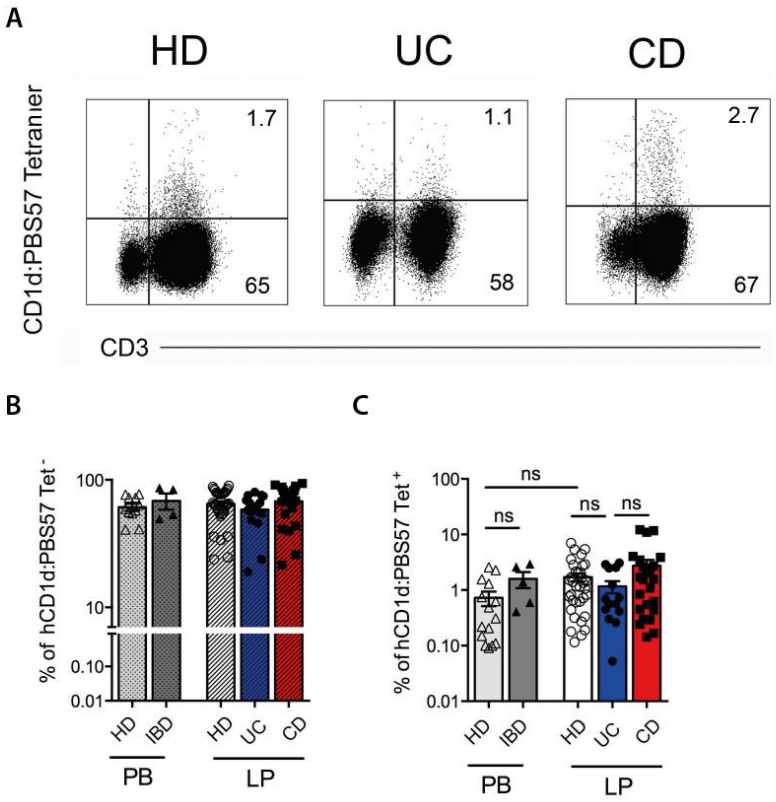

Figure 6.1 T cells infiltrate IBD patients colon lamina propria. (A) Representative dot plots and (B) summary of frequencies of (B) conventional T cells (hCD1d:PBS57 Tetramer-, dotted bars) and of (C) colonic iNKT (hCD1d:PBS57 Tetramer+, plain bars) among total CD3+ Lineage- cells in peripheral blood (PB) of healthy donors (HD, open triangles, n=15) or IBD patients (IBD, closed triangles, n=5) and in the lamina propria (LP) of uniflamed donors (HD, open circles, n=27), UC (closed circles, n=16) and CD patients (closed squares, n=24). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*) were regarded as statistically significant. Outliers were detected with Grubb's test.

6.1.2 Intestinal iNKT cells express high levels of CD161 and secrete pro-inflammatory cytokines

Since T cell surface expression of CD4 and CD161, a tissue-homing integrin highly expressed by gut-tropic T cells, have been associated to pathogenic functions in CD patients [109],

their expression was also evaluated on intestinal iNKT cells and tetramer^{neg} CD3^{+} T cells. Intestinal iNKT cells were mainly positive for CD4 (***Figure 6.2C-D***). The double positive CD4^{+} CD161^{+} population resulted the most abundant, as previously published [187], without significant differences among IBD patients and controls (***Figure 6.2C-D***). Likewise, intestinal conventional tetramer^{neg} CD3^{+} T cells were mainly CD4^{+} and the majority co-expressed CD161 (***Figure 6.2A-B***), confirming previously published data in CD patients [110] and in UC patients [115].

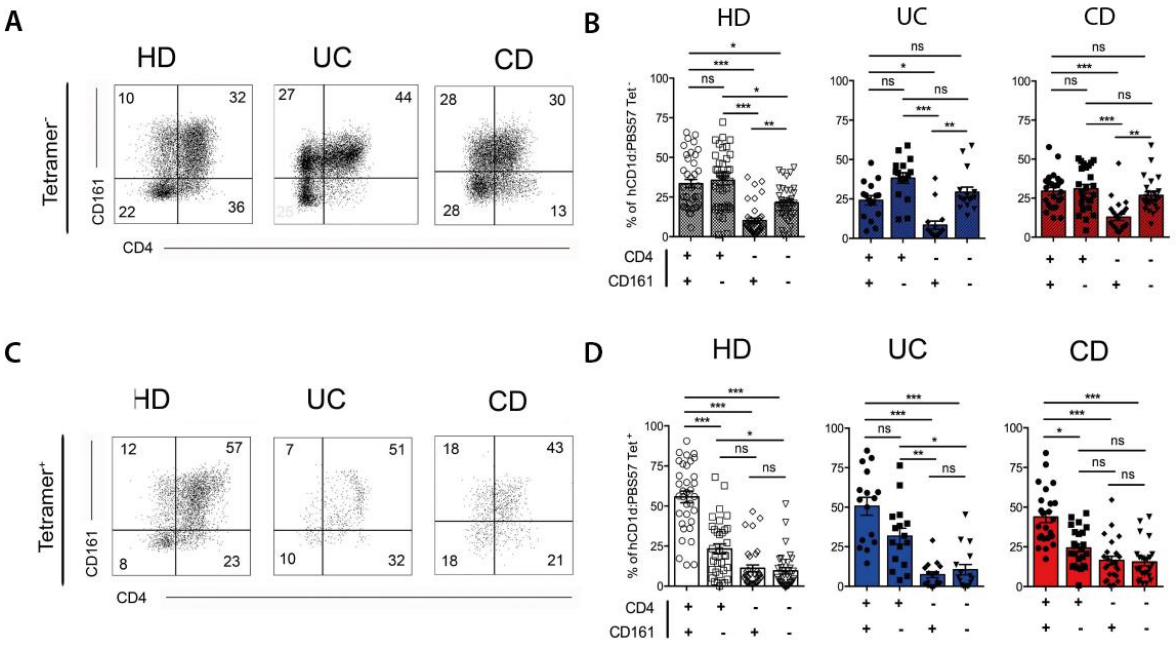

Figure 6.2 CD4 and CD161 expression on T cells in colon lamina propria of IBD patients. Representative dot plots (right panels) and cumulative frequencies of CD4/CD161 expressing cells among iNKT (Tetramer^{+}, D) and conventional T cells (Tetramer^{-}, E) isolated from the LP of HD (left panels), UC (middle panels) and CD patients (right panels). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

To note, colonic or ileal CD localization did not substantially influence neither the frequency (***Figure 6.3A-B***) nor the phenotype (***Figure 6.3C-D***) of lamina propria iNKT and conventional $CD3^+$ T cells.

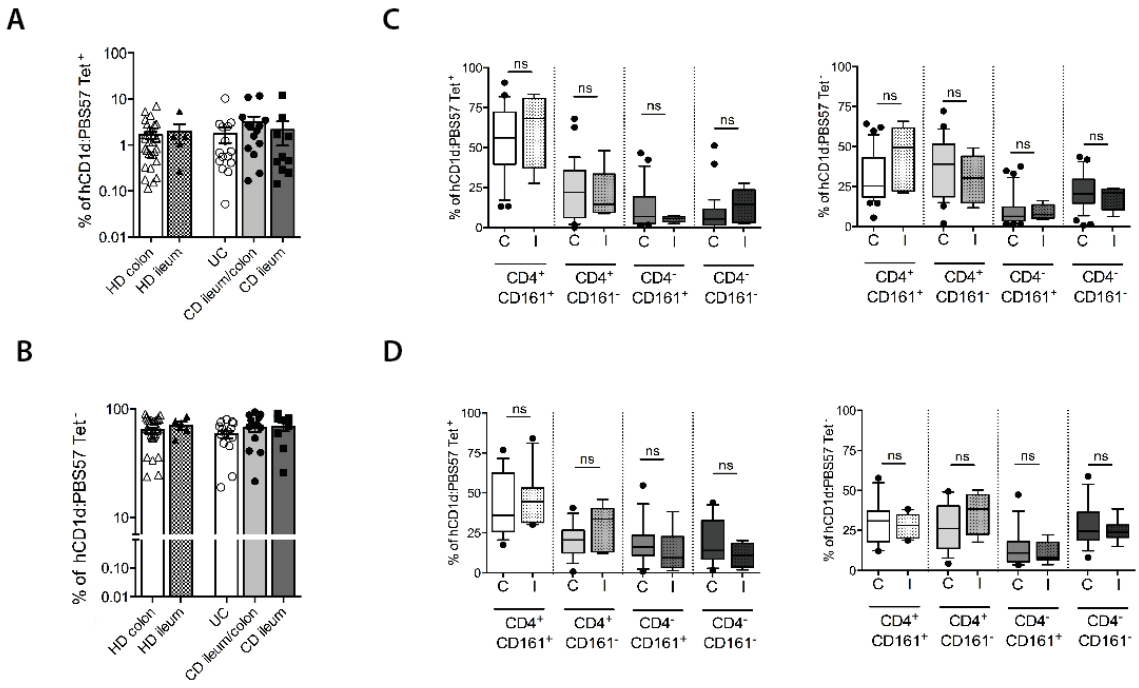

Figure 6.3 Analysis of frequency and phenotype according to colonic or ileal localization. Frequency of Tetramer+ (A) or Tetramer-(B) CD3+ LPMC, subdividing HD and CD patients for colonic or ileal disease localization. Frequency of CD161/CD4 expressing Tetramer+ (C) or Tetramer- (D) CD3+ LPMC subdividing HD and CD patients for colonic (C) or ileal (I) disease localization. HD colon, n=22; HD ileum n=5; CD colon n=13, CD ileum/colon n=11). Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

In previous studies the expression of the CD161 marker in conventional T helper cells from CD patients identified IL-17-secreting T cell subset (Th17 cells) [109]. In accordance, we

found that CD161 expression correlated with IL-17 secretion both in conventional T and in iNKT cells (***Figure 6.4A***), while CD161^{neg} cells secreted preferentially TNF and IFNγ (***Figure 6.4A***). Importantly, both CD161^{+} and CD161^{neg} iNKT cells secrete moderate levels of IL-13 when polyclonally re-stimulated *ex-vivo* (***Figure 6.4A***), especially those derived from UC patients. Interestingly, *ex-vivo* freshly isolated iNKT cells constitutively secreted relevant amounts of cytokines even in the absence of a re-stimulation (***Figure 6.4B***). They produced high amounts of IL-17, IL-13 and TNF, suggesting that the intestinal microenvironment can bestow a pro-inflammatory pre-stimulation *in vivo*. Specifically, CD4^{+} CD161^{+} iNKT cells derived from CD patients constitutively secreted higher levels of IFNγ and IL-17 compared to the one isolated from UC patients and HD.

