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MASTER'S THESIS

Impact of gut metabolites and antibiotics on immune responses

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Impact of gut metabolites and antibiotics on immune responses

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DECLARATION

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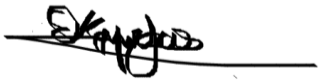

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ΠΕΡΙΛΗΨΗ

Το εντερικό μικροβίωμα - το σύνολο δηλαδή των συμβιοτικών μικροοργανισμών οι οποίοι διαβιούν στο έντερο- επηρεάζει με πολλαπλούς τρόπους το ανοσοποιητικό σύστημα του ξενιστή. Τα αντιβιοτικά, τα οποία είναι ευρέως χρησιμοποιούμενα στην κλινική πράξη, μπορούν να επηρεάσουν την επιβίωση και τον πολλαπλασιασμό των βακτηρίων του εντέρου, καθώς και τους μεταβολίτες που παράγονται από αυτά. Είναι ήδη γνωστό πως συγκεκριμένα βακτήρια μπορούν να επιδράσουν τόσο στην ανάπτυξη, όσο και στη διαφοροποίηση των Τ κυττάρων. Η παρούσα πειραματική διπλωματική εργασία διερευνά πώς κάποια αντιβιοτικά και βακτηριακοί μεταβολίτες –και πιο συγκεκριμένα λιπαρά οξέα βραχέας αλύσου- θα μπορούσαν να επηρεάσουν την αντιγονοπαρουσίαση και την ενεργοποίηση των Τ κυττάρων. Μέσω πολλαπλών τεχνικών ανάλυσης της αντιγονοπαρουσίασης στα CD8+ Τ κύτταρα, αυτή η εργασία έδειξε πως διαφορετικοί μεταβολίτες μπορούν να επηρεάσουν με διακριτό τρόπο την ανοσολογική απάντηση, καθώς κάποιοι μπορούν να την ενισχύσουν, ενώ άλλοι να την αναστείλουν. Με αυτή την εργασία προτείνεται επίσης πως είναι πιθανό να ενεργοποιούνται διαφορετικά μονοπάτια μεταγωγής σήματος με διαμεσολάβηση κυτταροκινών, ανάλογα με το είδος του μεταβολίτη, τη συγκέντρωση, το χρονικό σημείο, αλλά και τη διάρκεια έκθεσης των αντιγονοπαρουσιαστικών κυττάρων σε αυτόν. Μεταξύ των μεταβολιτών που εξετάστηκαν, το προπιονικό οξύ φάνηκε να έχει ανασταλτική δράση και ο μηχανισμός για αυτό είναι πιθανό να σχετίζεται με την αντιγονική επεξεργασία, ίσως μέσω υπερέκφρασης του γονιδίου της ιντερφερόνης α. Το βουτυρικό οξύ ίσως να επηρεάζει την έκφραση του γονιδίου του μείζονος συμπλέγματος ιστοσυμβατότητας τάξης ΙΙ. Αντιβιοτικά που εξετάσαμε δεν έχουν άμεση επίδραση στην ενεργοποίηση των Τ κυττάρων, *in vitro* και *in vivo*. Στο πλαίσιο διερεύνησης της πολύπλοκης αλληλεπίδραση του εντερικού μικροβιώματος με το ανοσοποιητικό σύστημα, προτείνουμε πως προσεκτικές ρυθμίσεις στους εντερικούς μεταβολίτες, μπορούν να διαφοροποιήσουν σημαντικά το αποτέλεσμα πολλών κλινικών θεραπειών.

Λέξεις – κλειδιά : εντερικό μικροβίωμα , ανοσοποιητικό σύστημα, μεταβολίτες, αντιγονοπαρουσίαση, ενεργοποίηση Τ κυττάρων

ABSTRACT

Gut microbiota –the assembly of commensal intestinal microorganisms - impacts the host immune system in several ways. Antibiotics -widely used therapeutic agents- are able to modulate the survival and the growth of gut bacteria, as well as the metabolites they produce. Some gut bacteria are already known to affect the T cell development and differentiation. This research thesis investigates the impact that certain antibiotics and metabolites (short chain fatty acids) could have on antigen presentation and T cell activation. Through series of assays evaluating the antigen presentation to CD8+ T cells, this thesis has shown that different metabolites have distinct effects, some are able to boost and others to inhibit the immune responses. We suggest that different metabolites may act through distinct signaling and cytokine transduction pathways. Factors like the concentrations, the timing and the duration of exposure of the APCs to those treatments, together with the type of the metabolite, might hold a role in the activation of different pathways as well. Among the metabolites we tested, propionate was shown to have inhibitory effects on antigen presentation, and the mechanism behind this is probably related to the antigen processing, through upregulation of the IFNα gene. Butyrate might be regulating the expression of the *MHC II* gene. Antibiotics were found to have no direct impact on T cell activation *in vitro* or *in vivo*. Even though the complexity of the gut microbiota impact on the immune system still needs further studies, we overall suggest that fine regulation of the gut metabolites can largely affect the clinical outcomes of multiple therapies.

Key words: gut microbiome; immune system; metabolites; antigen presentation; T cell activation

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
ACT	Adoptive Cellular Therapy
BATF3	Basic leucine zipper ATF-like transcription factor 3
BMDC	Bone marrow derived cells
C3	Propionate
C4	Butyrate
C5	Pentanoate
CD	Cluster of Differentiation
CP	Cyclophosphamide
CTLs	Cytotoxic T cells
DAMPs	Damage –associated molecular patterns
DC	Dendritic cells
ELISPOT	Enzyme-Linked Immunoabsorbent spot
FMT	Fecal Microbiota Transfer
GM-CSF	Granulocyte – macrophage colony stimulating factor
GF	Germ free
GPR	G- protein coupled receptor
HDAC	Histone deacetylase
HeLa	Henrietta Lacks
IBD	Inflammatory Bowel Disease
ICS	Intracellular staining
IEC	Intestinal Epithelial cells
IFNα	Interferon α
IFNβ	Interferon β
IFNγ	Interferon γ
IgA	Immunoglobin A
IgM	Immunoglobin M
IL-4	Interleukin-4
IL-12	Interleukin-12
IV	Intravenuous
LPS	Lipopolysaccharide
mAb	monoclonal Antibody
MIC	Minimum Inhibitory Concentration
MHC (I/II)	Major Histocompatibility Complex I/II
MTZ	Metronidazole
OVA	Ovalbumin
PAMPs	Pathogen –associated molecular patterns
PD-1	Programmed death-1
PD-L1	Programmed death- Ligand 1
PRR	Pattern Recognition Receptor
SCFAs	Short Chain Fatty Acids
SF	Stimulatory factor
SPF	Specific Pathogen-Free
TCR	T cell receptor
Th	helper T cells
TLR	Toll-like receptor
TME	Tumor microenvironment
T reg	T regulatory cells

TNFα	Tumor Necrosis Factor α
Utd/Ust	Untreated/ Unstained
VPA	Valproic acid (Pentanoic acid)

Chapter 1

Introduction

1.1 The immune system

The term immune system refers to all organs, cells and molecules which work coordinately to defend against foreign microorganisms and substances, building the immune response. This response is mediated by the collaboration of the innate and adaptive immune systems. In general, innate immunity provides the immediate mechanisms of defense after each encounter of the human body with a potent pathogenic intruder and typically does not depend on previous exposure to specific molecules or pathogens. On the contrary, adaptive immunity is characterized by specificity and memory. Specificity is the ability to distinguish different substances while memory refers to the more vigorous responses caused after repeated exposures to the same pathogen. (A. K. Abbas et al., 2022c, 2022a). However, a newly described form of adaptation of innate immunity (trained immunity or innate immune memory) has been also shown to improve the host's defense after successive exposures. Immune memory in innate and adaptive immunity is considered to have evolved by developing a more robust immune response first, that is mediated by epigenetic mechanisms, while specificity evolved later through gene recombination (Divangahi et al., 2021).

1.1.1 Antibodies and antigens

Adaptive immunity is named by its ability to respond specifically to the presence of infectious factors. The two major cell populations of adaptive immunity are the B and T lymphocytes. The antibodies, which are the secreted products of the B lymphocytes, also hold a key role in the development of the immune response. Molecules which can cause specific responses by binding on lymphocytes or antibodies (through T cell or B cell receptors) are called antigens.

1.1.2 Antigen presentation and MHC

The innate immune system consists of numerous components. Physical and chemical barriers, such as the skin, the normal body flora antigens, hair and multiple secreting substances, like enzymes, constitute the first line of defense. In addition to those components, major responses of the innate immune system are the elimination of the reservoirs of viral infections

through changes in cells (antiviral defense) and the process of inflammation, recruiting several types of cells, such as phagocytic cells, dendritic cells, natural killer, mast cells, among other innate lymphoid cells, but also proteins like the complement. Innate immunity is stimulated by limited molecular structures. The microbial structures, shared between microbes, are called pathogen – associated molecular patterns (PAMPs) and the endogenous molecules, derived by damaged or dying cells are called damage- associated molecular patterns (DAMPs).

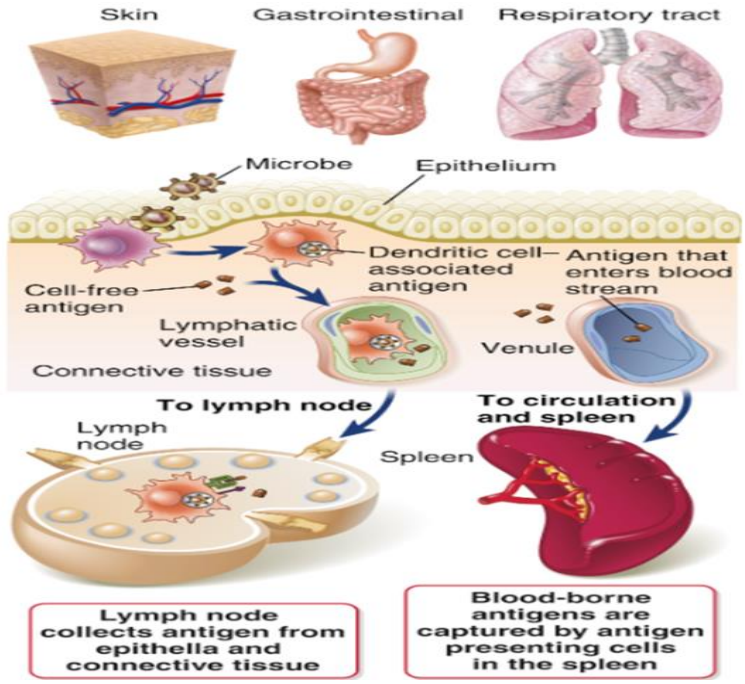


Figure 1.1 Routes of antigen entry and antigen recognition
Dendritic cells and other APCs capture and transfer antigens after they surpass the physical and chemical barriers in the most common entry sites. (A. K., Abbas et al, 2022).

Dendritic cells are unique cells because of their ability to connect the innate and adaptive immune system, transferring information from the former to the latter (Palucka & Banchereau, 1999). Being strategically located in tissues, they are able to respond rapidly to invading microorganisms or damaged cells. They express variable Toll-like receptors (TLRs) and other pattern recognition receptors, which can effectively recognize Pathogen-Associated and Damage-associated Molecular Patterns (PAMPs and DAMPs respectively). Following the

capture of antigens, DCs, along with some other antigen presenting cells (APCs), get activated and transfer the antigens to the lymph nodes or the spleen (Figure 1.1.). There they are able to present the antigens to naïve T cells, leading to the activation and differentiation of lymphocytes. Specialized proteins expressed on the surfaces of host cells, the Major Histocompatibility Complexes (MHCs), bind peptide fragments derived from pathogens, for recognition by T cells. All nucleated cells express MHC I molecules which display antigens to CD8+ cytotoxic T cells (CTLs), whereas the MHC II molecules are only expressed by professional APCs and present antigens to CD4+ helper T (Th) lymphocytes. DCs are essential for the bridging of innate and adaptive immunity because of their capability to promote T lymphocyte responses, after the initial activation of the immune system. After the recognition of the MHC I-antigenic peptides complexes on the target cells and the synapsis formation, CTLs cause lysis of infected cells which express the same class I MHC-associated antigen that triggered the proliferation and differentiation of the naïve CD8+ T cells they derive from. Apoptosis of the target cells is caused by the delivery of cytotoxic proteins, previously stored in cytoplasmic granules, to the target cells. The major cytotoxic proteins in the granules of CTLs (and NK cells) are granzymes and perforin. Simultaneously, CD8+ T cells also secrete IFNγ, another process which is considered to contribute to the phagocytic clearance of ingested microbes. IFNγ is also secreted by CD4+ Th cells after the recognition of the antigenic peptide and MHC II complexes. (A. K., Abbas et al., 2022)

1.1.3 T lymphocytes

T lymphocytes are the mediators of cellular immunity and they consist of subsets with distinct phenotypes and functions. The major subsets that contribute to the immune–mediated cell death are the CD4+ helper T cells and the CD8+ CTLs, while a third subset, the CD4+ regulatory T cells (T regs) control the immune responses. Naïve T lymphocytes (emerging from the thymus) are found in the peripheral lymphoid organs and circulation, where they can be activated by antigens to proliferate and differentiate into effector and memory cells. An antigen is the essential first signal for the activation of naïve T cells and ensures the specificity of the response. DCs and other cellular components of innate immunity are able to sense PAMPs and DAMPs through pattern recognition receptors (PRRs). T cell proliferation and their differentiation into effector and memory cells require also a second signal, provided by molecules on APCs (costimulators) and cytokines, in order to ensure that the adaptive

immune responses will be induced only against dangerous antigens. The co-stimulatory interactions take place at the time of the antigen presentation process. Some co-stimulatory interactions, such as CD28/B7, have a vital role in the T cell activation, while certain other co-stimulatory interactions, such as the CTLA-4/B7 or the PD-1/PD-L1 provide signals for inhibition of T cell activation. In the presence of co-stimulation, T cells recognize antigen–MHC complexes through T cell receptors (TCR), resulting in cytokine secretion and T cell expansion. Upon activation, helper T cells activate cells of the innate immune system to kill phagocytized microbes. CTLs directly kill infected cells after the formation of a peptide-MHC complex recognized by their TCRs (A. K. Abbas et al., 2022b). In the absence of costimulators, T cells might fail to respond to antigens, die by apoptosis or enter a state of unresponsiveness.