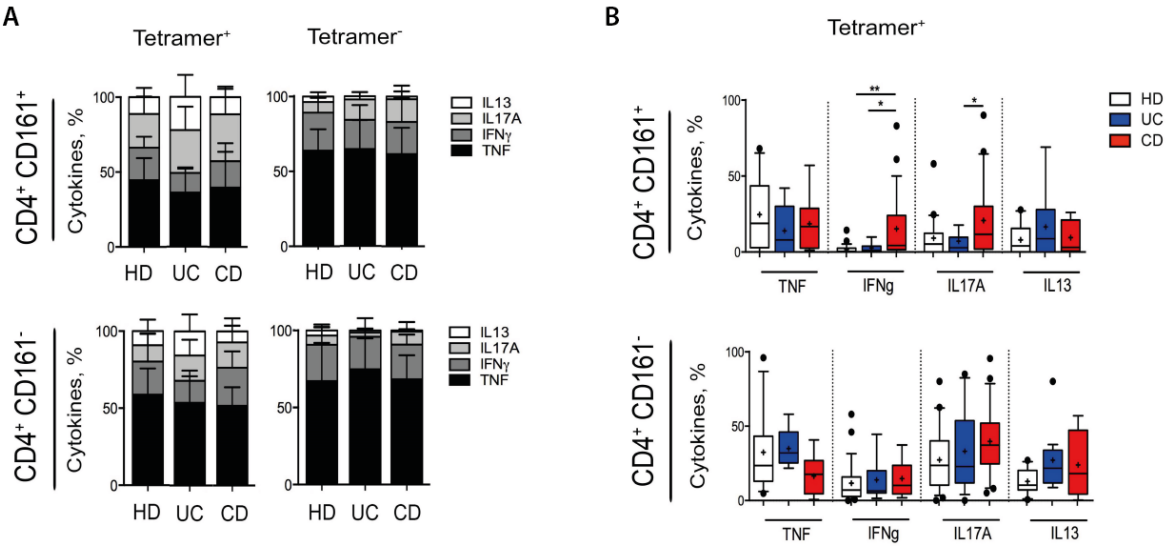

Figure 6.4 iNKT and conventional T cells are pro-inflammatory in IBD patients. *Frequency of pro-inflammatory cytokines (TNF, IFNγ, IL17A, IL13) produced (A) upon polyclonal stimulation or (B) without stimulation by CD4^{+}CD161^{+} (upper graphs) or CD4^{+}CD161^{-} (lower graphs) intestinal ex-vivo isolated iNKT cells (Tetramer^{+}) or conventional T cells (Tetramer-) from HD (n=27), UC (n=16) and CD patients (n=24). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Taken together, these data indicate that phenotypically distinct subsets of iNKT cells are present in the lamina propria of healthy donors and IBD patients, and that pro-inflammatory cytokines are secreted by these cells in the intestinal mucosa.

6.2 Generation of human intestinal iNKT cell lines and clones

To characterize iNKT cell role in IBD-associated inflammation through functional *in vitro* assays, iNKT cells were isolated from peripheral blood mononuclear cells, and from healthy intestine, ulcerative colitis and Crohn's disease LPMCs. Upon polyclonal re-stimulation, 5 stable iNKT cell lines were established. The lines were all positive for PBS57:CD1d tetramer staining (***Figure 6.5A***). The phenotype of these lines reflected in part the expression of CD4 and CD161 of *ex vivo* freshly isolated iNKT cells. While CD derived iNKT cell line uniformly co-expressed CD4 and CD161, the two different UC-derived lines differentially expressed CD161, as observed *in vivo* (***Figure 6.5B***).

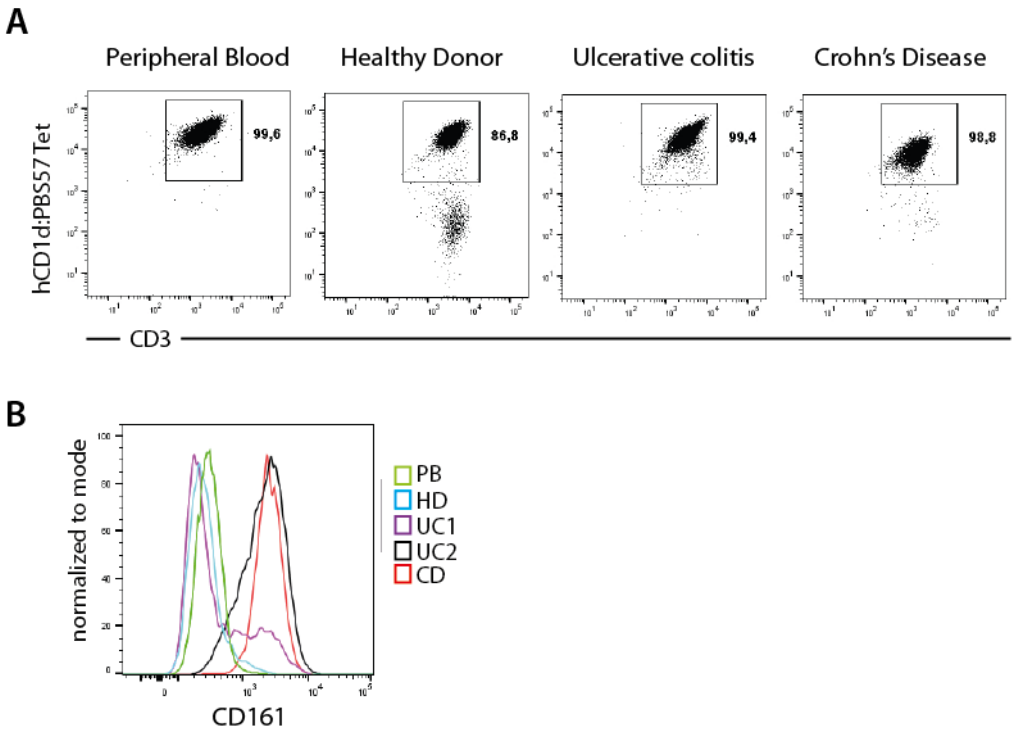

Figure 6.5 Intestinal iNKT cell lines characterization. *(A) Dot plots representing the staining with CD1d:PBS57 tetramer of iNKT cell lines derived from Peripheral blood, healthy intestine, ulcerative colitis (UC) and Crohn's disease (CD) surgical specimens.(B) Histograms of CD161 expression on iNKT cell lines.*

Intestinal iNKT cell lines were fully functional when tested *in vitro* for their response to both antigen-specific (***Figure 6.6A***) and polyclonal stimulation (***Figure 6.6B***). They resulted in a potent secretion of the inflammatory cytokines TNF, IFNγ and IL-13. To note, IL-17 production by iNKT cells is hardly detectable by ELISA. The addition of CD1d blocking antibody to the medium completely blocked the cytokine production induced by antigen presentation (***Figure 6.6A***).

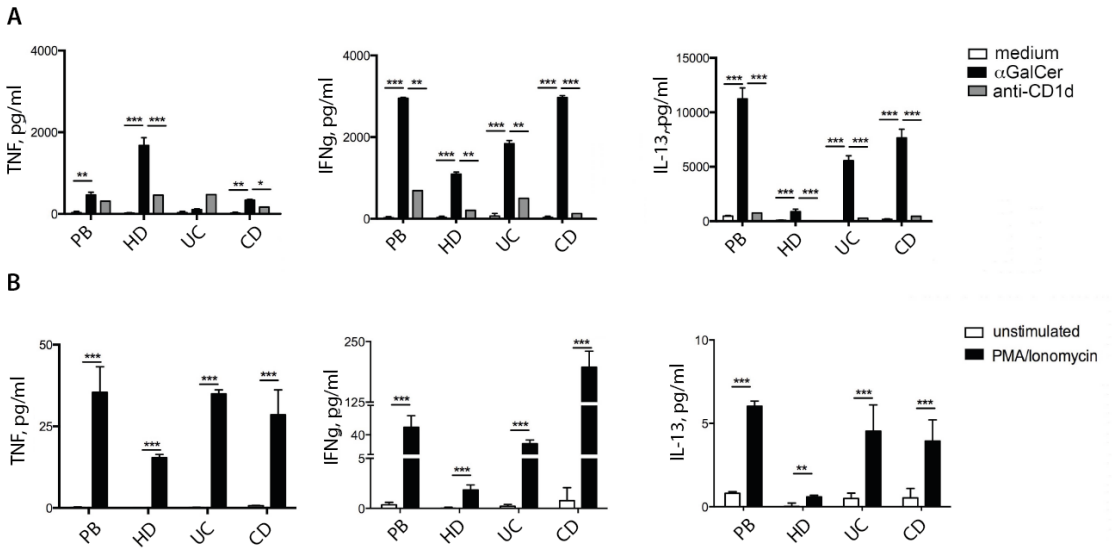

Figure 6.6 In vitro activation of intestinal iNKT cell lines. (A) TNF, IFNγ and IL-13 secretion upon co-culture of iNKT cell lines with moDC in the absence (white bars) or presence (black bars) of aGalcer and of concomitant CD1d blocking (gray bars). (B) Cytokine production by iNKT cell lines upon polyclonal (PMA/Ionomycin) stimulation. Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

More than 400 intestinal iNKT cell independent clones were also generated from IBD (UC, CD) patients and healthy donors (HD). These clones were characterized by different expression levels of TCR expression evaluated as PBS57:CD1d tetramer staining intensity (***Figure 6.7***). To note, obtaining iNKT cell clones from healthy intestinal mucosa was a challenging task. Indeed, only a very limited number of stable, but poorly viable, clones were obtained after several attempts of cloning.

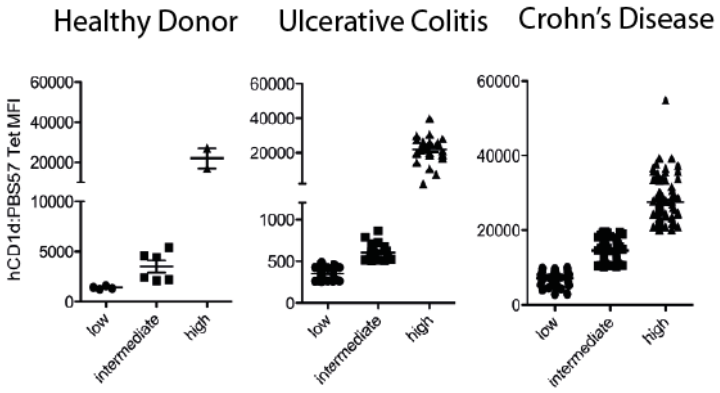

Figure 6.7 Intestinal iNKT cell clone characterization. Low, intermediate and high expression of the invariant TCR in iNKT cell clones derived from the intestine of healthy donors (N=12), UC (N=190) or CD (N=300) patients.

Similarly to what we observed with iNKT cell lines, antigen-specific (*Figure* 6.8***A***) and polyclonal stimulation (Figure 6.8***B***) induced a potent secretion of pro-inflammatory cytokines also on iNKT cell clones. Interestingly, polyclonally-stimulated UC-derived clones showed a more heterogeneous cytokine profile when compared to HD or CD-derived ones (*Figure* 6.8***B***). To note, IL17 production by iNKT cells is hardly detectable by ELISA.

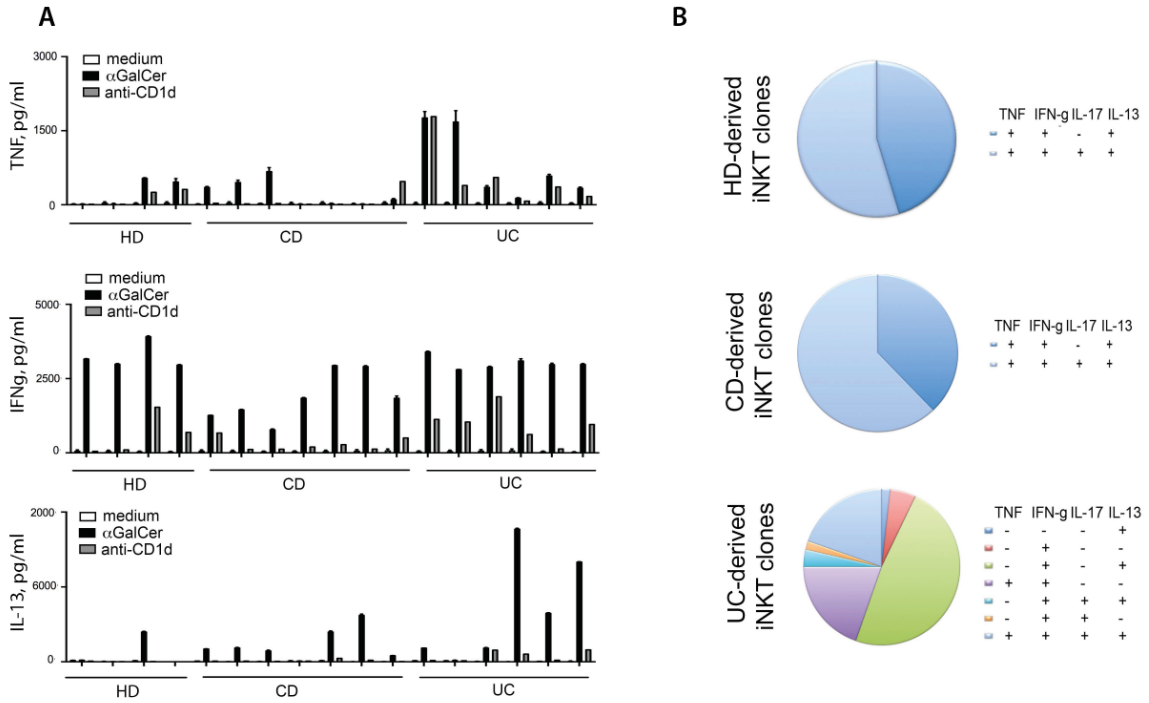

Figure 6.8 In vitro activation of intestinal iNKT cell clones. (A) TNF, IFNγ and IL-13 production by representative iNKT cell clones (HD, n=4; CD n=7, UC n=6) upon αGalCer stimulation in the presence (gray bars) or absence (black bars bars) of anti hCD1d-blocking antibodies. (B) Cumulative FACS analysis of the cytokine profile of 10 HD (out of 12), 50 UC (out of 196) and 50 CD (out of 210) intestinal-derived iNKT cell clones upon PMA/Ionomycin stimulation.

6.2.1 Intestinal iNKT cell line and clones from IBD patients are a valuable help to study iNKT cell function in IBD

Finally, to test if intestinal iNKT cells might acquire pathogenic functions against the intestinal epithelium upon *in vitro* activation, iNKT cells were polyclonally stimulated and their supernatants, containing pro-inflammatory cytokines (***Figure 6.9B***), were applied *in vitro* to polarized epithelial Caco2 monolayers (***Figure 6.9A***). Activated iNKT cells, independently from their origin, manifested a pathogenic potential affecting epithelial cell monolayer integrity, as demonstrated by decrease of Trans Epithelial Resistance (TEER)

(***Figure 6.9C***). Like conventional Th17 cells [44 and Appendix III] this effect could be inhibited upon neutralization of several T cell cytokines, with the notable exclusion of IL-13 (***Figure 6.9D***).

Collectively, these data indicate that the functional phenotype of intestinal iNKT cell lines reflects that of *ex-vivo* isolated intestinal iNKT cells. Hence, these lines and clones can be used as an innovative tool to study human intestinal iNKT cells *in vitro* and assess their potential role in gut inflammation.

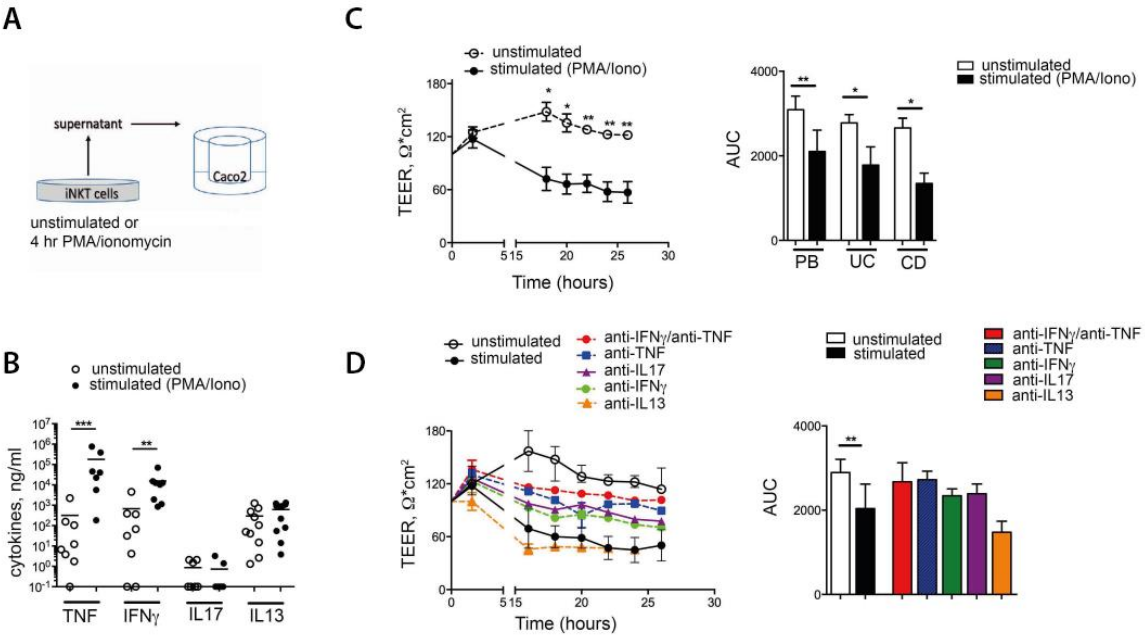

Figure 6.9 Human iNKT cells are pathogenic against epithelial cells. (A) Schematic representation of the experiment. (B) Multiplex analysis of TNF, IFNγ, IL-17A and IL-13 concentrations in the supernatants of polyclonally stimulated iNKT cells. (C) Trans-epithelial resistance (TEER) measured upon co-culture of Caco-2 cells with supernatants of unstimulated (open circles) or polyclonally stimulated (closed circles) iNKT cells. Left panel, representative plot; right panel, Area under the curve (AUC) of n= 5 independent experiments with iNKT lines. (D) Trans-epithelial resistance (TEER) measured upon co-culture of Caco-2 cells with supernatants of unstimulated (open circles) or polyclonally stimulated iNKT cell lines in the absence (closed circles) or presence of anti IFNγ/TNF (red

circles), anti TNF (blu squares), anti IL17 (purple triangle), anti IFNγ (green circles) and anti IL13 (orange triangles) inhibitors. Left panel, representative plot; right panel, Area under the curve (AUC) of n= 3 independent experiments.

6.3 iNKT cell response to mucosa-associated microbiota

A current hypothesis holds that aberrant activation of pathogenic T lymphocytes in IBD patients depends on gut microbiota recognition [153] and it is known that gut microbes are potent stimulators of iNKT cell responses [90]. Hence, we asked whether the gut microbiota directly activates human intestinal iNKT cells leading to the pro-inflammatory phenotype of iNKT cells in IBD patients.

6.3.1 IBD patients experience gut microbial dysbiosis

We collected the gut mucosa-associated microbiota surgical specimens of IBD patients and HD and we evaluated the bacterial composition by 16S rRNA sequencing (***Figure 6.10***). Unweighted UniFrac-based comparisons of the samples isolated from the colon of 9 HD, 7 UC and 6 colonic CD patients was performed (***Figure 6.10A***). Principle component analysis (PCoA) differentiated healthy microbiota samples from IBD patients, but no differences between UC and CD samples were detected (***Figure 6.10A***). Also, mucosa-associated microbiota derived from IBD patients showed a lower α-diversity when compared to HD microbiota (***Figure 6.10C***), confirming previous published data [188]. The taxonomic composition of the mucosa-associated microbiome of IBD patients showed an increase of

Proteobacteria and *Fusobacteria* and a decrease in *Firmicutes* compared to HD. As previously reported [189], these alterations were more evident in microbiota samples from CD as compared to UC patients (***Figure 6.10C***). To note, around 15% of the microbial ecology at genus level was significantly changed in the samples analyzed. Specific variations between colonic IBD and HD-derived samples included the increase of *Actinomyces*, *Enterococcus* and *Streptococcus* and the decrease of *Roseburia*, *Blautia*, *Odoribacter* and *Lachnospinaceae ND3007* (***Figure 6.10D***).

These observations confirm that IBD patients are characterized by a state of microbial dysbiosis profoundly altering the healthy gut microbiota composition.

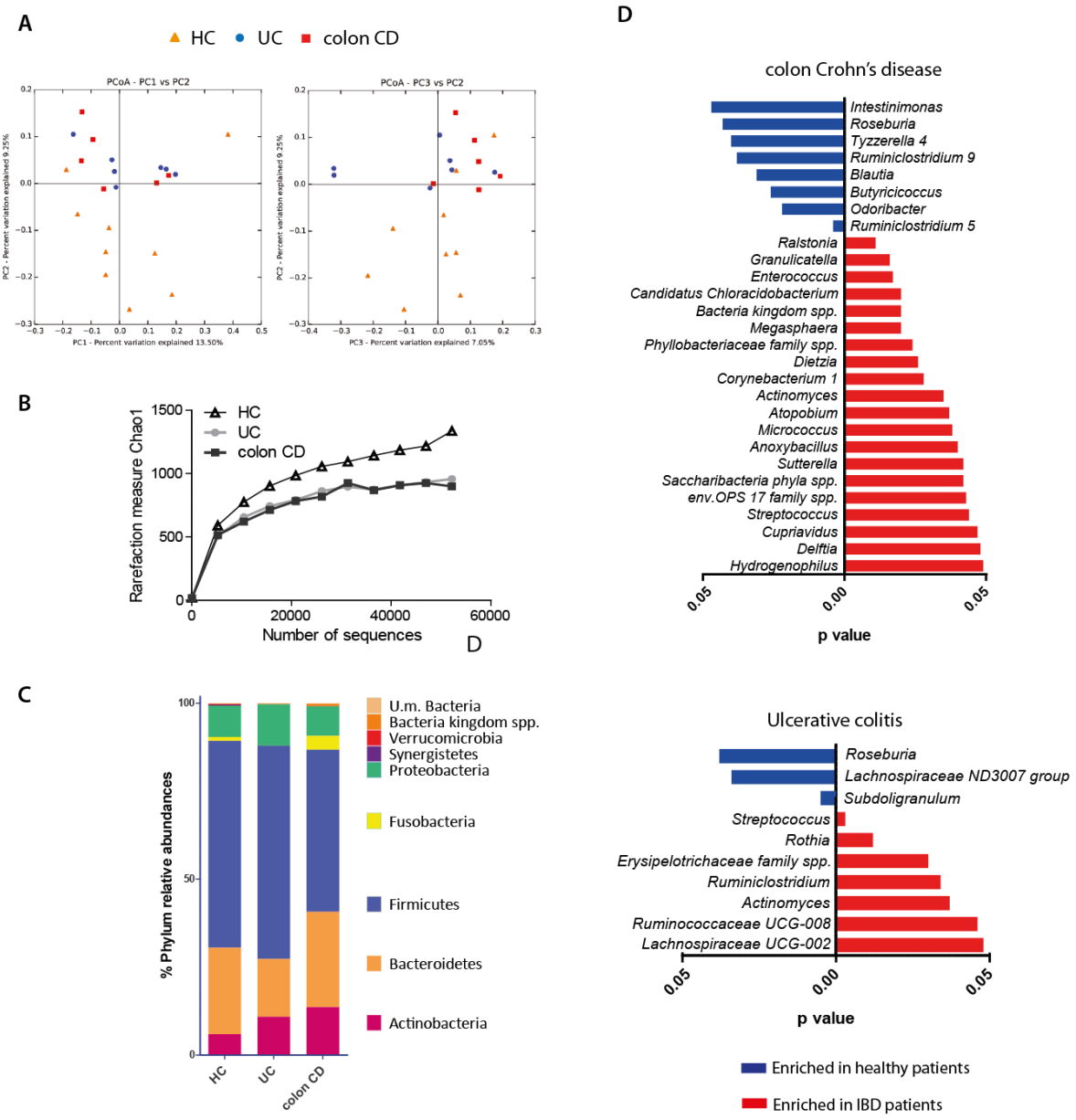

***Figure 6.10 Mucosa-associated microbiota analysis in IBD patients and HD**. (A) Microbiome clustering based on unweighted Principal Coordinate Analysis (PCoA) UniFrac metrics of mucosa-associated microbiota derived from HD (orange triangles), UC (blue dots) and colon CD (red squares) patients. (B) Rarefaction curves showing microbial richness based on the Chao1 index. (C) Bar plots of the taxonomic composition showing relative abundances >1% of bacterial phyla (D) P value of the comparison of the relative abundances of different taxa between colon Crohn's disease patients (upper panel) or Ulcerative colitis patients (lower panels) and healthy donors. Blue bars, taxa enriched in healthy donors, red bars, taxa enriched in IBD patients. Statistical significance was assessed with Kruskal Wallis test with LSD post-hoc test for more than two groups.*