As far as DCs are concerned, the absence of the secondary stimuli results in immature DCs behaving like tolerogenic bystanders (Isaacs et al., 2018). The tolerance against the self-antigens can be lost and then the immune system becomes responsive to self-antigens and tumors.

1.2 The gut microbiome

The term human microbiome describes the total of microorganisms – bacteria, fungi, archaea, viruses, along with their genes and products – which live in and on the human body. A "reference human" contains on average about 30 trillion human cells and 39 trillion bacteria (Abbott, 2016) and the gene repertoire present in these microbes is 100-fold higher than the number of genes present in the human genome (Nogal et al., 2021).The highest number of microbes has been found to colonize the gut which has been extensively studied and proved to be one of the key elements contributing to the regulation of host health (de Vos et al., 2022). The gut microbiome has been proven essential for the homeostasis and the regulation of the immune system, through local interactions and indirect long-range systemic effects (Uribe-Herranz et al., 2020).

1.2.1 Gut microbiome and immune system

The gastrointestinal (GI) system, a tube – like structure, consisting of a continuous epithelial cell layer on top of a basic membrane, is one of the physical barriers of the human body to the external environment (Figure 1.2). There are numerous types of intestinal epithelial cells (IECs), the goblet cells that secrete mucus, the M cells which sample antigens and the Paneth cells which secrete antimicrobial peptides (AMPs) and are located at the bottom of the crypts. All the above-mentioned cells derive from a common precursor in the crypts of the intestinal glands. The GI barrier though does not consist only of the epithelial layer and its cells. Right underneath the epithelial layer there is the lamina propria, a loose connective tissue layer which contains blood and lymphatic vessels and mucosa–associated lymphoid tissue (MALTs), the submucosa, a dense connective tissue layer and the layers of smooth muscle. There are also organized gut-associated lymphoid tissues (GALTs), such as the Peyer's patches, and various immune cells including APCs, DCs, T and B lymphocytes. Draining mesenteric lymph nodes (mLNs) lie in the mesentery of the small bowel and the colon (A. K. Abbas et al., 2022d; Gopalakrishnan et al., 2018).

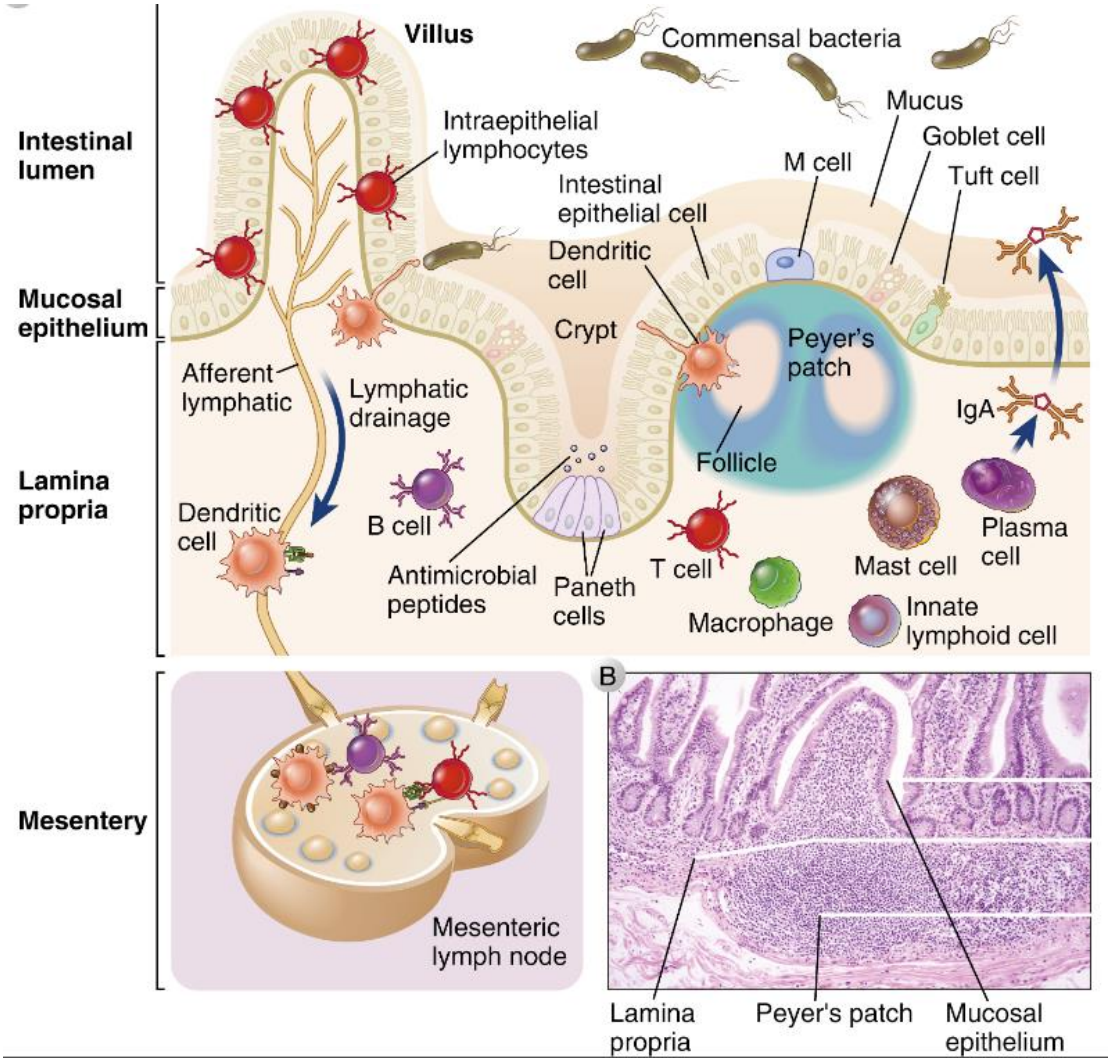


Figure 1.2 The gastrointestinal immune system
Physical and cellular components of the immune system in the intestine. The epithelial barrier is presented covered by secreted mucus, while DCs and M cells sample antigens. Several innate cells and lymphocytes, organized MALTs, such as Peyer's patches, draining mesenteric lymph nodes, and plasma cells that secrete IgA, also depicted. DCs, dendritic cells; MALT, mucosa-associated lymphoid tissue (A. K. Abbas et al., 2022d)

1.2.1.1 Local immunity

The intestinal bacteria are involved in maintaining host homeostasis and they do so by modulating innate and adaptive immune responses, both locally and systemically. The gut has three major mechanisms to prevent infections: a) the presence of the mucus layer that keeps the most organisms away of the epithelium, b) antibiotic peptides which are produced by IECs and are able to kill or restrict pathogens in the lumen and, c) the IgA that is produced by plasma cells in the lamina propria and transferred into the lumen where it is able to neutralize pathogens before they reach the epithelial barrier. IgA class switching, the process through

which B cells switch from IgM production to IgA expression, occurs via both T-cell-dependent and T-cell-independent pathways. Notably, the IgA antibody targets both pathogenic and commensal microorganisms.

To maintain a balance between defense against intestinal pathogens and tolerance to commensals and food antigens, local immunity is promoted when IECs and immune cells like DCs, recognize pathogen associated molecular patterns (PAMPs) through their pattern recognition receptors (eg. Toll-like receptors, TLRs) (A. K. Abbas et al., 2022d; Gopalakrishnan et al., 2018). Subsequently, DCs migrate to mLNs where they interact with and stimulate naïve T cells to differentiate into CD4+ T regulatory cells (T regs) and T helper 17 cells (Th17) (Bekiaris et al., 2014). Educated T reg cells play multiple roles in gut homeostasis. They provide tolerance against commensal bacteria and food antigens, they protect the host from possible unnecessary inflammation by producing the immunosuppressive cytokine IL-10 (Gopalakrishnan et al., 2018) and it is also known that they can suppress and limit antitumor immunity by targeting tumor specific effector T cells (Curiel, 2007). The presence of effector Th17 cells in the lamina propria is also essential, as they ensure protection against bacterial and fungi infections, while they produce cytokines, such as IL-17 and IL-22, which stimulate IECs to form tighter junctions and to secrete AMPs (Weaver et al., 2013).

Besides PAMPs, metabolites produced by the gut microbiota such as short chain fatty acids (SCFAs) are able to affect local immunity. They act as major energy source for IECs (Chilakapati et al., 2020), they can modulate T reg responses (Smith et al., 2013), activate DCs and macrophages (Trompette et al., 2014), modulate cytokine secretion (Iraporda et al., 2015)and affect B Cell IgA class switching (White et al., 2014).

1.2.1.2 Gut microbiome and systemic Immunity

Systemic immunity can be modulated by the gut microbiota in various ways. Distant innate immune cells are able to recognize bacterial nucleic acids, peptidoglycans, flagellins, lipopolysaccharide and other PAMPs when they have entered the circulation (Chilakapati et al., 2020). DCs, after being primed by commensal microbes, are able to enter the systemic circulation and to migrate into distant lymph nodes. B or T cells primed by intestinal DCs can also circulate systemically at distant sites and induce immune responses against the same or similar (cross-reaction) antigens (Gopalakrishnan et al., 2018).

1.2.2 Dysbiosis

The sheer enormity of the microbial biomass in the human intestinal tract, the co-evolution between humans and the microbiota, and the established function of the gut microbes regulates normal host physiologic functions. Germ-free (GF) mice that lack gut microbiota suffer from immune defects, including absent mucus layer, smaller Peyer's patches, decreased IgA secretion and smaller mLNs, which can cause further damage of the intestinal barrier (Fessler et al., 2019). Alterations on the host microbiome homeostasis can create a less diverse, less stable and potentially enriched with opportunistic pathogenic bacteria gut microbiome, a state called dysbiosis. Several diseases, such as inflammatory bowel disease (IBD), neurologic diseases, asthma, obesity, diabetes and cancer have been associated to dysbiosis (Levy et al., 2017). Improved characterization of metabolites and other microbial effectors, coupled with computational pathway analyses, takes us further than the "one microbe, one response" approach and offers the opportunity for optimized therapeutic combinations in order to shape appropriate immune responses.

1.2.3 Antibiotics

The term antibiotic means "against life". Any substance that can kill microbes is technically an antibiotic, but the term is broadly used to refer to the medicines which fight bacterial infections in people and animals. They do so either by killing them or by not allowing them to grow and multiply fast (Figure 1.3). Antibiotics can be administered orally, topically or through either intravenous or intramuscular injections. Antibiotic treatments can save lives by fighting local as well as systemic disease, but they also have side effects, while their overuse has led to resistance because of the bacterial adaptation to them.

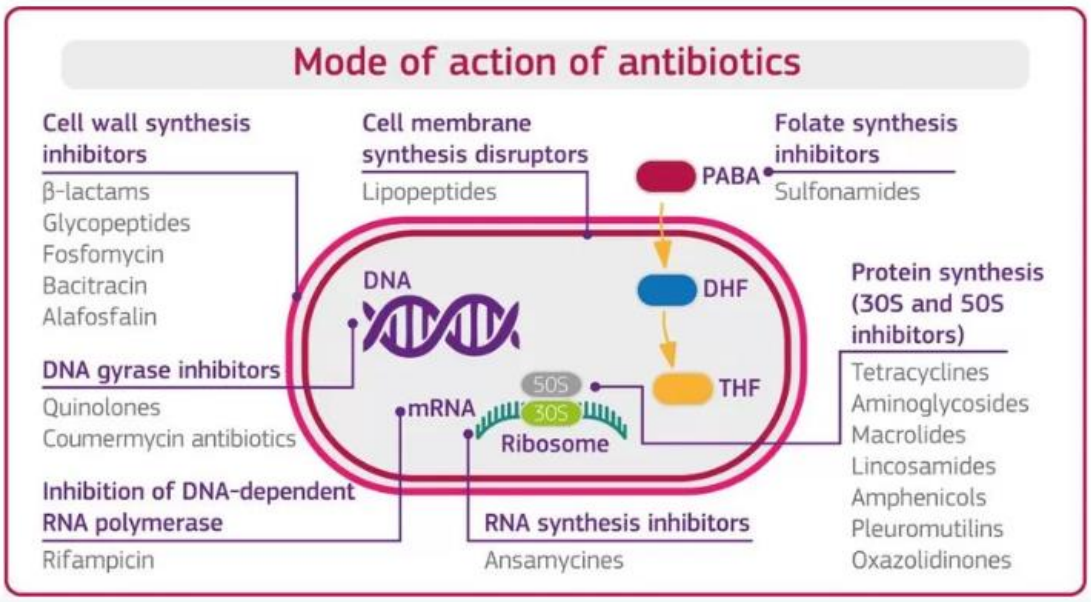


Figure 1.3 Classes of antibiotics and their modes of actions
Main classes of antibiotics and their mechanisms of action (Lettieri et al., JRC Technical Reports, 2018)

Antibiotics affect bacterial survival and growth and thus they have a great impact on the gut microbiome. They cause changes on the bacteria species which can consequently affect the levels of their products, such as the short chain fatty acids and other metabolites. Certain bacterial species can drive different immune responses, by favoring some immune cell types or changing their balance. For example, bacteria of Clostridium species induce outstanding Treg accumulation in the colon. At this end, changing the gut microbiota composition in a way that favors the effector T cell and Treg balance may have a promising impact on immune therapies (Atarashi et al., 2011).

The field of bacterial-based therapies keeps expanding since 1891, when Coley introduced the toxins as treatment methods for bone and soft-tissue sarcomas (McCarthy, 2006). Various bacteria have been proved to have the capacity of selectively colonizing tumors, primarily localizing to the hypoxic tumor core and even leading to tumor regression. Antibiotics have been proven capable to modify the immune responses, through regulations of the tumor microenvironment (TME). Bacterial pathogens trigger the activation of cell signaling pathways through the stimulation of pattern-recognition receptors (PRRs), such as the TLRs (Leventhal et al., 2020) and specific bacteria have been found to target the STING agonist to APCs as they are actively phagocytosed.

Among many other antibiotics, ampicillin, metronidazole and vancomycin have been studied extensively, based mainly on the frequency, the spectrum and their known side effects.