6.3.2 iNKT cells acquire a pro-inflammatory phenotype upon exposure to mucus-associated microbiota

In order to test whether the pro-inflammatory phenotype of ex-vivo analyzed iNKT cells was linked to the distinguished gut microbiota profile of IBD patients, we exposed iNKT cells lines in vitro to the mucosa-associated microbiota samples from IBD and HD that we had characterized by metagenomic analysis (*Figure 6.10*). As shown in ***Figure 6.11***, we loaded antigen presenting cells (APC) with the microbiota samples that were pre-killed with several rounds of heat inactivation. After 2 hours, iNKT cells were added to the well and left in co-culture for 36 hours.

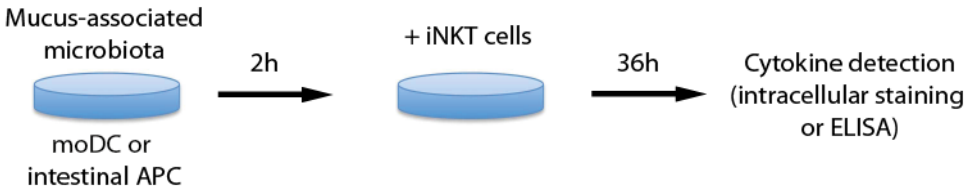

Figure 6.11 Antigen presentation assay with human intestinal iNKT cells. Schematic representation of the experiment.

All the iNKT cell lines cultured with monocyte-derived dendritic cells in the presence of gut microbiota, regardless their origin, were activated and secreted high amounts of pro-inflammatory cytokines such as TNF, IFNγ and IL-13 (***Figure 6.12***). Nonetheless, qualitatively and quantitatively different responses were observed when iNKT cell lines were exposed to the mucosal microbiota isolated from IBD patients compared to that of healthy donors (***Figure 6.12***). These results are in line with the microbial differences we

observed in the microbiota of these patients (*Figure 6.10*) and with the possible presence of different TCR-specificities among iNKT cell lines [42]. Interestingly, also HD-derived microbiota stimulated iNKT cells and induced the secretion of pro-inflammatory cytokines. However, exposure of CD-derived iNKT cell lines to IBD-derived microbiota triggered a strong production of pro-inflammatory cytokines, i.e. TNF and IFNγ. Conversely, exposure of iNKT cell lines derived from intestinal tissue of HD to the microbiota samples (from IBD or HD) did not result in a marked pro-inflammatory cytokine-skewing. In addition, IL-17 secretion by UC iNKT cell lines was decreased upon exposure to UC-derived microbiota (***Figure 6.12***).

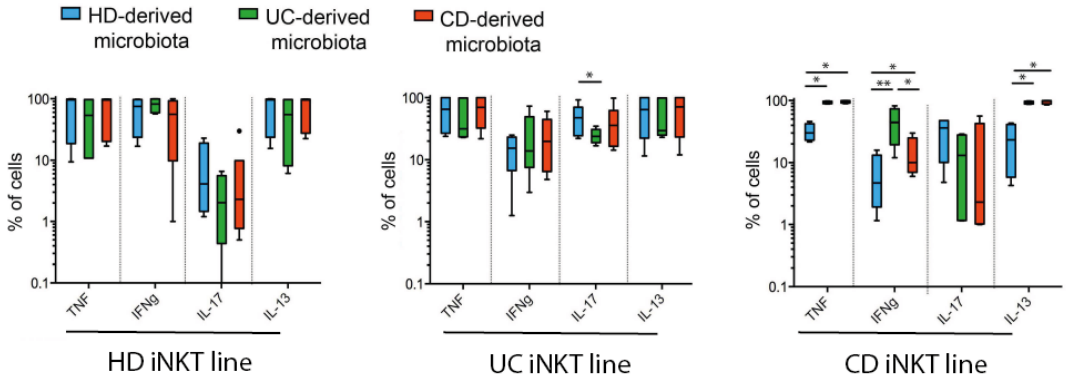

Figure 6.12 iNKT cells react to mucosa-associated microbiota. (B) Cytokine profile of iNKT cell lines (HD, UC, CD) co-cultured 36 hours with monocyte-derived dendritic cells exposed to 100 ng of mucosa-associated microbiota isolated from 8 HD (blue bars), 7 UC (green bars) and 8 CD patients (red bars). Quantification of pulled experiments with n=6/8 independent donors per group. Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.

Next, we tested whether the microbiota-induced pro-inflammatory effect on iNKT cells was associated to APC modulation. *In vitro* differentiated moDC or *ex-vivo* sorted CD1d^{+} HLADR^{+} intestinal antigen presenting cells (APC) were exposed to mucosa-associated microbiota samples and then their capacity to induce different cytokine profiles in iNKT cells was evaluated. Similarly to what observed with moDC, the exposure of CD1d^{+} sorted intestinal APC to gut microbiota, induced a pro-inflammatory activation of iNKT cells with a relevant TNF production. Interestingly, only iNKT cells challenged with CD-derived microbiota loaded on intestinal APC induced a sustained IL-13 production (***Figure 6.13***). To note, unstimulated intestinal APC were sufficient to induce an IL17 response by iNKT cells (***Figure 6.13***).

In conclusion, iNKT cells become functionally activated upon exposure to human mucosa-associated microbiota and display a highly skewed pro-inflammatory phenotype.

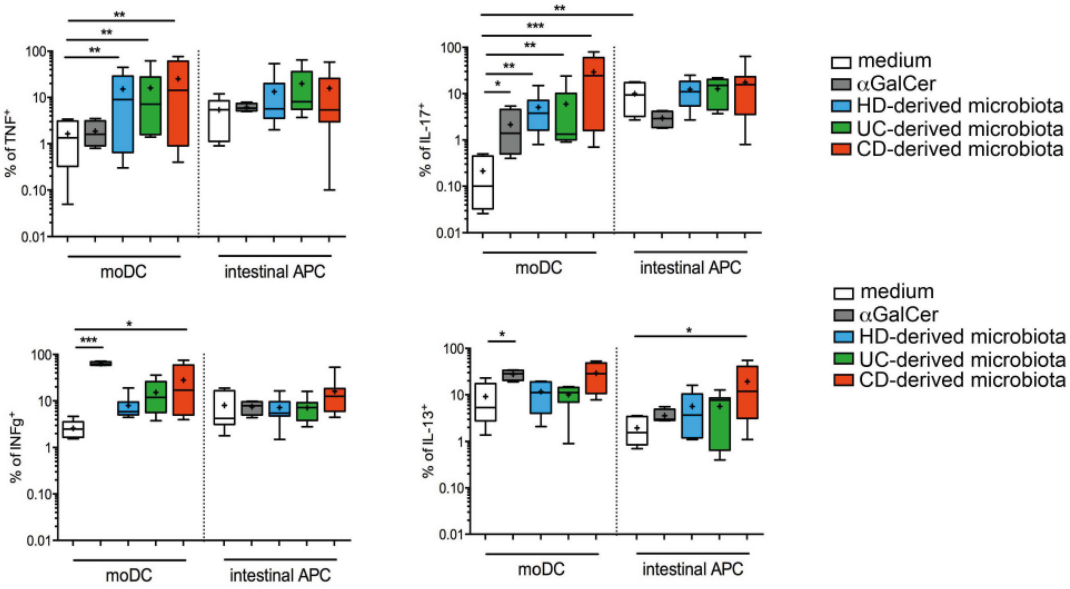

Figure 6.13 Intestinal antigen presenting cells present bacterial antigens. Frequency of TNF, IFNγ, IL17, IL13 positive iNKT cells co-cultured 36 hours with moDC or ex vivo sorted CD1d^{+}MHCII^{+}CD3^{-} intestinal

APC in the absence (white bars) or presence of aGalCer (grey bars) or to 100 ng of mucosa-associated microbiota isolated from at least 6 HD (blue bars), 4 UC (green bars) and 6 CD patients (red bars) in 3 independent experiments. Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.

6.3.3 iNKT cells recognize intestinal pathobionts by innate and adaptive mechanisms

Functional activation of iNKT cells is mediated by CD1d-dependent and/or independent mechanisms [19]. To explore whether microbiota-induced iNKT cell activation requires CD1d presentation, we exposed iNKT cells *in vitro* to two well-characterized purified intestinal pathobionts, known to be present in IBD patients and to mediate inflammatory responses [190], [191], i.e. Adherent Invasive *Escherichia coli* (AIEC) strain LF82 (***Figure 6.14A,C***) and *Salmonella thyphimurium* (B,D). Both strains induced a potent dose-dependent pro-inflammatory activation of iNKT cells (***Figure 6.14A,B***). This response resulted from a combination of both antigenic and not-antigenic (innate) stimulation, as demonstrated by its only partial inhibition after CD1d blockade (***Figure 6.14C,D***).

Noteworthy, in our cohort of patients the genus Salmonella was detected almost exclusively in mucus-associated IBD samples (***Figure 6.14E***), while the Escherichia/Shighella genus was detected also at lower levels in HD samples (***Figure 6.14F***).

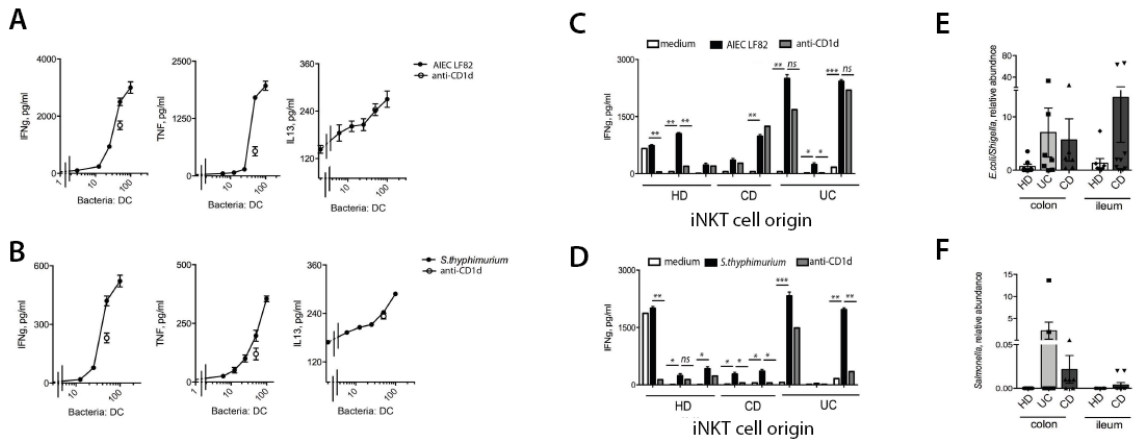

Figure 6.14 Human iNKT cells respond to stimulation with pathogenic intestinal bacterial strains. (A, B) IFNγ (left panels), TNF (middle panels) and IL-13 (right panels) in the supernatants of iNKT cells co-cultured 36 hours with monocyte-derived dendritic cells exposed to increases doses of the Adherent-invasive E. coli LF82 (AIEC, A) and of Salmonella thyphimurium (B). White dots, stimulation in the presence of anti-CD1d blocking antibody. (C,D) Cumulative representation of IFNγ production upon exposure to AIEC (B) or Salmonella thyphimurium (D) in at least 3 independent experiments with 2 different HD clones, 3 different CD and 3 different UC clones (E,F). Escherichia/Shigella (E) and Salmonella (F) genus in colonic HD (n=9), UC (n=7), colonic CD (n=6), ileal HD (n=7), and ileal CD (n=7). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**) were regarded as statistically significant. Outliers were detected with Grubb's test.

6.3.4 iNKT cells become pathogenic against intestinal epithelial cells upon microbiota stimulation

Further, to test if the pro-inflammatory iNKT cell activation driven by mucosa-associated microbiota might induce specific pathogenic functions against the intestinal epithelium, iNKT cells were exposed to the mucosa-associated IBD-derived microbiota. After 24 hours of activation iNKT cells were magnetically sorted and left in culture for 4 hours. Their supernatant was then collected and put on Caco2 epithelial monolayers in transwells

(***Figure 6.15A***). Consistent to our previous findings, microbiota-activated iNKT cells secreted pro-inflammatory cytokines such as TNF and IFNγ (***Figure 6.15B***).

Microbiota- activated iNKT cells, regardless of their origin, manifested pathogenic activities affecting epithelial cell monolayer integrity, as demonstrated by decrease of Trans Epithelial Resistance (TEER) (***Figure 6.15C***). Additionally, specific inhibition of iNKT-cell derived TNF and IFNγ abolished their pathogenic functions towards epithelial cell integrity (***Figure 6.15D***).

These findings suggest that exposure to mucosa-associated bacteria is sufficient to drive both innate and adaptive iNKT cell pro-inflammatory activation. Once activated, intestinal iNKT cells secrete pro-inflammatory cytokines conferring pathogenic features towards the intestinal epithelium.

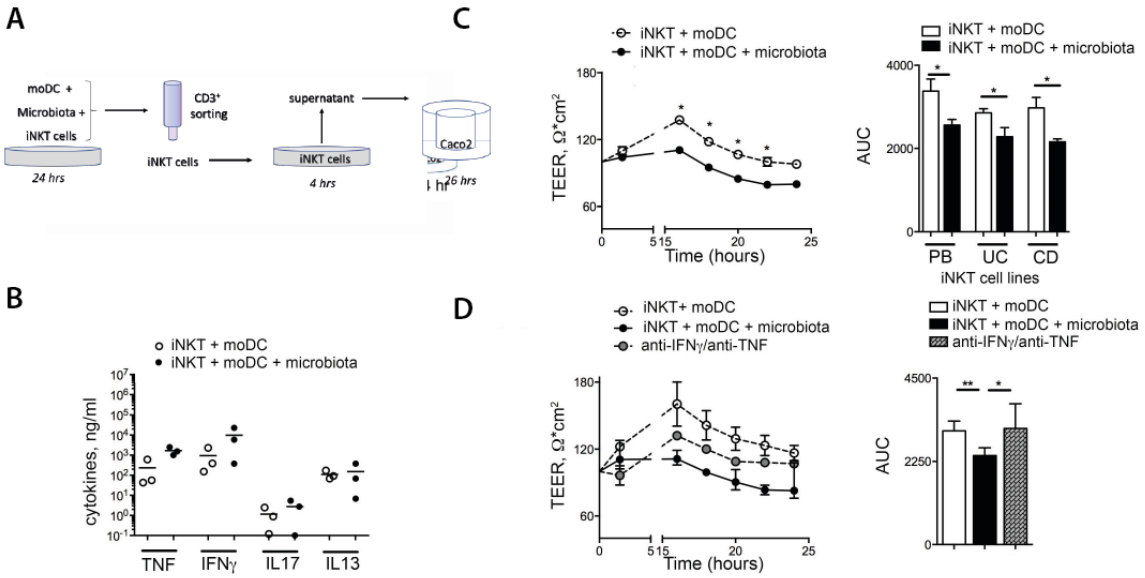

Figure 6.15 Microbiota-stimulated human iNKT cells are pathogenic against epithelial cells. (A) Schematic representation of the experiment. (B) Multiplex analysis of TNF, IFNγ, IL-17A and IL-13 concentrations in the supernatants of iNKT cells co-cultured with moDC alone (open circles) or with moDC stimulated with intestinal microbiota (closed circles) (C) Trans-epithelial resistance (TEER)

measured upon co-culture of Caco-2 cells with supernatants of iNKT cells co-cultured with moDC alone (open circles) or with moDC stimulated with intestinal microbiota (closed circles). Left panel, representative plot; right panel, Area under the curve (AUC) of n=4 independent experiments with iNKT cell lines. (D) Trans-epithelial resistance (TEER) measured upon co-culture of Caco-2 cells with supernatants of iNKT cells with moDC alone (open circles) or with moDC stimulated with intestinal microbiota (closed circles) in the absence (closed circles) or presence of anti IFNγ/TNF (grey circles) inhibitors. Left panel, representative plot; right panel, Area under the curve (AUC) of n=3 independent experiments. Statistical significance was calculated with Mann-Whitney test for comparison between two groups and Kruskal wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

6.3.5 Gut microbiota is responsible for iNKT cell pro-inflammatory activation in vivo during intestinal inflammation

Finally, we aimed at recapitulating *in vivo* the effects of bacteria exposure on pro-inflammatory activation of iNKT cells during intestinal inflammation. To this goal, we depleted gut microbiota in mice by the use of a broad-spectrum antibiotics cocktail (Ampicillin, Neomycin, Metronidazole, Vancomycin) during DSS-induced colitis (***Figure 6.16A***). For this setting, we adopted the DSS-induced chronic colitis model in which DSS was administered in three serial cycles of DSS and water alternation. This model better resembles the IBD patient intestinal inflammation due to its chronic timeframe and its massive epithelial disruption and bacterial translocation [155].

Chronic administration of DSS resulted in dramatic weight loss (***Figure 6.16B***), epithelial barrier ulceration and hyperplasia (***Figure 6.16C***) and upregulation of a panel of pro-inflammatory genes (***Figure 6.16D***). All these signs of inflammation were strongly reduced upon microbiota depletion (***Figure 6.16B-D***), consistently to reported data [192].

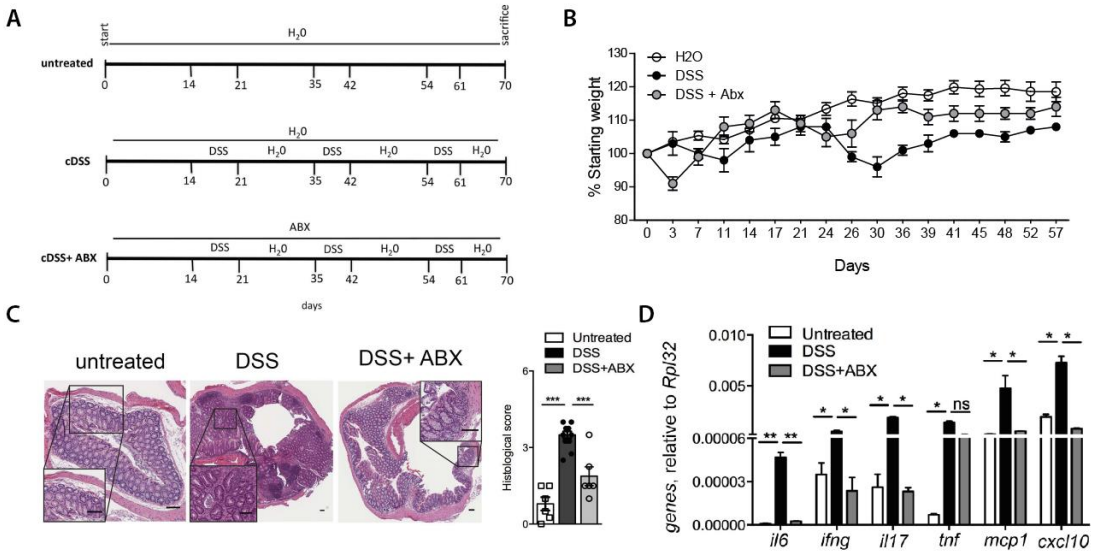

Figure 6.16 Antibiotics driven gut microbiota depletion reduces intestinal inflammation in a chronic model. (A) Representative scheme of the treatment. cDSS, chronic DSS. ABX, mix of ampicillin, neomycin, vancomycin and metronidazole. (B) Weight curve, (C) H&E staining (scalebar 100µm) and cumulative colonic histological score and (D) colonic expression of pro-inflammatory genes in mice untreated (white bars), DSS-treated (dark grey bars) and DSS+ ABX-treated (light grey bars). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.

After having performed a fluorescent *in situ* hybridization of the colonic tissues, we observed that DSS induced a marked bacterial translocation into the intestinal lamina propria, while antibiotics treatment abrogated this effect (***Figure 6.17***).

This result confirmed that in this experimental setting the colocalization, and thus the direct interaction, between gut microbiota and the mucosal immune system is abrogated.

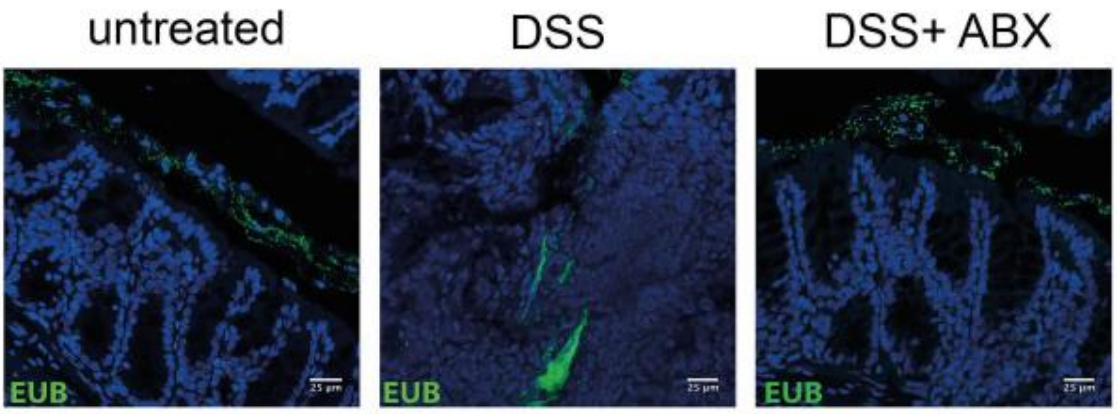

Figure 6.17 Antibiotic treatment reduces bacteria translocating into the lamina propria during intestinal inflammation. Fluorescent in situ hybridization of bacterial DNA detected with with Eubacteria probes (Eub-488, green) on paraffin embedded colonic tissues obtained from untreated, DSS- and DSS+ ABX-treated mice.