Ampicillin is a third generation penicillin and it belongs in the beta-lactam class of antibiotics (Pandey & Cascella, 2022). In rodents it can be used to temporarily deplete microbiota. Even though several weeks after the treatment the gut can be recolonized, the treatment is able to reduce innate cytokine expressions and have a non – lasting reducing impact on regulatory T cells and a longer term impact in natural killer T cells (Castro-Mejía et al., 2018). Ampicillin causes alterations in gut microbiota composition, reducing the abundance of gram – positive and increasing the abundance of gram – negative bacteria. It has been shown to reduce the colon motility as well, compared to mice given only water, and to promote colonic neurogenesis through TLR2 (Yarandi et al., 2020).

Metronidazole (MTZ) is a nitroimidazole antibiotic used mostly against infections caused by anaerobic bacteria and protozoa (Tally & Sullivan, 1981). Besides the antibiotic and antiprotozoal effects, it displays immunopharmacological behavior. MTZ has been shown to induce immunosuppression by downregulating cytokines of both the innate and the adaptive immune system. It has anti-inflammatory effects, but is able to cause neutropenia and reduction in the number and functions of macrophages. MTZ causes damages to the DNA of lymphocytes and therefore inhibits their proliferation and leads to immunosuppression (Shakir et al., 2011).

Vancomycin is a glycopeptide antibiotic that acts mainly on gram-positive bacteria. In accumulating recent data which prove that antibiotics can regulate effects of immune therapies and radiotherapies, vancomycin has been found to modulate multiple mechanisms. Antibiotics have been shown to inhibit the clinical beneficial effect of PD-1/PD-L1 mAb treatment in patients with advanced cancer (NSCLC, RCC or urothelial carcinoma), while oral supplementation of the bacteria to antibiotic-treated mice can restore the response to immunotherapy (Routy et al., 2018). Other studies have shown that the combination of antibiotics with Cyclophosphamide (CP) chemotherapy and ACT immunotherapy diminished the endogenous T cell responses elicited by CP and dampened the therapeutic effects of adoptively transferred tumor specific CD4+ T cells in mice colorectal tumors (Kuczma et al., 2017). Uribe-Herranz demonstrated that administration of oral vancomycin is able to boost the antitumor effect of tumor radiotherapy, enhancing dendritic cells' ability to cross present tumor specific antigens (Uribe-Herranz et al., 2020). In a different murine study, Uribe-

Herranz demonstrated that vancomycin-mediated depletion of bacteria enhanced the antitumor effects of the ACT in an IL-12 dependent manner (Uribe-Herranz et al., 2018). Therefore, combined these studies demonstrate that the gut microbiome can influence the ACT efficacy. Vancomycin is retained locally within the gut when administered orally; it does not enter the systemic circulation and impacts the gut microbiota directly without any known systemic effects. It is strongly suggested that vancomycin induces local interactions between the gut microbiota and the immune system, resulting in local and long-range systemic effects.

Butyrate-producing bacteria are among the preferable targets of vancomycin treatment. Sodium butyrate, a key metabolite of gram-positive gut bacteria, abrogates the vancomycin effect. It has been shown that butyrate, a by-product of the digestion of dietary fiber by gut microbes, acts as epigenetic switch inducing transcription remodeling and anti-inflammatory effects (Furusawa et al., 2013). The Weischelbaum group reported a little later than Uribe (Uribe-Herranz et al., 2020) that gut microbiota–derived butyrate impairs the antitumor effects of Radiotherapy (RT) through the suppression of local type I IFN production (Yang et al., 2021).

1.2.4 SCFAs and metabolites

The gut microbiota aids host digestion and generates a large repertoire of metabolites, including short-chain fatty acids (SCFAs) that constitute the main products of bacterial fermentation. SCFAs are carboxylic acids defined by the presence of an aliphatic tail of two to six carbons. Although SCFAs can be produced naturally through host metabolic pathways particularly in the liver, the major site of production is the colon which requires the presence of specific colonic bacteria explaining their absence in germ-free mice (Hoverstad & Midtvedt, 1986). Acetate (C2), propionate (C3), and butyrate (C4), being the major SCFA released through fermentation of fiber and resistant starches, are mostly released in the proximal colon in very high concentrations (70– 140 mM) while their concentrations are lower in the distal colon (20–70 mM) and in the distal ileum (20–40 mM) (Wong et al., 2006). The molar ratio of acetate, propionate, and butyrate production in the colon is 60:25:15, respectively (Tazoe et al., 2008), although proportions can vary depending on factors such as diet, microbiota composition, site of fermentation, and host genotype (Hamer et al., 2008). Butyrate is mostly utilized by colonocytes while acetate and propionate reach the liver through the portal vein. The process involved in the production of SCFAs from fiber

involves complex enzymatic pathways that are active in an extensive number of bacterial species. The main pathway of SCFA production in bacteria is via the glycolytic pathway. The production of SCFAs is a highly complex and dynamic process. Dietary changes can alter the composition of the gut microbiota in as little as a day(Wanders et al., 2012) and even minute alteration of dietary factors such as fiber content could shape microbial communities (Donohoe et al., 2011) . SCFAs enter cells through passive diffusion and carrier-mediated transportation through specific transporters (Na^+/glucose cotransporters and H^+-coupled transporters) which have been documented to be expressed in the apical membrane of colonocytes, DCs, kidney cells, and/or brain cells (Kim, 2014) .The ability of SCFAs to modulate biological responses of the host depends on two major mechanisms. The first involves the direct inhibition of histone deacetylases (HDACs) to directly regulate gene expression. Intrinsic HDAC inhibitor (HDACi) activity is particularly characteristic of the SCFAs butyrate and propionate. The second mechanism for SCFA effects is signaling through G-protein-coupled receptors (GPCRs). The major GPCRs activated by SCFAs are GPR41, GPR43, and GPR109A (Figure 1.4).

1.2.4.1 SCFAs and the immune system

Of all the SCFAs, butyrate is considered to be the most potent inhibitor of HDAC activity. Recent studies suggest that butyrate is able to regulate CD8+ T cells through HDAC inhibition (Luu et al., 2018) and promote the memory of CD8+T cells through the regulation of GPR41/43 dependent metabolism (Bachem et al., 2019). Studies on both HeLa (Boffa et al., 1978) and colon cancer cell lines have demonstrated that butyrate exhibits a stronger HDAC inhibitory activity than propionate whereas acetate appeared to have very little or no or effect (Hinnebusch et al., 2002; Kiefer et al., 2006; Waldecker et al., 2008). SCFA-mediated HDAC inhibition has been observed to result in an anti-inflammatory immune phenotype. Indeed, treatment of human macrophages with 1mM of acetate, in vitro, significantly reduced their global HDAC activity and increased global histone acetylation correlating with decreased production of inflammatory cytokines IL-6, IL-8, and TNFα (Kendrick et al., 2010). Similarly, butyrate and propionate decreased LPS-induced TNFα production in vitro from human peripheral blood mononuclear cells (PBMCs) in a similar manner to trichostatin A (TSA) (Usami et al., 2008). These results suggest an active control of the release of proinflammatory cytokines by SCFAs through HDAC inhibition in both rodents and humans (Figure 1.5).

Recent studies (Luu et al., 2019) showed that also pentanoate, an abundant metabolite which is also an HDAC inhibitor, is a potent regulator of immunometabolism. Pentanoate induces IL-10 production in lymphocytes by reprogramming their metabolic activity towards elevated glucose oxidation. Mechanistically, this reprogramming is mediated by supplying additional pentanoate-originated Acetyl-CoA for histone acetyltransferases, and by pentanoate-triggered enhancement of mTOR activity. In experimental mouse models of colitis and multiple sclerosis, pentanoate-induced regulatory B cells mediate protection from autoimmune pathology. Additionally, pentanoate shows a potent histone deacetylase-inhibitory activity in CD4+ T cells, thereby reducing their IL-17A production (Luu et al., 2019). *In vitro* and *in vivo* studies, as well as results of early clinical trials have shown antitumor effects of pentanoate through epigenetic regulations. Moreover, microarray analysis from tumors of patients treated with Valproic Acid (VPA, derivative of pentanoic acid) has shown significant up-regulation of many genes involved in multiple pathways including ribosomal proteins, oxidative phosphorylation, MAPK signaling; focal adhesion, cell cycle, antigen processing and presentation, proteasome, apoptosis, PI3K, Wnt signaling, calcium signaling, TGF-beta signaling, and ubiquitin-mediated proteolysis and others (Duenas-Gonzalez et al., 2008) .

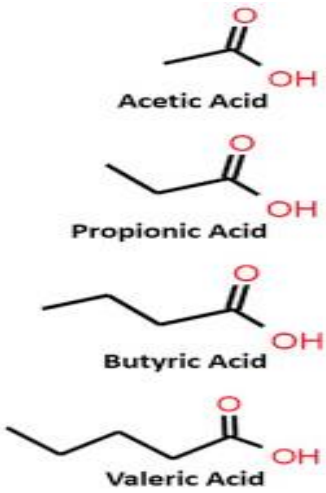


Figure 1. 4 Base short chain fatty acids
Key short chain fatty acid molecules. C2 through C5 short chain fatty acid 2 dimensional structures are shown for acetic acid, propionic acid, butyric acid and valproic acid (pentanoate) which are discussed throughout the chapter (Matthew Stratton, Science direct(Mallappa et al., 2022))

SCFAs dictate the extrathymic differentiation of peripheral regulatory T cells. Butyrate acts within T cells to enhance acetylation of the Foxp3 locus and Foxp3 protein, as well as in DCs

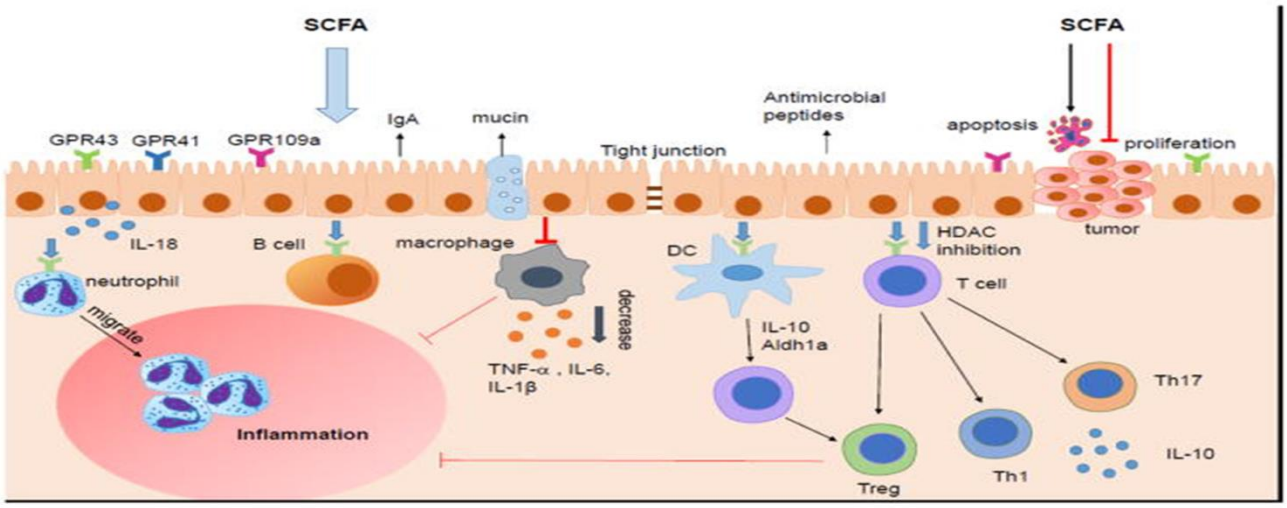


Figure 1.5 SCFAs and immune system regulation
The SCFAs modulate the immune system, mainly through the HDAC inhibition and the GPR41/43 signaling. They promote immunity by inducing mucin and AMP production, IgA secretion, Th1 differentiation. They are also able to suppress inflammatory responses and affect the tumor microenvironment (Sun et al., 2016)

to decrease their proinflammatory NF-κB-dependent cytokine secretion profile, through an HDAC inhibitory activity. Butyrate and propionate, but not acetate, induce the production of gut hormones and reduce food intake (Lin et al., 2012). Butyrate, also, promotes increased mucin production, providing a positive feedback loop for the maintenance of the mucous barrier and its colonization by butyrate-producing commensals. SCFAs have been shown to be protective against colon and mammary cancer in human cell lines and in mice (Viaud et al., 2013) .

Moreover, SCFAs, such as butyrate, have been shown to induce IL-18 production in intestinal epithelial cells by activating the GPR109a receptor, and to act directly on DCs, macrophages, and T cells. SCFAs have also been shown to induce the expansion of T reg cells, producing the anti-inflammatory cytokine IL-10, thus suppressing colonic inflammation and carcinogenesis. Other studies demonstrated that butyrate and propionate treatment of DCs significantly reduced LPS-induced IL6 mRNA and IL12 gene expression (Nastasi et al., 2015). Interestingly, cytotoxicity via the perforin/granzyme pathways and Th1 cytokines such as IFNγ and IL-12 has been shown to be critical anti-tumor mediators (Braumüller et al., 2013; Trapani & Smyth, 2002; Trinchieri, 2003).

1.3 Aim of the study

The administration of antibiotic treatments has been proven to cause gut barrier disruption and dysbiosis. Gut dysbiosis involves loss of diversity, changes in the prevailing of certain taxa and consequent events on their metabolic capacity. Antibiotic induced changes in the gut microbiota composition could trigger weakening of the gut barrier and alteration to the immune response through changes in mucin, cytokine and antimicrobial peptide production by intestinal epithelial cells. (Duan et al., 2022)

Antibiotic treatments are often administered in parallel with multiple other therapies, like immunotherapies, chemotherapies or radiotherapies. These treatments can frequently cause apoptosis, dsDNA damages, autophagy, switch the type of the immune responses, the activation and balance of immune cells and the activation of variable pathways. The gut microbiome composition and its regulation by antibiotics significantly impact multiple therapies. The cross talk between the effects of plausible alterations in bacterial species -and consequently the regulations of bacteria- produced metabolites- and the immune responses needs to be elucidated.

The aim of this study was to explore the impact of antibiotics and metabolites, such as SCFAs, on antigen presentation. Several studies have shown the influence of the gut microbiome on T cell function, but much less have been proven about its influence on the dendritic cell function and the antigen presenting mechanisms. Moreover, there is no established knowledge on plausible direct effects of antibiotics on the immune cells. To address these questions, we examined the effects of gut metabolites on the interaction between DCs and T cells *in vitro*. We interrogated the importance of timing and duration, as well as the optimization of the concentrations of our treatments. Moreover, potent changes on the gene expression of DCs caused by the treatments of interest have been examined. On top of testing the most abundant and well-studied SCFAs, effects of antibiotics were examined both *in vitro* and *in vivo*. For the *in vitro* experiments, Vancomycin with activity mainly against Gram+ and preferentially butyrate- producing bacteria, Metrondiazole which targets mainly Gram- bacteria and Ampicillin which targets both Gram+ and Gram- were tested. Effects of vancomycin treatments have been studied *in vivo* as well. The impacts on the DCs maturation and T cell activation have been addressed separately, aiming to distinguish the impact on their different mechanisms and functions.

Chapter 2

Materials and methods

2.1 Mouse strains

Eight to twelve weeks old C57BL/6 female mice, purchased from the Jackson Laboratory, were used to generate DCs in vitro for these experiments. Mice of the same strain had undergone treatments with vancomycin, ad libitum or intravenously, and DCs were generated from their bone marrow. The aim of all experiments was to identify possible differences in the immune responses, so all mice were maintained in the same vivarium and under special conditions upon arrival. Only one person was handling the mice, changing the cages and adding autoclaved food and autoclaved water bottles in order to avoid contamination and control as much as possible the exposure of the mice to a variety of antigens which could affect the immune system and therefore lead to inaccurate results. The C57BL/6 strain was chosen because the DCs had to be generated from mice syngeneic to the OT-1 strain, a strain vital for immunological studies as the present.

The C57BL/6-Tg(TcraTcrb)1100Mjb or OT-1 mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes. The transgenic T cell receptor is designed to recognize ovalbumin peptide residues 257-264 (OVA257-264) in the context of H2Kb (CD8 co-receptor interaction with MHC class I). This results in MHC class I-restricted, ovalbumin-specific, CD8+ T cells (OT-I cells). That is, the CD8 T cells of this mouse primarily recognize OVA257-264 when presented by the MHC I molecule. Immune response dynamics can be studied by in vivo adoptive transfer or in vitro co-culture with cells transgenic for ovalbumin or by direct administration of ovalbumin. These mice are often used to study CD8+ T cell response to antigen, positive selection, and in any research requiring CD8+ T cells of defined specificity and allow researchers to efficiently study T cell-mediated immune responses according to antigen-specific TCR activity by synchronizing all T cell responses to one epitope.

Eight weeks or older male and female C57BL/6-Tg(TcraTcrb)1100Mjb mice (also known as OT-I) were used to obtain splenocytes and isolate OT-1 T cells with a mouse Pan T Cell isolation kit according to the manufacturer’s instructions. Those mice were initially purchased from the Jackson Laboratory and had been bred as a colony ever since.

All animal studies were approved by the IACUC and the University Laboratory Animal Resources at the University of Pennsylvania. Mice were treated in accordance with University of Pennsylvania guidelines.

2.2 *In vitro* Dendritic cell generation

Bone marrow derived dendritic cells were isolated from the femur and tibia of C57BL/6 mice and were differentiated and matured according to a 7-days long protocol (Chiang et al., 2011). Immediately after their isolation, the bmDCs were plated in R10 media [RPMI 1640 media containing 10% FBS, 55mM 2-mercaptoethanol (1:1000), 200 mM L-Glu 1:100 and Penicillin/Streptomycin (1:100)]. 2.5 million cells / ml were cultured with 10ng/ml of GM-SCF and IL-4 on day 0. On days 1, 3 and 5 floating cells representing granulocytes were removed and replenished with fresh media and GM-CSF and IL-4. On day 7 in the morning, DCs were harvested, counted and plated again with fresh media and cytokines. At this time point that the cells are supposed to be differentiated DCs, they had to be stimulated and matured. Half groups were stimulated with OVA protein, before the maturation. The maturation cocktail (1ug/ml of LPS and 100ng/ml of IFNγ was added and left overnight, along with fresh cytokines, after 6 hours of incubation at 37°C. Early on day 8 OVA peptide was added to the rest of the groups as stimulating factor and after 6hours incubation at 37°C the DCs were harvested in separate groups to proceed with the assays.

2.3 OVA antigens: Ovalbumin and OVA peptide

The OVA system is commonly used as a model for antigen specific immune responses in mice. The chicken ovalbumin (OVA) is the major protein of chicken egg whites, a glycoprotein very well studied, nontoxic and mildly immunogenic. It can be used for in vivo vaccinations but also for cell stimulation in vitro, as in our experiments. Ovalbumin can bind on both MHC I and MHC II at positions 257–264 and 323–339, respectively, and can be presented to T cells via the MHC class II antigen processing pathway. OVA 257-264 is a class I (Kb)-restricted peptide epitope of ovalbumin (OVA), presented by the class I major histocompatibility complex (MHC) molecule, H-2Kb (Diebold et al., 2001) . It has been demonstrated that OVA 257-264 peptides can be used to detect a strong CD8+ cytolytic T cell response (Cho & Celis, 2009).

Based on the literature and to be consistent with previous experiments in the lab, I ended up using the following concentrations: 100ug/ml for OVA protein and 0.1ng/ml for the OVA peptide.

2.4 Cell isolation and purification

Spleens of C57BL/6-Tg(TcraTcrb)1100Mjb (OT-1) mice were collected right after the mice were euthanized, always according to the University of Pennsylvania guidelines. The spleens were digested using 2 mg/mL of collagenase type D (Sigma-Aldrich), resuspended in HBSS (Gibco) and the spleen cell suspension was used to isolate T cells with mouse Pan T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions

2.5 SCFAs and antibiotics

The SCFAs were stored in powder form at room temperature. Fresh aliquots of the desired concentrations were prepared every three days and were then stored at 4°C. The aliquots were prepared by diluting the SCFAs in sterile PBS. The SCFAs I used were Sodium butyrate 98%, Sigma Aldrich, Sodium Propionate ≥99%, Sigma Aldrich and Sodium Pentanoate, AmBeed.

The antibiotics were stored in powder form at 4°C and fresh aliquots of the desired concentrations were prepared on the day of the co-culture and were kept at 4°C. The different concentrations were achieved by serial dilutions in R10 media. The antibiotics which were used are Vancomycin hydrochloride from Streptomyces orientalis (Sigma- Aldrich, V2002), Metronidazole (Sigma- Aldrich, M3761) and Ampicillin sodium salt (Sigma- Aldrich, A9518).

2.6 Flow cytometry

Single cell suspensions were subjected to up to 8-parameter flow cytometry on a FACS Canto flow cytometer using BD FACS Diva software (BD Biosciences) and data were analyzed using FlowJo version X (Tree Star). Flow cytometry was used to investigate the impact of the treatments on the DCs phenotype and to specify the T cell specific immune responses.

The DC phenotyping was carried out using the following monoclonal antibodies against surface mouse markers on 100.000 DCs: anti-CD11b APC-Cy7 (BioLegend, clone M1/70), anti-CD11c PERCP (BioLegend, clone N418) or FITC (BioLegend, clone N418), anti-CD40 PE/Cy7 (BioLegend, clone 3/23), anti-CD80 APC (BioLegend, clone 16-10A1), anti-PDL1 PE (BioLegend, clone 10F.9G2), anti-MHCII FITC (BioLegend, clone M5/114.15.2), anti-

CD103 PERCP/Cy5.5 (BioLegend, clone 2E7), anti- XCR1 PE (BioLegend, clone ZET) and anti- GR1 APC (BioLegend, clone RB6-8C5).

For the functional characterization of T cells, we performed intracellular staining on co-cultures of DCs and OT-1 T cells. The chosen ratio for the co-culture was DCs: T cells 1:5 (50.000 DCs: 250.000 T cells in absolute numbers). After staining for surface markers, the samples were incubated in Fixation/Permeabilization buffer (Fixation/Permeabilization concentrate: Fixation/ Perm Diluent in a ratio 1:3, Invitrogen 00-5123-43 and Invitrogen 00-5223-56, respectively). Subsequently, the antibodies for the intracellular staining were added in Permeabilization buffer (Invitrogen 00-8333-56). The surface markers for the determination of the T cell population were anti- CD45 Pacific Blue (BioLegend, clone S18009F), anti- CD3 APC/Cy7 (BioLegend, clone 17A2), anti-CD8 FITC (BioLegend, clone 53-6.7), while for the intracellular staining anti-IFNγ PE (BioLegend, clone XMG1.2) and anti-TNFα PerCP/Cy5.5 (BioLegend, clone MP6-XT22) antibodies were used.

In all stainings, LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies) was used to detect live cells.

No trypsin was ever used while harvesting cells for FACS, in order to maintain the integrity of the extracellular matrix. Instead, cells were detached using manual cell lifters (scrapers).

2.7 Gating strategy

In all analyses lymphocytes were identified by the side and the forward scatter profile (SSC-A/ FSC-A), while the singlets were recognized by the forward scatter profile (FSC-H/FSC-A). The LIVE/DEAD gating identified the live cells within the singlet cells subset (Figures 3.3, panel A and 3.5, panel A).

The gating for the T cells assessment was CD45+/CD3+ (all T cells) and CD8+ to target the cytotoxic CD8+ T cells. The quantification of the IFNγ and TNFα percentages was determined as SSC-A/IFNγ and SSC-A/TNFα respectively within the CD45+/CD3+/CD8+ subset (Figure 3.5, panel A).

In order to look for possible differences on the DCs phenotype, DCs were identified as CD11b+ (Myeloid)/ CD11C+ (plasmacytoid). Percentages of the maturation markers CD40, CD80, PD-L1, MHC I, MHC II, CD103, GR1 and XCR1 where quantified (Figures 3.3, panel A and 3.7, panel A).

The parameters of the side and the forward scatter profile (SSC/FSC) were set respectively to the size of the cells which were targeted in each experiment, since the size and the density between DCs and T cells differ.

2.8 ELISPOT

ELISPOT (Enzyme-Linked Immunoabsorbent spot) assays detecting mouse IFNγ were performed. The medium used in all assays was the R10 medium containing RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and 0.1% β-mercaptoethanol. Ninety- six well multiscreen immobilon – P (MAIP) plates (Millipore) were coated with 2.5μg/ml rat anti-mouse IFNγ (BD biosciences) diluted in sterile PBS and were stored at 4°C overnight. The day after the plates were washed 3 times with sterile PBS and blocked with R10 medium for at least 2 hours at 37°C. DCs and OT -1 T cells were plated in a ratio 1:5 respectively and in equal volumes (50ul each per well). All conditions were plated in triplicates and were incubated overnight at 37°C. Anti-MHC I antibody was used as a positive control of inhibition in a concentration of 20ug/ml and the incubation conditions were 30minutes in room temperature, just before seeding the cells in an ELISPOT plate. The last day of the protocol, the plates were first washed 6 times with PBS containing 0.05% Tween-20 (Bio-Rad) and then incubated with anti-mouse biotin-conjugated anti–IFN-γ antibody (BD Biosciences) for 3 hours at room temperature. After this incubation, the washes with the washing buffer (PBS+ 0.05% Tween-20) were repeated and were followed by an incubation with streptavidin-alkaline phosphatase conjugate (BD Biosciences) for 30 minutes at 37°C. The plates were then washed 3 times with washing buffer and 3 times with PBS. Finally, the plates were developed by adding nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Pierce), filtered through 22μm filter in order to decrease the false positive spots and equilibrated at room temperature. The spots were measured using an automated ELISPOT reader (Autoimmun Diagnostika GmbH) and the numbers were analyzed using GraphPad Prism 8.

2.9 Gene expression analysis

Relative quantification of expression levels of selected genes was carried out by real time reverse transcription PCR (q PCR). Total RNA from treated and untreated DCs was extracted using TRIzol reagent (Invitrogen, catalog 15596018) according to the manufacturer's instructions. To determine the quality of RNA samples intact 18s bands were visualized

following agarose gel electrophoresis with ethidium bromide staining. 1µg of total RNA was reverse transcribed with random primers using the High capacity cDNA Reverse Transcription kit (Thermo Fischer Scientific, catalog 4368814). The PCR conditions, optimized for the cDNA Reverse Transcription kit, were 10min at 25 °C, 120min at 37°C (transcription of RNA to cDNA), 5min at 85°C (primers annealing). 20ng of cDNA were used in each qPCR reaction with a QuantStudio 6 Flex Real- Time PCR System (Thermo Fischer Scientific). The expression levels of the following mouse target genes were quantified using TaqMan Gene Expression Master Mix (Applied Biosystems, 4369016) and TaqMan gene expression assays (*MHC II:* Mm00439216_m1 H2-Ab1*, IFNα:* Mm03030145_gH*, IFNβ:* Mm00439552_s1*, Batf3:*Mm01318274_m1, Applied Biosystems). mRNA expressions were normalized against the housekeeping gene *18s*: Mm03928990_g1.

2.10 Statistics

All statistical analyses were conducted using GraphPad Prism 8. Statistical analyses for all indicated data were performed using paired two-tailed *t* tests. A P value less than 0.05 was considered significant (*P < 0.05, **P < 0.01, ***P < 0.001). All experiments were performed a minimum of two times including experimental duplicates or triplicates for each sample; all figures portray one representative experiment.

2.11 Study approval

All animal studies were approved by the IACUC and the University Laboratory Animal Resources at the University of Pennsylvania. Mice were treated in accordance with University of Pennsylvania guidelines.

Chapter 3

Results

3.1 The impact of SCFAs on antigen presentation depends on the timing of exposure and the stimulatory factor

To investigate the effects of SCFAs on antigen presentation in vitro, treated DCs were co-cultured with CD8+ T cells in the presence of SCFAs. Both ovalbumin and ova peptides were used to stimulate DCs. The rationale behind this was that DCs recognize and process protein antigens differently than the peptide antigens. Immature DCs capture and phagocytose pathogens or proteins, degrade the proteins and display the fragments (peptides) on their surface, as MHC-peptide molecules. On the other hand, mature DCs load their MHC molecules with exogenous peptides. Upon maturation, DCs can no longer capture or phagocytose. They are able to present their MHC- peptide complexes and express higher levels of co-stimulatory molecules. This way they activate T cells specific for the displayed complexes. To determine the number of individual activated T cells after stimulation with a specific antigen, we utilized a sensitive immunoassay (ELISPOT) that detects IFNγ production. The strength of the immune response is depicted by a proportionally increased number of spots.