DSS + Abx treated mice showed also a strong decrease in colonic expression levels of CXCL16, the chemokine responsible for iNKT cell chemoattraction into the gut, and of its receptor CXCR6 were strongly diminished (***Figure 6.18A***).

To assess whether the lack of bacteria in the lamina propria could impact on the mucosal T cell compartment activation status, we analyzed the lamina propria infiltrate by flowcytometry. As expected, colonic iNKT and $CD4^+$ T cell frequencies (***Figure 6.18B-C***) and absolute numbers (***Figure 6.18B-C***) were decreased in mice treated with DSS in the presence of antibiotics.

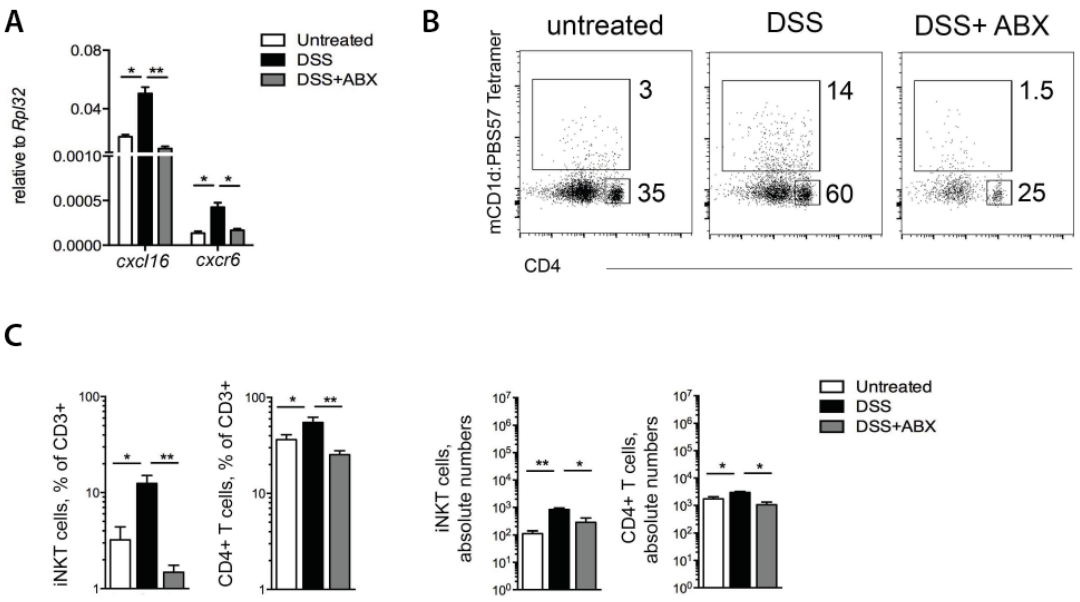

Figure 6.18 Antibiotic treatment reduces CD4⁺ T and iNKT cell accumulation into the lamina propria during intestinal inflammation. (A) *Colonic expression levels of cxcl16 and cxcr6 in mice untreated (white bars), treated with DSS (black bars) or with DSS + ABX (dark grey bars). (B) Representative dot plots (C) frequency and absolute numbers of colonic iNKT cells and CD4⁺ T cells in mice untreated (white bars), treated with DSS (black bars) or with DSS + ABX (dark grey bars). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Most importantly, the reduction of intestinal bacterial translocation into the lamina propria during intestinal inflammation significantly inhibited proliferation and activation, determined through Ki67 and CD69 staining, respectively (***Figure 6.19A***), of both iNKT and CD4⁺ T cells.

Interestingly, also the cytokine profile of iNKT cells, but not the one of CD4⁺ T cells, was affected. The secretion of the pro-inflammatory TNF, IFNγ and IL-17 cytokines were

enhanced upon DSS administration and greatly impaired in the absence of translocating bacteria in the lamina propria (***Figure 6.19B***).

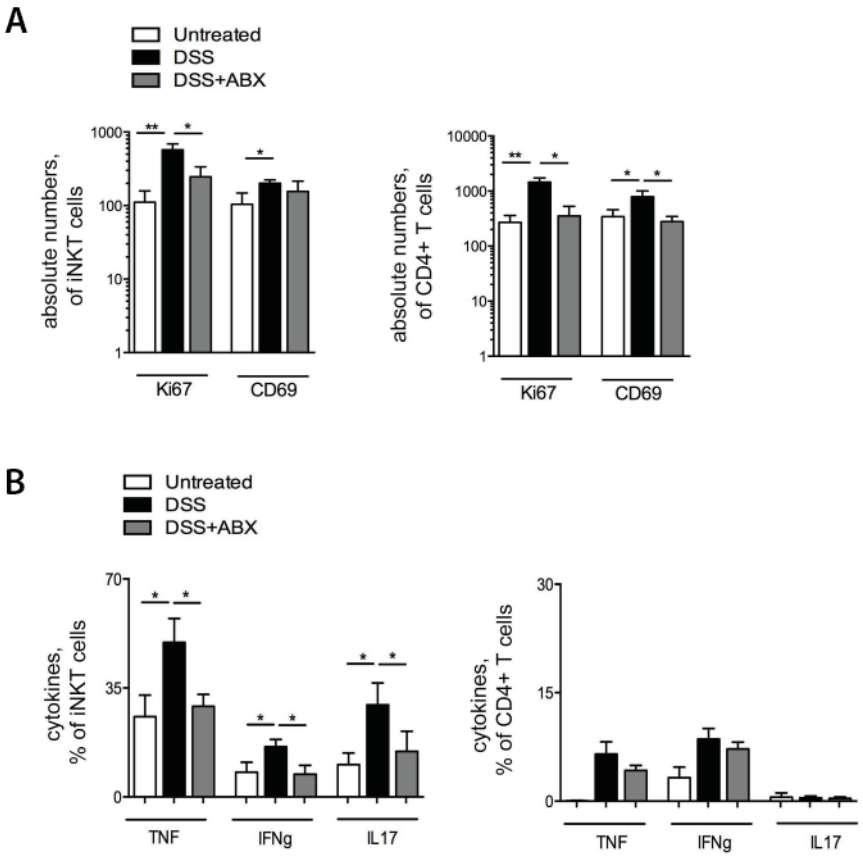

Figure 6.19. Intestinal microbiota elimination reduces pro-inflammatory activation of iNKT cells during experimental chronic colitis. *(A) Absolute numbers of Ki67⁺ and CD69⁺ colonic iNKT cells (left panel) and CD4⁺ T cells (right panels) and (B) frequency of TNF, IFNγ and IL-17 producing iNKT cells (left panels) and CD4⁺ T cells (right panels) in mice untreated (white bars), treated with DSS (Black bars) or with DSS + ABX (dark grey bars). Statistical significance was calculated with Kruskal Wallis test with Dunn's post-test for multiple comparison. P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) were regarded as statistically significant. Outliers were detected with Grubb's test.*

Collectively, these data indicate that exposure to the intestinal microbiota is required for iNKT cell pro-inflammatory activation in the colonic mucosa, and that iNKT and CD4+ T cells manifest *in vivo* similar functional properties during intestinal inflammation.

7 Discussion II

A functional involvement of iNKT cells has been suggested for a wide variety of human autoimmune disorders including multiple sclerosis [193], rheumatic diseases [194] and asthma [195]. Conversely, iNKT cell contribution to IBD pathogenesis is still incompletely understood. By studying human intestinal tissues, we demonstrated that iNKT cells infiltrate the intestinal mucosa of IBD patients (Section 6.1) and that they display a pro-inflammatory phenotype and pathogenic features upon exposure to intestinal mucosa-associated microbiota (Section 6.3). Thus, to our knowledge, this is the first comprehensive report of human intestinal iNKT cells in IBD patients.

Technical difficulties hampered for long time univocal attribution of iNKT cell pathogenic or protective roles in human diseases. Before the advent of the Tetramer technology, iNKT cells could be identified either by the co-expression of NK-related markers (CD56 and/or CD161, the human counterpart of the murine NK1.1) with CD3, by the reaction to CD1d stimulation, or by Vα24 TCR expression. It is believed therefore that old studies on iNKT cell functions most likely included different subsets, such as CD1d-restricted Type2 NKT, MAIT and γδ T cells [196].

In addition, the frequency of circulating and tissue-resident iNKT cells is greatly variable among individuals [19]. The analysis of the LPMC isolated from IBD patients and non-IBD donors confirmed this relevant intra-individual variability also for intestinal iNKT cell frequencies (*Figure 6.1*), that did not correlate to any epidemiologic or clinical parameter.

Functionally different subsets of iNKT cells can be distinguished according to the expression of CD4 [197], whose engagement by CD1d molecules potentiates iNKT cell activation [19]. Human and murine tissue-derived iNKT cells are mostly $CD4^+$ [51], and we here observed that up to 80% of human intestinal iNKT cells express CD4 both in HD and IBD patients (***Figure 6.2***). CD161 expression, instead, has been specifically associated to intestinal tissue distribution of several T cell subsets, including MAIT cells and TCR γδ cells [198]. In our study, intestinal iNKT cells from IBD patients and HD mainly co-expressed CD4 and CD161 and secreted substantial amounts of pro-inflammatory cytokines (***Figure 6.2*** and ***Figure 6.4***). These data are in accordance with previous reports indicating that $CD4^+$ $CD161^+$ intestinal T cell subsets might exert specific pathogenic functions, both in CD [109] and UC [115] patients. Those reports, though, either excluded iNKT cells from the analysis [109] or focused on type II sulfatide-specific NKT subsets [24], [115].

Here we also reported that, both in mice and men, pro-inflammatory cytokine secretion is a key functional attribute of intestinal iNKT cells. Distinct Th subsets in the gut are known to secrete specific Th1 or Th2 cytokines, although context-dependent functional plasticity has been demonstrated for murine and human intestinal Th subsets [35], [181]. Similarly to murine [51] and PB-derived cells [199], in our analyses intestinal iNKT cells possess an intrinsic capability to secrete a broad array of Th1, Th2 and Th17 cytokines. This characteristic, shared with other innate populations, could be an evolutionary conserved functional requirement for cells endowed with patrolling of mucosal surfaces [96].

Healthy and IBD-derived mucosa-associated microbiota were capable to efficiently activate *in vitro* human intestinal iNKT cells, stimulate cytokine secretion and induce pathogenic

functions (***Figure 6.12***). Several evidences suggest a reciprocal influence of iNKT cells and the intestinal gut microbiota [71], [89], [94]. iNKT cells affect murine gut colonization by commensal microorganisms [94] while during early neonatal and postnatal stages of development, commensals bacteria negatively shape iNKT cell repertoire [71]. The commensal *B. fragilis*, known to produce lipid antigens controlling homeostatic iNKT cell proliferation and activation [21] was detected only in two healthy donors and three colon CD-derived samples. Further studies are required to understand if a reduction of *B. fragilis* in IBD patients might correlate with pro-inflammatory functional skewing of human intestinal iNKT cells.

As previously discussed, IBD patients harbor significant variations in the intestinal microbiota composition as compared to non-IBD controls [118]–[122], defined by an overall decrease of α-diversity but also by alterations of microbial taxa relative abundances, i.e. specific increase in *Proteobacteria* (such as adherent invasive *E. coli* and *Enterobacteriaceae* in CD) and decrease in *Firmicutes* (such as *F. prausnitzii*) [125]. These variations were confirmed in our samples (***Figure 6.10***), including the decrease in the butyrate-producing *Roseburia, Blautia* [118] and *Odoribacter* [119] in IBD versus HD donors, as well as the increase of *Erysipelotrichaceae* in UC patients [121].