The first question we had to answer was the timing and duration of the treatments. In our experiments DCs cultures were treated with butyrate and propionate for either 24hours or 8 days (total duration of the protocol). Concentrations in a range of 0.01mM to 0.5mM were used, based on the literature (B. Wang et al., 2008). As stimulatory factors of the DCs, OVA protein or OVA peptide were used. On day 8, DCs were co-cultured with OT-1 T cells and IFNγ ELISPOT assays were developed. DCs that had been stimulated with either ovalbumin or OVA peptide and matured with LPS and IFNγ but had not been exposed to any treatment were used as control groups. DCs that had received neither ovalbumin nor OVA peptide were used as controls of the stimulation. We observed that all treatments were causing inhibition of the IFNγ secretion, thus inhibition of the immune response, after 24 hours exposure, which was not the case for the 8 days exposure. Interestingly, we observed inhibitory effects after the addition of butyrate in all tested conditions. However, the inhibition was stronger when the stimulatory factor was the ovalbumin after 24 hours exposure, but with OVA peptide as stimulatory factor the inhibition was stronger when the treatment was added for 8 days. The data shown in Figure 3.1 are collected from two experiments. The data are merged for all three concentrations (0.01mM,0.1mM and 0.5mM) of each treatment to present the overall picture of the inhibition focusing on the different time points. the Despite the fact that the inhibition was much stronger after 24h administration, we decided to continue by adding

SCFAs in the culture for 8 days, since that emulates better the physiological events which take place in the human body during the administration of antibiotics and their long-term effect on the immune system regulation.

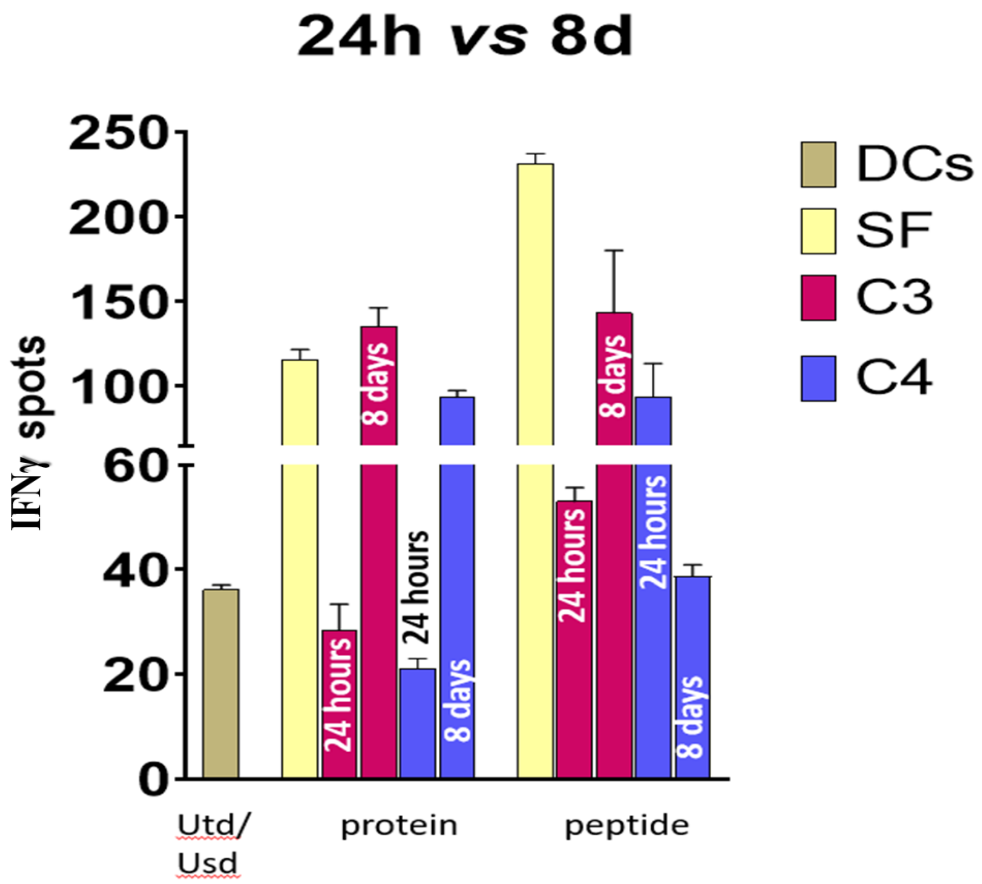


Figure 3. 1 Impact of SCFAs on antigen presentation *in vitro*. Comparison of 24hours versus 8 days *in vitro* treatments.
Shown are IFNγ spots detected in ELISPOT assays after administration of propionate and butyrate in DCs and OT-1 T cells co- cultures. Ovalbumin or OVA peptide were used as stimulatory factors (SF) of the DCs. Data merged for concentrations 0.01mM, 0.1mM, 0.5mM in two experiments. SEM is shown.

3.2 Propionate inhibits the antigen presentation ability of DCs *in vitro*

After deciding to work with 8 days exposure, different concentrations of butyrate, propionate and pentanoate were added to DC cultures, in order to further investigate the effects of SCFAs on antigen presentation *in vitro*. Co-cultures with OT-1 CD8+ Tcells were preformed subsequently.

The OT-1 CD8+ T cells used for the assays are MHC I restricted T cells. Since we are investigating for possible inhibitory effects of the SCFAs on antigen presentation, a blocking anti- MHC class I (H-2Kb) antibody (BioXCell, Y-3, be0172) was used as a positive control. Ovalbumin (OVA protein) or OVA peptide have been used as stimulatory factors (SF) in the DCs culture.

Interestingly, propionate significantly inhibits antigen presentation by DCs (Figure 3.2, A). The inhibition capability of butyrate is not statistically significant (Figure 3.2, B). When it comes to the combination of the two treatments, we observe statistically significant inhibition again. According to the results of exposures to only propionate or only butyrate, the inhibitory effects after adding both SCFAs simultaneously seem to be caused by the addition of propionate (Figure 3.2, C). The pentanoate treatments show some inhibitory capability only in the peptide groups, not statistically significant nevertheless (Figure 3.2, D).

There are differences in some groups that seem as significant, but the statistic tests used were paired t- tests and concluded as non-significant.

The 0.5 mM concentration was considered toxic for the in vitro cultures and for this reason this concentration was subsequently excluded.

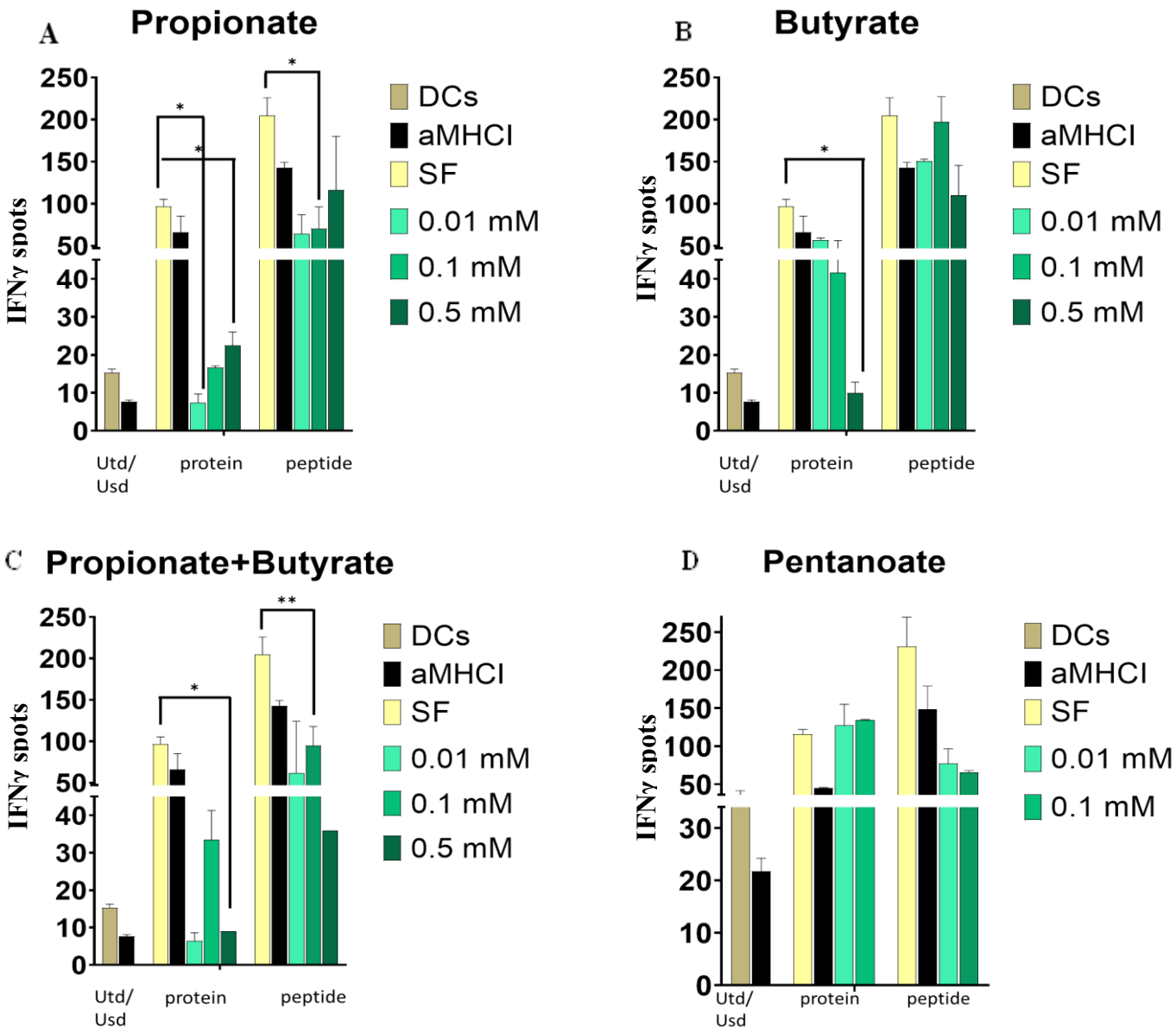
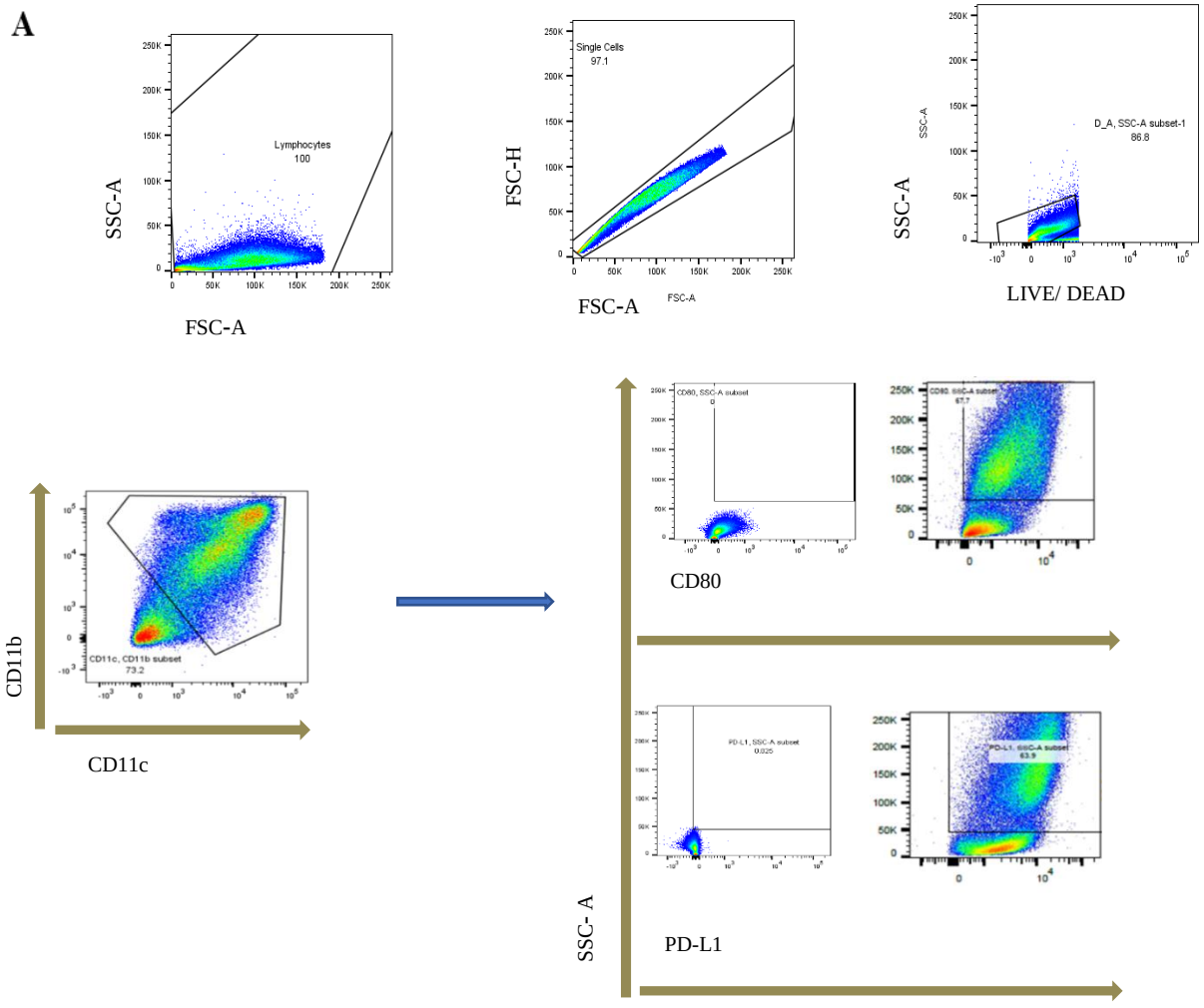


Figure 3. 2 Impact of SCFAs on antigen presentation and T cell activation
Shown are IFNγ spots detected in ELISPOT assays after administration of propionate (A), butyrate (B), pentanoate (D) and combinational treatments (C) in different concentrations. SEM is shown. Statistical significance was assessed by 2-tail paired t-tests. *P-value < 0.05 ** P-value < 0.01. Representative experiment of 4 repetitions. Only the differences that were confirmed in all the experiments are depicted as statistically significant and all results are from paired tests.
Utd/Ust: untreated/ unstimulated, SF: Stimulatory factor