From a mechanistic point of view, our data suggest that the loss of the barrier integrity might be the critical event exposing iNKT cells to the mucosa-associated microbial ecology and to luminal metabolites. Once in contact with the microbiota, as we showed (***Figure 6.17***), intestinal iNKT cells could be activated through both TCR-dependent and independent mechanisms.

So far, few bacteria-derived glycosphingolipid antigens capable to specifically activate iNKT cells have been identified, including those isolated from the cell wall of Gram-negative *Sphingomonas spp* [200], *Borrelia burgdorferi* [201] and *Mycobacteria* [202]. Conversely, no iNKT-specific lipid antigens have been isolated from adherent invasive *E. coli* and *Salmonella*, two well-known intestinal pathobionts consistently present in our IBD and non-IBD-derived samples and which efficiently stimulated iNKT cell responses partially through CD1d-dependent mechanisms (***Figure 6.14***). Abundant evidences instead exist that iNKT cells can be activated in an innate fashion by microbial products such as LPS [203], either directly through TLR4 binding [204] or after IL-12/IL-18-mediated activation of LPS-stimulated dendritic cells [45]. We speculate that in addition to an innate microbiota-dependent iNKT cell activation, endogenous lipid antigens might be induced or upregulated in bacteria-stimulated moDC, thus explaining the observed antigen-specific activation by iNKT cells.

Upon exposure to the commensal intestinal microbiota, activated intestinal iNKT cells secreted a broad array of cytokines, including TNF and IFNγ, which are known to increase intestinal permeability [96], and that were directly responsible for *in vitro* iNKT cell pathogenic functions. We have recently demonstrated that human Th17 cells isolated from the ileum of CD patients co-secrete pro-inflammatory IFNγ and TNF conferring pathogenic properties against the intestinal epithelium (Appendix III and [35]), suggesting that during intestinal inflammation iNKT cells and conventional $CD4^+$ T cells might manifest a similar behavior.

Differently to conventional Th cells, though, iNKT cells can also behave as innate cells that rapidly and massively respond to the commensal microbiota-driven activation. Abolishment of bacterial translocation by broad-spectrum antibiotic treatment during experimental chronic colitis efficiently blocked iNKT (and conventional CD4+ T cells) activation and cytokine secretion, recapitulating *in vivo* what observed *in vitro* and providing a rationale for possible targeted interventions aimed at containing immune cell responses in IBD patients.

In conclusion, our study sheds novel light on the pathogenic functions of iNKT cells during intestinal inflammation in IBD patients. Moreover, it suggests that a wider knowledge of the human microbiome at the community-level, rather than on single microbial species, can better contribute to the final understanding of the mechanisms of immune modulation. Finally, targeting the mucosa-associated microbiota recognition by iNKT cells might be a promising approach to control their pro-inflammatory activity and intestinal inflammation.

8 Conclusions

With this thesis we provided solid evidence that a strict interaction between the gut microbiota and intestinal conventional and non-conventional T cells is in place. We observed that inflammation-induced and antibiotic-driven dysbiosis affect T and iNKT cell phenotype and functions (Sections 4.1 and 4.2). Moreover, a drastic manipulation of the microbiota community through Faecal Microbiota Transplantation (FMT) results in a modulation of the whole mucosal immune response, encompassing both epithelial cell functions, and innate and adaptive branches of mucosal immunity (Sections 4.3-4.6). Further, we performed a comprehensive analysis on the phenotypic and functional shaping of human iNKT cells mediated by IBD-associated dysbiotic bacterial communities (Chapter 6).

In the past years, pivotal studies revealed the existence of mutual mechanisms of regulation between the intestinal microbiota and iNKT cells [21], [71], [89], [94]. These observations were mainly obtained by taking advantage of murine experimental models completely devoid of commensal microbiota communities, i.e. the Germ free (GF) mice. GF mice proved to be essential in demonstrating causative associations between single-strain bacteria and specific T cell subsets [58], [75], [76], [205] and to gain functional insights into host-microbe interactions in health and disease [62], [127]. Nonetheless, it must be kept in mind that the absence of the microbiota in the early phases of life importantly impacts on the development of a mature host immune system, thus shaping immune functions in the adults [71], [88]. This caveat needs to be taken in consideration when using GF mice to study microbiota-related immune responses. In the study presented in this thesis, we faced

the need to bypass this vicious circle and to find suitable experimental models to assess the effects of gut microbiota manipulation on immune cells that had undergone a normal development and a correct shaping from bacterial components. For these reasons, we decided to adopt antibiotics treatment on mice reared in specific pathogen free (SPF) conditions, being aware that this model, as well, bears some pitfalls. The antibiotics used in our experiments had a wide spectrum of action, but their specificity could not cover the whole microbial population, and antibiotic administration does not affect other non-microbial components of the microbiota, such as viruses and fungi. In addition, in experimental FMT, antibiotics *per se* could negatively influence the engraftment niches of bacteria. Nonetheless, keeping these caveats in mind, we adopted this type of experimental setting to study at least some aspects of microbiota-driven immune response in adults.

In this work we observed the presence of a negative regulation on the intestinal iNKT cell repertoire exerted by the gut microbiota under homeostatic conditions (***Figure 4.6***), confirming what previously shown in GF mice [21], [71]. Several mechanisms have been shown to explain this regulation, i.e. modulation of CXCL16 production by intestinal epithelial cells (IEC) [71], bacterial antigen presentation inhibiting iNKT cell proliferation [21] and expansion of cytotoxic $CD8^+$ T cells [92].

Moreover, we also described a marked expansion of the intestinal iNKT cell population in another context of dysbiosis, i.e. DSS-driven inflammation. The murine model of DSS induced colitis is characterized by epithelial toxicity, disruption of the intestinal physical barriers and massive translocation of luminal dysbiotic bacteria and metabolites in the

lamina propria. These events, in turn, trigger the recruitment and expansion of both innate and adaptive immune cells. Thus, it is not surprising, though nobody explicitly addressed it before, that also iNKT cells accumulate in the lamina propria during this chemically induced inflammation. Many indirect evidences suggested an iNKT cell involvement during DSS-induced inflammation, i.e. αGalCer administration was shown to ameliorate experimental colitis in a CD1d dependent manner [206], and $J\alpha18^{-/-}$ mice were shown to experience a worsening of colitic symptoms that were ameliorated by WT iNKT cell adoptive transfer [207]. In contrast, in another murine model of intestinal inflammation, i.e. the oxazolone induced colitis model, iNKT cells were indicated as the main drivers of inflammation due to a marked Th2 (or NKT2) activity [114], [208]. Here, we observed that during DSS-induced colitis, iNKT cells massively secreted Th1/17 (or NKT1/17) types of cytokines such as IL-17, IFNγ and TNF (***Figure 4.3*** and ***Figure 6.19***). These data perfectly recapitulated what observed in the lamina propria of IBD patients (***Figure 6.4***) and upon *in vitro* stimulation of human iNKT cells with IBD-associated microbiota (***Figure 6.12***).

DSS-induced intestinal inflammation strictly requires the presence of gut microbiota, since both DSS-treated GF mice [192] and antibiotics treated mice (Appendix II and [91]) have been shown to display reduced signs of inflammation. Consistently, when we blocked luminal bacterial translocation administering a broad-spectrum antibiotic mix in combination to chronic (***Figure 6.17***) or acute (Appendix II and [91]) DSS treatment, we also observed an overall reduction in both macroscopic tissue inflammation (histopathology and pro-inflammatory gene expression, ***Figure 6.16***) and T and iNKT cell accumulation in the intestinal lamina propria (***Figure 6.18***). Importantly, also iNKT cell pro-inflammatory activity was significantly decreased (***Figure 6.19***). Altogether these findings indicate that

the pathological breach of epithelial barrier and the following entrance of luminal bacteria are additional triggers of intestinal iNKT cell expansion and activation. These results also suggest that microbiota depletion during pathologic conditions might not lead to iNKT cell pool expansion as it happens during intestinal homeostasis. One possible explanation for these findings is that the cell types involved in microbiota-associated iNKT cell regulation (i.e. epithelial cells, antigen presenting cells and/or other T cell subsets) lose their full homeostatic activity and fail in regulating iNKT cell repertoire during pathologic conditions.

Conversely, when antibiotic-treated mucus-associated and luminal bacteria were transplanted in mice after DSS administration, intestinal iNKT cell population boomed, becoming significantly more abundant than upon DSS administration alone (***Figure 4.28***). We believe that the key to provide a possible explanation for these apparently conflicting results is to take into consideration the chronological timing of the events in the different experimental settings. DSS-induced inflammation, antibiotic administration and reconstitution of the gut microbiota by FMT generate distinct and independent microbiota-associated functional events that, if occurring with a precise timing, can work in synergism and lead to intestinal iNKT cell expansion and/or recruitment.

In the attempt to reconcile the observations obtained in the different experimental conditions, we here propose a model (Figure 8.1) describing the events ("triggers") occurring in the intestinal mucosa and involving epithelial cells, gut microbiota and iNKT cells in homeostatic and inflammatory conditions.

During DSS-induced inflammation, the highly destabilizing conditions of epithelial breach and the following microbiota entry induce iNKT cell expansion in a very short time (trigger

1). Upon antibiotic treatment, instead, reduction of the luminal bacterial load and α-diversity occurs, likely triggering the already described microbiota-associated release of the negative regulation of iNKT cell pool (trigger 2) [21], [71]. After recolonization with a normal microbiota achieved through FMT, normal iNKT cell levels are restored. Consistently, when colitis is induced during broad spectrum antibiotic treatment, even if the DSS has caused epithelial breach, the absence of bacteria impedes the induction of the events associated to trigger 1. Conversely, when the DSS-induced microbial translocation into the lamina propria is sequentially followed by the transplant of an antibiotic-treated donor microbiota with low α-diversity and bacterial load, trigger 1 and trigger 2 add together and induce a boom in iNKT cell pool.