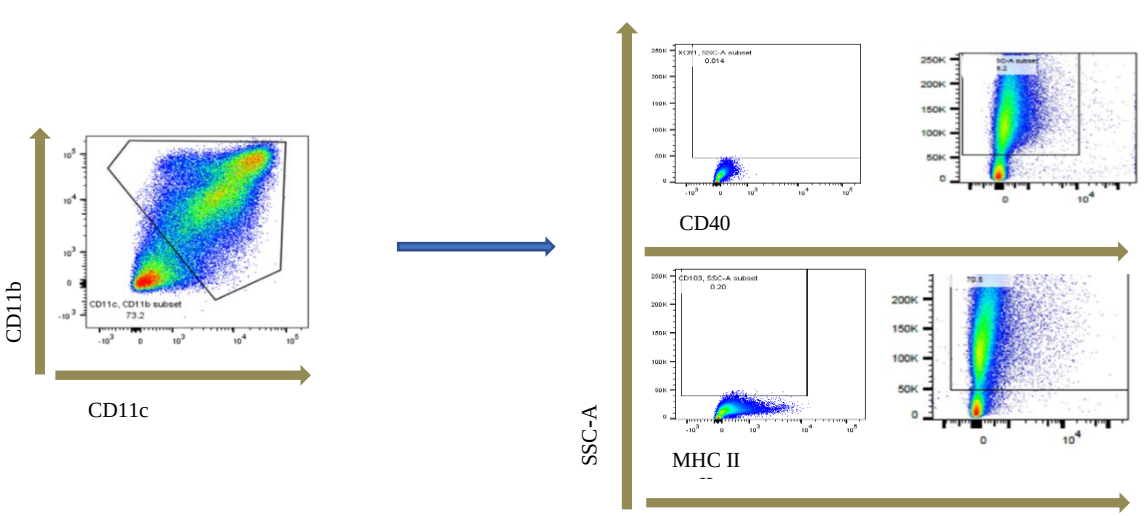
3.3 The maturation process of DCs is affected by their exposure to SCFAs

In order to understand if the observed inhibitory effects were due to a possible negative impact of the SCFAs on the maturation process of the DCs, Fluorescence-Activated Cell Sorting (FACS) analysis was performed. DCs were stained against the surface markers of DCs maturation CD11b, CD11c, CD40, CD80, PDL1 and MHCII.

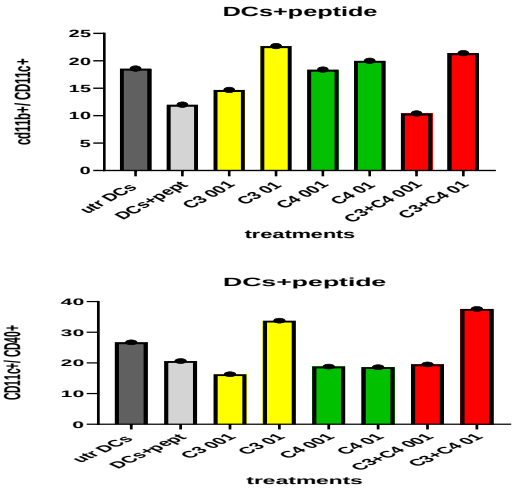
Although we hypothesized that the SCFAs might hinder the maturation of the DCs leading to the inhibition of antigen presentation which was predominant in the protein groups in the previous experiments, the maturation process per se was not negatively influenced by the administration of SCFAs, at least for the surface markers we tested. The expression of all the markers, despite varying between different treatments, was always higher than the control groups (Figure 3.3), indicating a positive impact of the metabolites on the maturation process. Similar patterns were observed both after protein (Figure 3.3, C) and peptide (Figure 3.3, B) stimulation.



A


CD11b
CD11c
CD11c, CD11b subset
73.2
CD40
SSC-A
MHC II

B


DCs+peptide
cd11b+/ CD11c+
utr DCs
DCs+pept
C3 001
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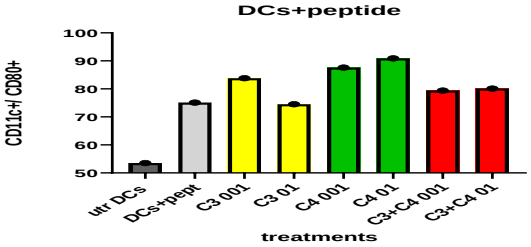
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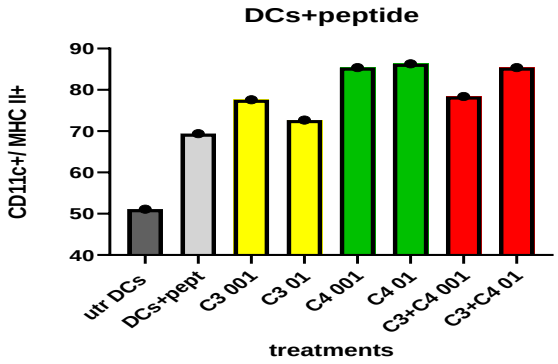
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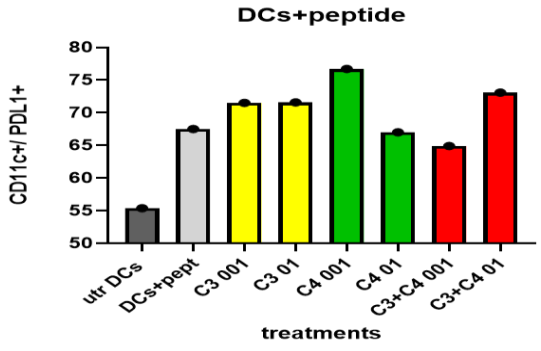
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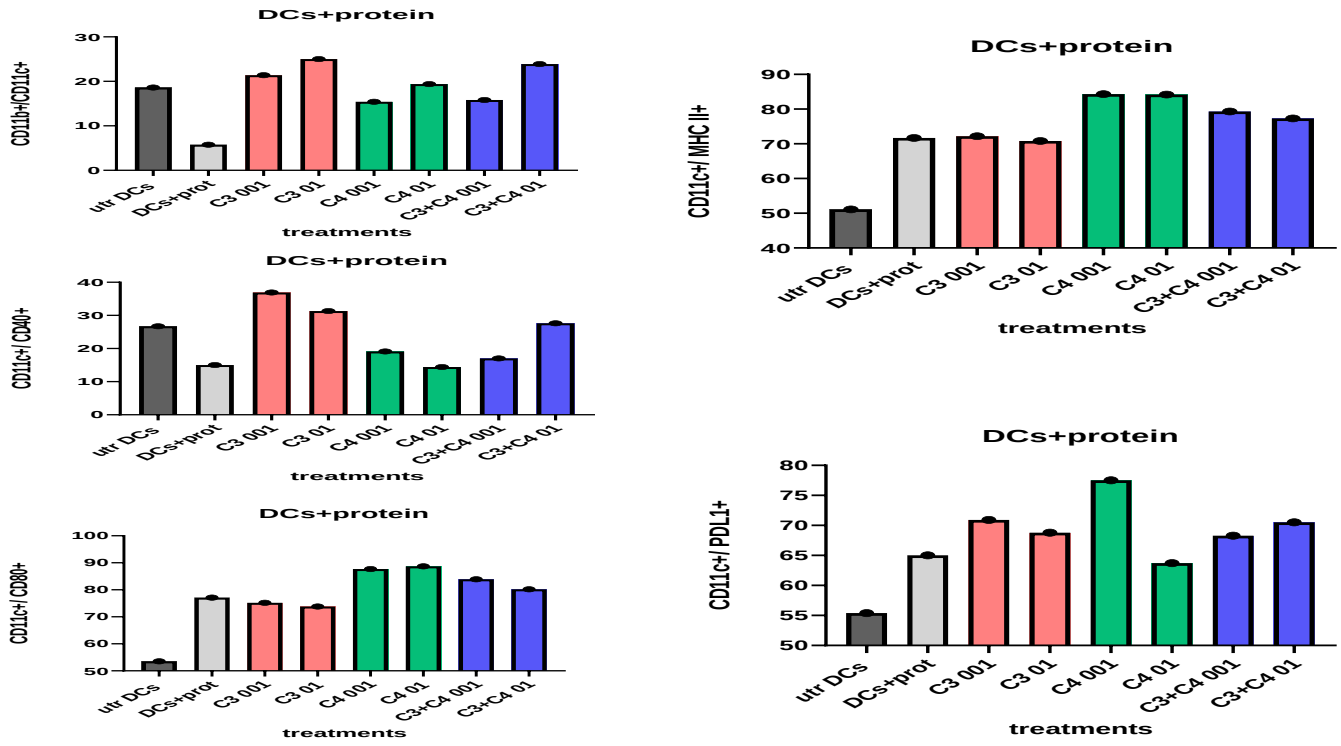


Figure 3. 3 The maturation process of DCs is affected by exposure to SCFAs
A. Gating strategy for DCs maturation markers. **B.** DCs maturation markers expression in DCs stimulated with OVA peptide, all treatments **C.** DCs maturation markers expression in DCs stimulated with OVA protein, all treatments. All data are from the same experiment. The experiment was performed twice.
Utd/Ust: untreated/ unstimulated, C3: propionate, C4: butyrate

3.4 Propionate increases the expression of *IFNα* gene and butyrate the expression of *MHC II* gene

To inquire the mechanisms through which SCFAs affect the antigen presentation, the spotlight was put on the gene expression. DCs cultures were treated with propionate, butyrate or their combination and the expression of certain genes was tested by qPCR. The genes we tested were the *Batf3* (transcription factor required for the development of conventional DCs), the *MHC II* and *IFN (α* and *β*) (Figure 3.4). The expression of *IFNα* is increased after C3 administration (Figure 3.4, C) and that of *MHC II* after C4 administration (Figure 3.4, A). These results might be indicating that propionate is able to enhance the activation of DCs and butyrate their antigen-presenting ability.

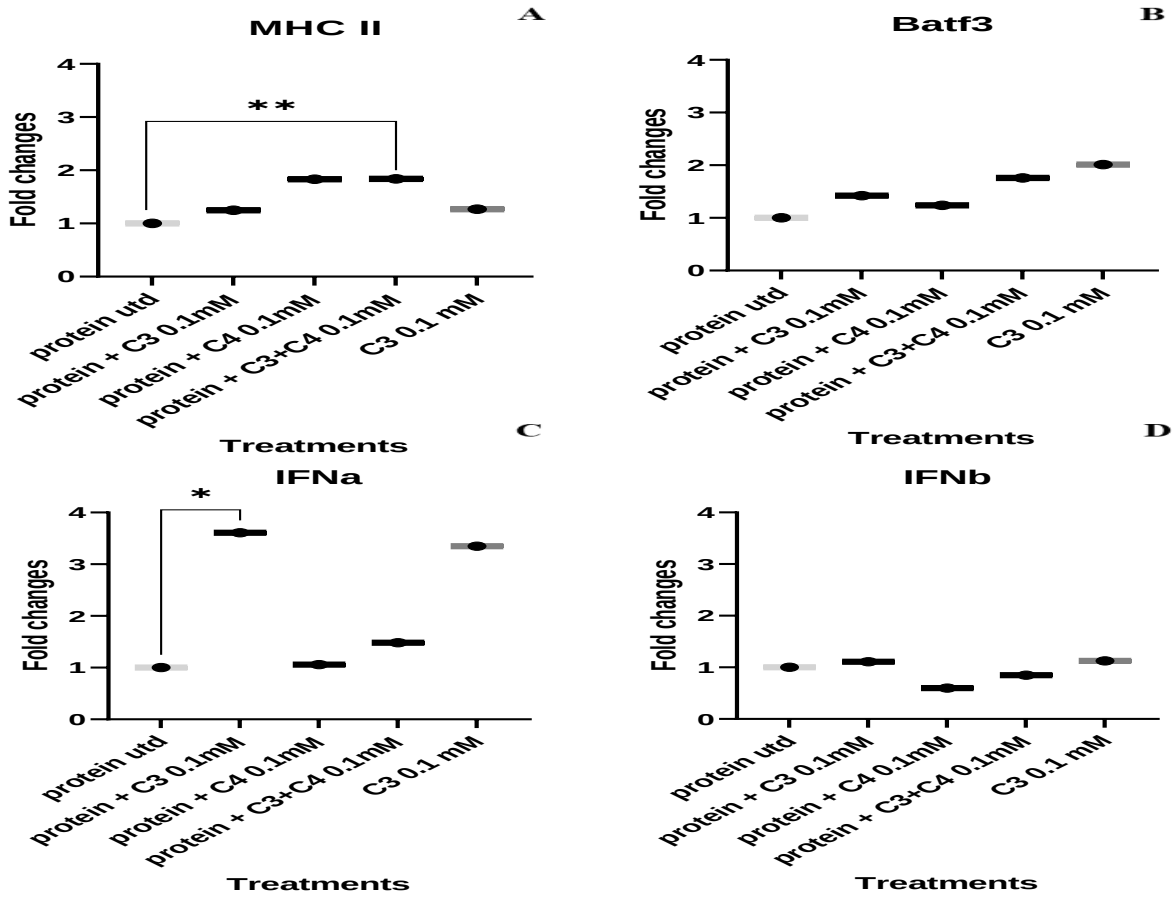


Figure 3.4 Gene expression. *IFNα* expression is increased after C3 administration and *MHC II* after C4 administration

(A) MHC II, **(B)** batf3, **(C)** IFN α, **(D)** IFN β mRNA expression levels in matured and stimulated DCs treated with SCFAs. RNA extracted on day 8 of the protocol

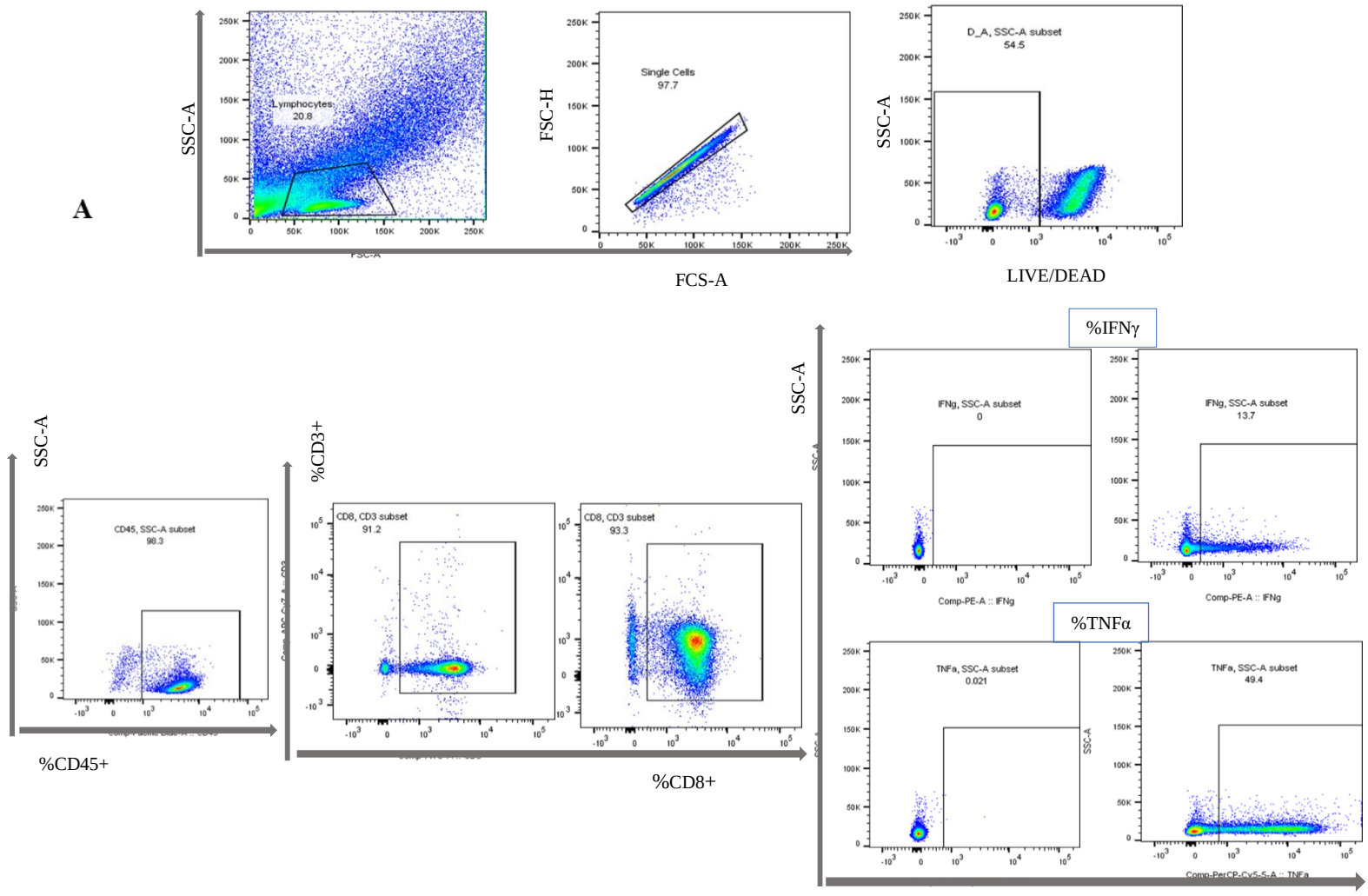
Utd: untreated, C3: propionate, C4: butyrate,

group C3 0.1mM refers to treated DCs that had not received stimulation with protein (ovalbumin)

Statistical significance was assessed by 2-tail paired t-tests. *P-value < 0.05 ** P-value < 0.01. Experiment (with triplicate measurements) was repeated two times and the results from one experiment are shown. Only the differences that were confirmed in both experiments are depicted as statistically significant and all results are from paired tests

3.5 Cytotoxic T cell activation is inhibited by propionate and butyrate *in vitro*

Following our inquiry on the DCs, T cell activation was the next to investigate. To do so, DCs exposed to different treatments were co- cultured with OT-1 T cells and Golgi plug protein inhibitor (BD GolgiPlug protein transport inhibitor, 51-2301KZ) was added 2 hours after the co-culture. The addition of the Golgi plug protein inhibitor allows us to trap the proteins intracellularly, before their secretion, in order to enhance their detectability. IFNγ and TNFα production from CD8+ T cells was quantified, since both cytokines are secreted by activated CD8+ T cells (Figure 3.5). The cytotoxic cells were recognized as CD45+ (lymphocyte common antigen), CD3+ (pan- T cell marker), CD8+ (Cytotoxic T cells). A blocking anti- MHC I antibody was used as positive control for the investigated inhibitory effects. Butyrate and propionate were shown to inhibit the production of IFNγ in the groups pulsed with ovalbumin but not in the groups pulsed with OVA peptide (Figure 3.5, B). TNFα production was also significantly decreased after propionate treatments (Figure 3.5, C).



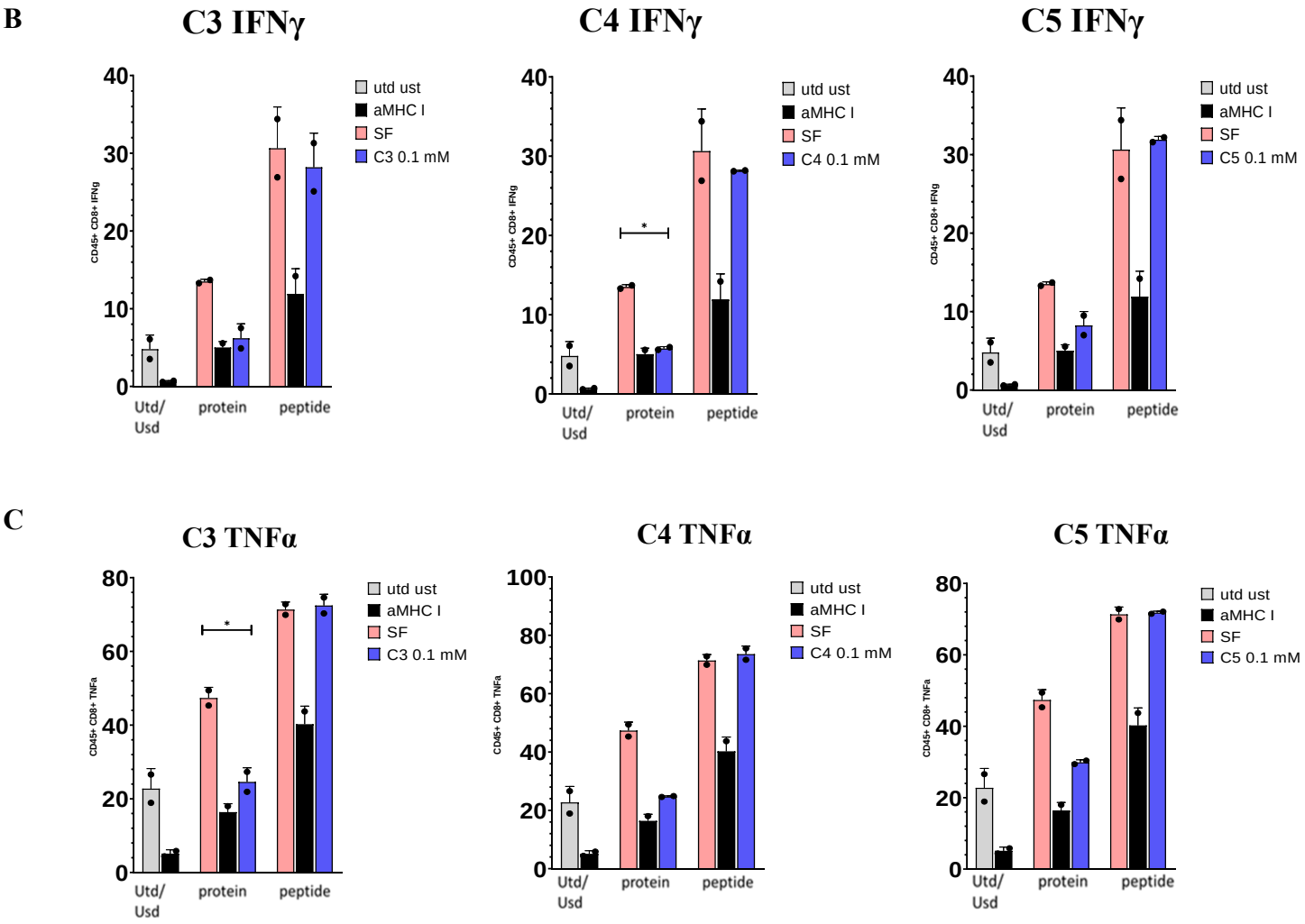


Figure 3.5 Cytotoxic T cell activation is inhibited by butyrate and propionate *in vitro*
Intracellular staining for IFNγ and TNFα on CD45+CD3+CD8+ T cells co-cultured with DCs that have been treated with C3, C4 and C5 respectively. **(A)** Gating strategy for IFNγ and TNFα on CD45+CD3+CD8+ T cells **(B)** %IFNγ production **(C)** % TNFα production. Statistical significance was assessed by 2- tail paired t-test *P < 0.05, **P < 0.01, ***P < 0.001
Utd/Ust: untreated/ unstimulated, C3: propionate, C4: butyrate, C5: pentanoate, SF: stimulatory factor. Representative results of five experiments

3.6 Antibiotics do not have direct impact on antigen presentation *in vitro*

SCFAs availability is correlated with antibiotics treatments. After observing that *in vitro* addition of SCFAs causes inhibition on T cell activation, we decided to investigate if treatments with antibiotics would have any effects on immune cells functions as well. The rationale was that we should be able to distinguish between the effects of antibiotics on immune cells and those of SCFAs on the same cells and processes. In *an vivo* model this distinction is important as the former is the direct impact of antibiotics while the latter is their indirect impact through the modulation of the levels of SCFAs. ELISPOT and ICS were initially performed after the co-culture of DCs with OT-1 T cells. Starting with concentrations of 0.0001mg/ml, 0.01 mg/ml and 0.1 mg/ml of vancomycin, in order to observe the in vitro response in a larger spectrum, eventually the 0.01 mg/ml was used for the latest experiments. The working concentrations for metronidazole were 0.01 and 0.04 mg/ml. Ampicillin has very variable minimum inhibitory concentrations in order to achieve therapeutic efficacy while at the same time it is believed that its efficacy depends on a minimum serum concentration which has to be at least twice as much as the MIC for each bacteria (Giachetto et al., 2004). Ampicillin MICs can vary from 0.00003mg/ml to 0.004 mg/ml, while the mean serum concentration can be measured in a range of 0-58mg/ml. We decided to work with three different concentrations, 0.01mg/ml, 0.02mg/ml and 0.05mg/ml. The antibiotics were diluted in R10 media. Percentages of IFNγ and TNFα secretion were measured in CD45+CD3+CD8+ T cells. No inhibition was observed with any of the antibiotics. (Figure 3.6)

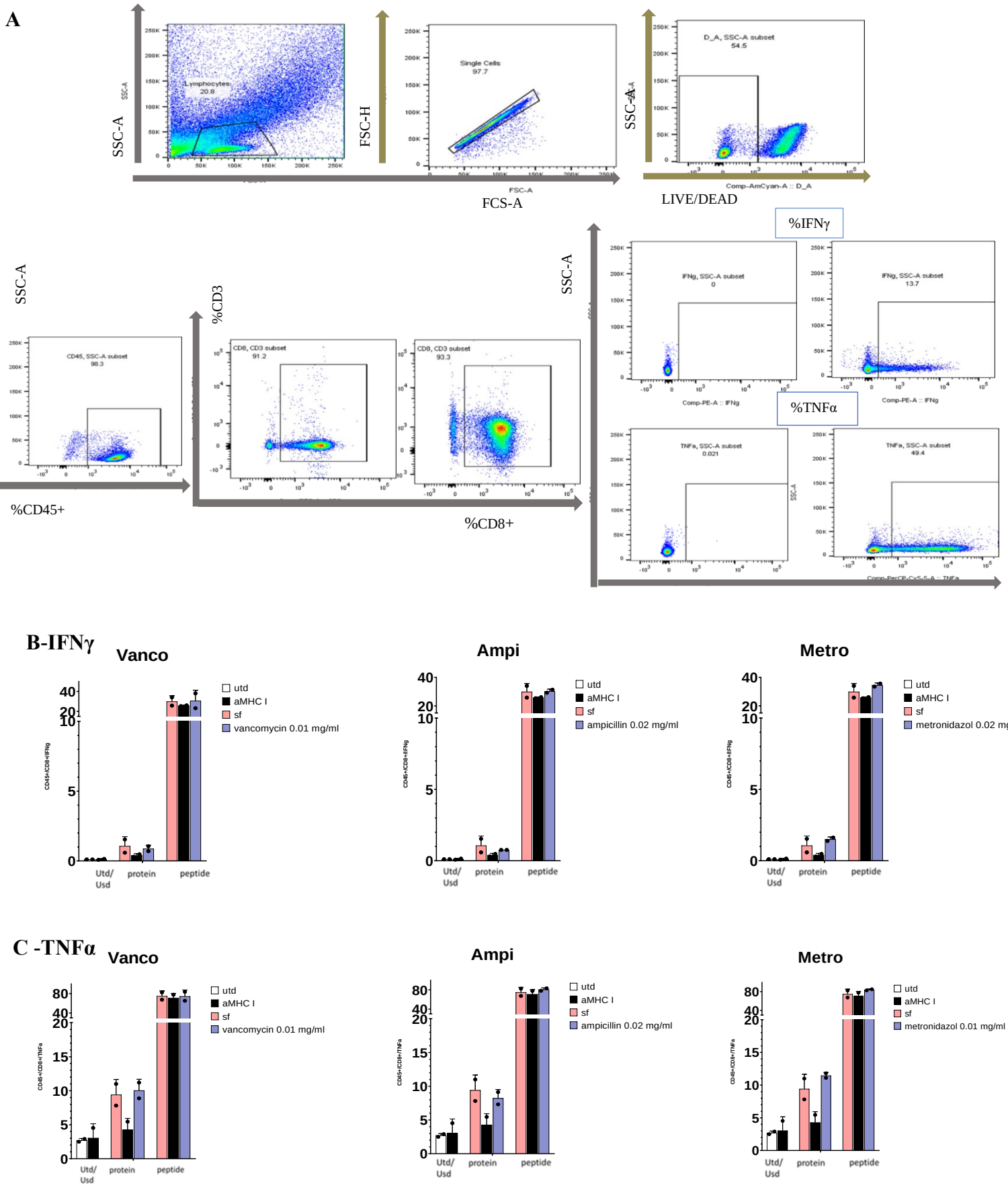


Figure 3.6 Antibiotics administration does not have any direct impact on T cell activation *in vitro*