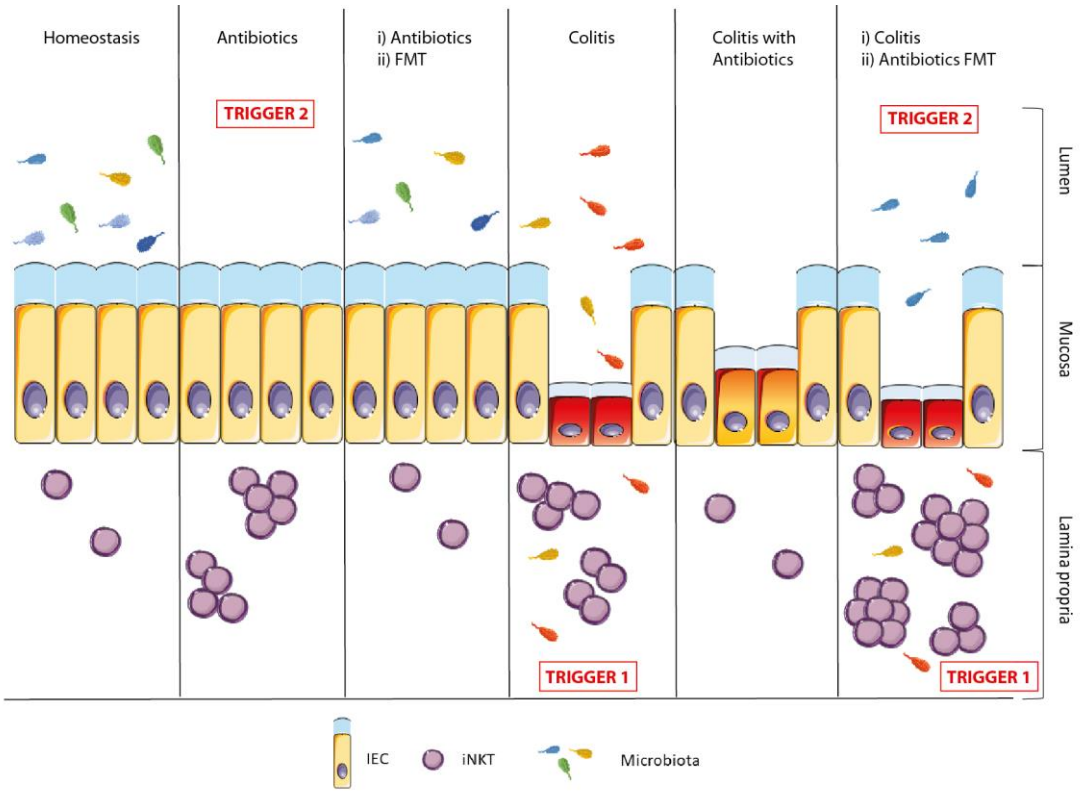

Figure 8.1 Schematic representation of intestinal iNKT cell regulation by gut microbiota in health and disease

In order to prove the validity of this model further experiments are needed. It would be interesting to understand whether the regulation of intestinal iNKT cells would be affected by a switch in the timing between trigger 1 and trigger 2 occurrence. Indeed, we plan to treat mice with antibiotics mix or with the transplant of antibiotic-treated mucus-associated and luminal bacteria, prior to the induction of colitis with DSS. In this way we believe we will be able firstly to trigger iNKT cell expansion, thanks to the release of microbiota dependent-iNKT cell inhibition, and then induce epithelial disruption and translocation of the remaining bacteria into the lamina propria. We will likely have to wait for a period of rest between the two treatments to allow a bacterial recolonization sufficient to trigger colitis without restoring a complete inhibition on iNKT cells.

This experiment will give us additional information on microbiota-iNKT crosstalk, hinting also at the possibility to translate this knowledge into clinics. Knowing that iNKT cells have been shown to drive IBD pathogenesis in patients and in murine models [24], [209] and that they can importantly contribute to intestinal inflammation through epithelial toxicity (Chapter 6), it would be relevant to shed light on their mechanisms of regulation during both intestinal homeostasis and disease.

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Appendix I

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Original Research Article

Implementation of an automated inclusion system for the histological analysis of murine tissue samples: A feasibility study in DSS-induced chronic colitis

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Fulvia Milena Cribiù,[1] Claudia Burrello,[2] Giulia Ercoli,[1] Federica Garavaglia,[2] Vincenzo Villanacci,[3] Flavio Caprioli,[4,5] Silvano Bosari[1] and Federica Facciotti[2]

Abstract

Animal models are powerful tools to expand our understanding of human diseases. Histopathological evaluation of murine experimental models is often required to support further research; thus, a more rigorous evaluation of murine histological samples is strongly advocated. Indeed, the overall quality of tissue sections is critical to draw reliable and accurate conclusions. As several methodological variables may reduce the reliability of the pathological analysis, a standardization of the procedural steps required for the processing of histological murine tissues is advisable. Here, we describe a method to standardize the technical procedure from the initial preparation to the paraffin embedding of murine samples. Specifically, we have implemented an automated inclusion system, that is, the SAKURA Tissue-Tek inclusion instrument, which is routinely used for paraffin inclusion of human samples, to process murine specimens of intestinal inflammation. Colitis severity was assessed in chronically Dextran Sodium Sulphate (DSS)–treated mice by cytofluorimetric analysis of colonic cellular infiltrates, expression of inflammatory genes and histopathological analysis of tissue samples, comparing manual and automated tissue preparation systems. We here conclude that implementation of this technique can significantly increase the quality and the reliability of histopathological examination of murine tissues.

Keywords

automation, chronic colitis, histopathological analysis, murine models

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Introduction

In the last decades, animal models have been increasingly utilized as a tool to expand our understanding of the pathogenesis of human diseases.[1,2] During the experimental process, researchers are often faced with the need to perform detailed histopathological analyses to assess the severity of a disease, the effect of a treatment, or to demonstrate, in a specific experimental setting, a variation in the architecture of a tissue or of its infiltration by immune cells.[3]

The increasing complexity of translational research often implies the concomitant evaluation of human and murine samples.[4] A detailed histopathological

[1]Pathology Unit, Fondazione IRCCS Cà Granda, Ospedale Policlinico di Milano, Milan, Italy
[2]Department of Experimental Oncology, European Institute of Oncology (IEO), Milan, Italy
[3]Pediatrics Clinic, Department of Clinical and Experimental Sciences, Spedali Civili, University of Brescia, Brescia, Italy
[4]Gastroenterology and Endoscopy Unit, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milan, Italy
[5]Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy

Corresponding author:
Claudia Burrello, Department of Experimental Oncology, European Institute of Oncology (IEO), Via Adamello 16, 20139 Milan, Italy.
Email: Claudia.burrello@ieo.it

Appendix II

frontiers in Medicine

ORIGINAL RESEARCH
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Short-term Oral Antibiotics Treatment Promotes Inflammatory Activation of Colonic Invariant Natural Killer T and Conventional CD4+ T Cells

*Claudia Burrello[1,2], Federica Garavaglia[2], Fulvia Milena Cribiù[3], Giulia Ercoli[3], Silvano Bosari[3], Flavio Caprioli[4,5] and Federica Facciotti[2]**

[1] Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milan, Italy, [2] Department of Experimental Oncology, European Institute of Oncology, Milan, Italy, [3] Pathology Unit, Fondazione IRCCS Cà Granda, Ospedale Policlinico di Milano, Milan, Italy, [4] Gastroenterology and Endoscopy Unit, Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milan, Italy, [5] Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy

The gut mucosa is continuously exposed to a vast community of microorganisms, collectively defined as microbiota, establishing a mutualistic relationship with the host and contributing to shape the immune system. Gut microbiota is acquired at birth, and its composition is relatively stable during the entire adult life. Intestinal dysbiosis, defined as a microbial imbalance of gut bacterial communities, can be caused by several factors, including bacterial infections and antibiotic use, and has been associated with an increased risk to develop or exacerbate immune-mediated pathologies, such as allergic reactions, asthma, and inflammatory bowel diseases. Still, the mechanisms by which antibiotic-induced gut dysbiosis may lead to development of mucosal inflammation are still matter of debate. To this end, we aimed to evaluate the impact of antibiotic treatment on phenotype and functions of intestinal immune cell populations, including invariant natural killer T (iNKT) cells, a subset of lipid-specific T cells profoundly influenced by alterations on the commensal microbiota. To this aim, a cocktail of broad-spectrum antibiotics was administered for 2 weeks to otherwise healthy mice before re-colonization of the intestinal microbial community with oral gavage of eubiotic or dysbiotic mucosa-associated bacteria and luminal colonic content, followed or not by intestinal inflammation induction. Here. we showed that short-term antibiotic treatment alters frequency and functions of intestinal iNKT cells, even in the absence of intestinal inflammation. The presence of a dysbiotic microbiota after antibiotic treatment imprints colonic iNKT and CD4+ T cells toward a pro-inflammatory phenotype that collectively contributes to aggravate intestinal inflammation. Nonetheless, the inflammatory potential of the dysbiotic microbiota decreases over time opening the possibility to temporally intervene on the microbial composition to re-equilibrate dysbiosis, thus controlling concomitantly mucosal immune T cell activations.

Keywords: iNKT cells, antibiotics, microbiota, T cells, intestinal inflammation

Abbreviations: iNKT, invariant natural killer T cells; GF, germ free; ABX, antibiotics; Th, T helper; IBD, inflammatory bowel diseases; FMT, fecal microbiota transplantation.

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****Correspondence:***
Federica Facciotti
federica.facciotti@ieo.it

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Appendix III

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Original Article

Original Article

Pathogenicity of *In Vivo* Generated Intestinal Th17 Lymphocytes is IFNγ Dependent

Giulia Nizzoli[a,b,*], Claudia Burrello[c,d,*], Fulvia Milena Cribiù[e], Giulia Lovati[d], Giulia Ercoli[e], Fiorenzo Botti[b,f], Elena Trombetta[g], Laura Porretti[g], Katia Todoerti[h], Antonino Neri[c,i], Maria Rita Giuffrè[b], Jens Geginat[j], Maurizio Vecchi[a,k], Maria Rescigno[d], Moira Paroni[l,#], Flavio Caprioli[a,b,#], Federica Facciotti[d,#]

[a]Gastroenterology and Endoscopy Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy [b]Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy [c]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy [d]Department of Experimental Oncology, European Institute of Oncology, Milan, Italy [e]Pathology Unit, Fondazione IRCCS Ca' Granda Ospedale Policlinico di Milano, Milan, Italy [f]General and Emergency Surgery Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy [g]Flow Cytometry Service, Clinical Chemistry and Microbiology Laboratory Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy [h]Laboratory of Pre-clinical and Translational Research, IRCCS-CROB, Referral Cancer Center of Basilicata, Rionero in Vulture, Italy [i]Hematology Unit, Fondazione IRCCS Ca ' Granda, Ospedale Maggiore Policlinico, Milan, Italy [j]INGM – National Institute of Molecular Genetics "Romeo ed Enrico Invernizzi" Milan, Italy [k]Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy [l]Department of Biosciences, Università degli Studi di Milano, Milan, Italy

Corresponding author: Flavio Caprioli, MD PhD, Department of Pathophysiology and Transplantation, University of Milan, Via Francesco Sforza, 35, 20122 Milan, Italy. Tel: +39-02-5503-3368; fax: +39-02-5503-2140; email: flavio.caprioli@unimi.it

[#]GN and CB contributed equally to this paper.

[*]FF, MP, and FC contributed equally to this paper.

Abstract

Background and aims: T helper 17 [Th17] cells are crucially involved in the immunopathogenesis of inflammatory bowel diseases in humans. Nevertheless, pharmacological blockade of interleukin 17A [IL17A], the Th17 signature cytokine, yielded negative results in patients with Crohn's disease [CD], and attempts to elucidate the determinants of Th17 cells' pathogenicity in the gut have so far proved unsuccessful. Here, we aimed to identify and functionally validate the pathogenic determinants of intestinal IL-17–producing T cells.

Methods: *In vivo*–generated murine intestinal IL-17–producing T cells were adoptively transferred into immunodeficient *Rag1*$^{-/-}$ recipients to test their pathogenicity. Human IL-17, IFNγ/IL-17, and IFNγ actively secreting T cell clones were generated from lamina propria lymphocytes of CD patients. The pathogenic activity of intestinal IL-17–producing T cells against the intestinal epithelium was evaluated.

Results: IL-17–producing cells with variable colitogenic activity can be generated *in vivo* using different experimental colitis models. The pathogenicity of IL-17–secreting cells was directly dependent on their IFNγ secretion capacity, as demonstrated by the reduced colitogenic activity of IL-17–secreting cells isolated from *IFNγ*$^{-/-}$ mice. Moreover, IFNγ production is a distinguished attribute of CD-derived lamina propria Th17 cells. IFNγ secretion by CD-derived IL-17–producing

Abbreviations: IBD, Inflammatory bowel disease; CD, Crohn's Disease.

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