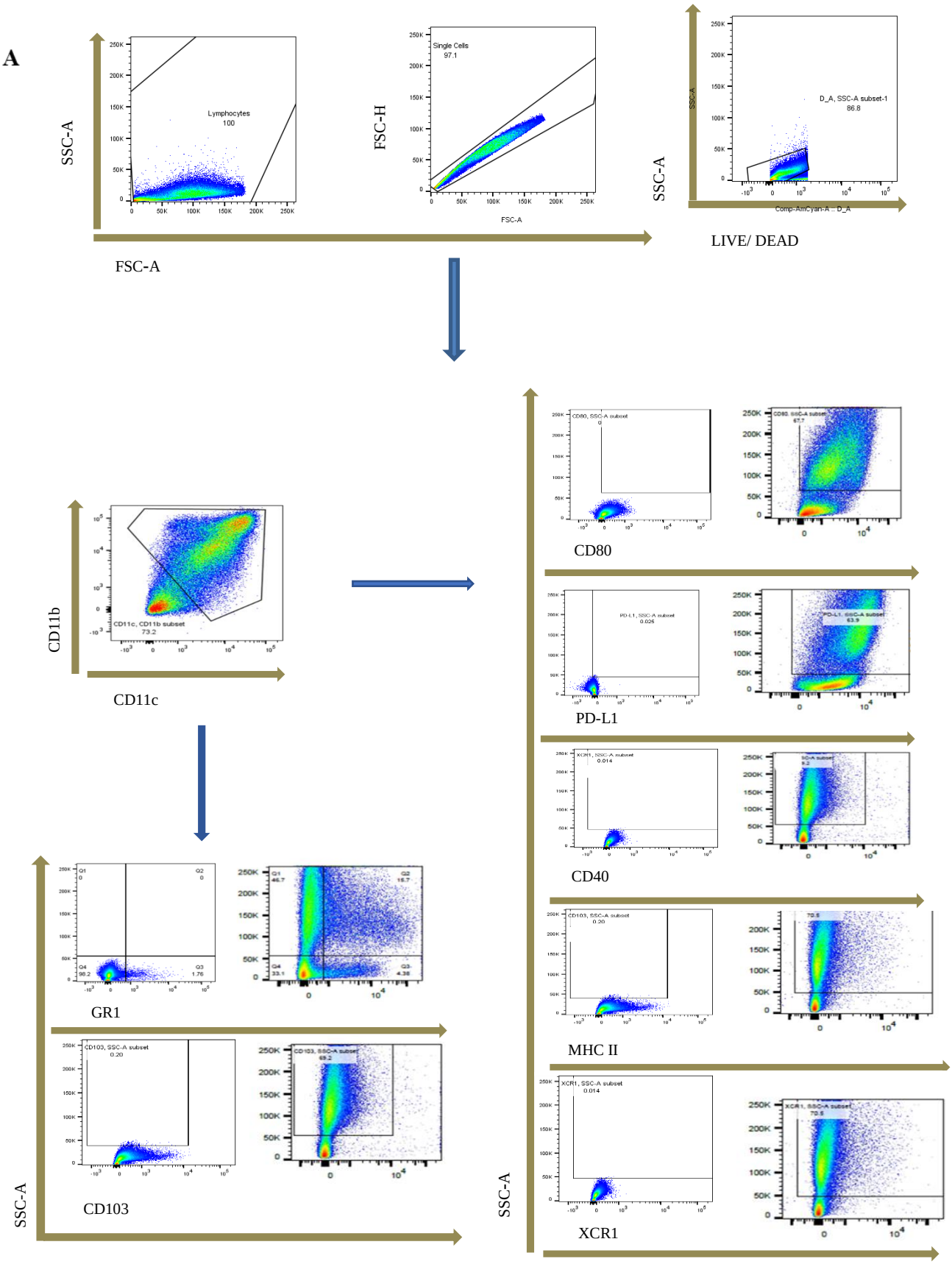
Intracellular staining for IFNγ and TNFα on CD45+CD3+CD8+ T cells co-cultured with DCs that have been treated with vancomycin, ampicillin and metronidazole respectively. **(A)** Gating strategy **(B)** %IFNγ production **(C)** % TNFα production. Representative results of five experiments

3.7 Vancomycin does not affect the DCs differentiation process nor the cytotoxic T cell activation when administered *in vivo*, regardless the route of administration

Based on our observations, we proceeded with *in vivo* experiments to further investigate the impact of treatments on antigen presentation and T cell activation. Vancomycin has been proven to impact the immune response and is also directly correlated to changes on the butyrate concentrations in the gut, so it was the antibiotic of choice for the first *in vivo* experiments. Two routes of administration were selected, oral and intravenous. Vancomycin cannot be absorbed by the gastrointestinal tract, when administrated orally. The rationale for the two routes of administration was to examine the effects of oral vancomycin limited in the gut and impacting the gut microbiome, in comparison to the systemic effects the iv administration could possibly cause. For the intravenous route, four shots were injected in the lateral tail veins, three with five days interval one from the other and one booster one week after the latest shot, all in the concentration of 15mg/kg in PBS. For the oral route, vancomycin was diluted in drinking water for 18 days in the concentration of 500mg/L. At day 19, all groups were sacrificed and bone- marrow derived cells were collected and seeded in order to be differentiated to DCs. On day 7, flow cytometer analysis was performed for the maturation status of DCs. In addition to the previously examined markers CD80 and PD-L1, this time we stained additionally for the surface markers CD103, XCR1, and GR1. CD103+ and XCR1+ DCs promote CD8+ T cell responses (Cao et al., 2016, Audsley et al., 2020) and GR1 marker is important for the identification of different APCs and the type of immune response they induce (Hammond et al., 2012). This analysis concluded in no phenotypical differences between the treated and untreated groups (Figure 3.7).

The rest of the DCs were pulsed either with ovalbumin or OVA peptide, while half received maturation cocktail and half didn't. Consequently all groups were co-cultured with OT-1 T cells and ICS was performed for IFNγ and TNFα production. The vancomycin treatments had no impact on the cytotoxic T cell activation from BMDCs, regardless the route of administration. Both IFNγ and TNFα production decrease much more for the mice treated with oral vancomycin and stimulated with protein when the DCs had not been matured (Figure 3.7, B). No other differences were observed for either of the targeted cytokines between the two routes of administration. Neither spleen derived DCs cause enhancement to the CD8+ T cell activation (Figure 3.8). Overall, no differences were observed in the CD8+ T

cell activation after the administration of vancomycin, no matter what was the route of the administration or the origin of the DCs that were used as APCs.



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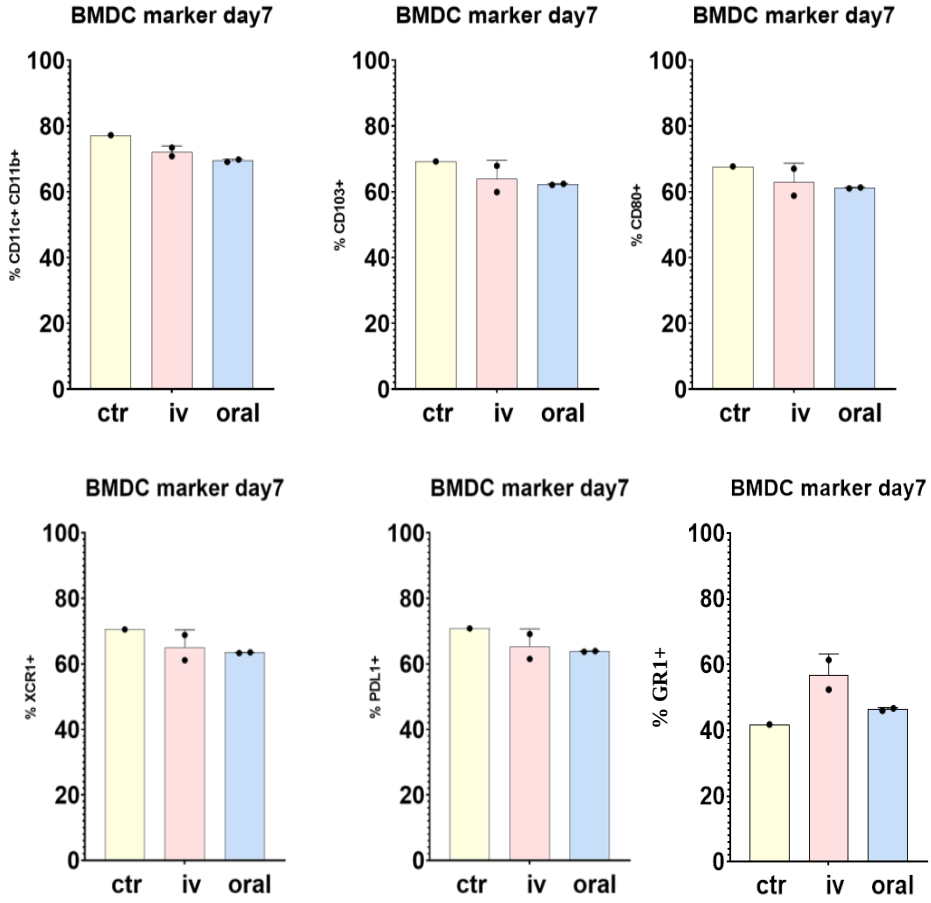

Figure 3.7 DCs phenotype after in vivo vancomycin treatments
Shown are the percentages of maturation markers expressed on CD11c+ CD11b+/- bone marrow derived DCs, after 7 days of in vitro differentiation. **(A)** Gating strategy. **(B)** Percentages of the maturation markers CD103, CD80, XCR1, PD-L1 and GR1 on CD11c+ CD11b+/- DCs. DCs derived from bone marrows of untreated mice (ctr) and mice which received vancomycin either intravenously (iv) or orally (oral). SEM is shown. Representative results of two experiments.

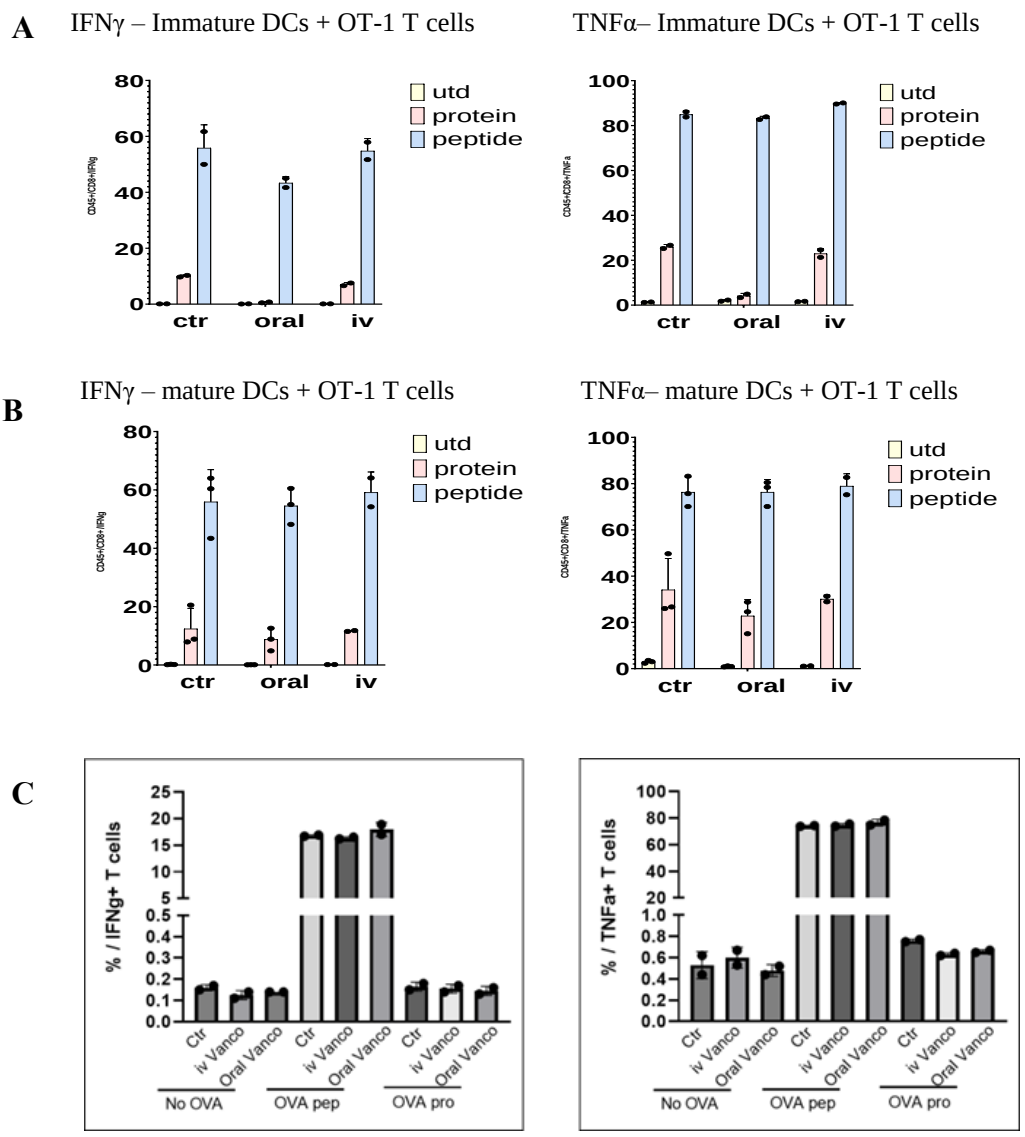

Figure 3.8 In vivo vancomycin administration orally and intravenously had no impact on cytotoxic T cell activation

Shown are percentages of IFNγ and TNFα production by CD45+CD3+CD8+ T cells after co-culture with DCs quantified with FACS, after intracellular staining (ICS) **(A)** ICS was performed on the co-culture of T cells with immature bone marrow derived DCs **(B)** ICS was performed on the co-culture of T cells with mature bone marrow derived DCs. **(C)** ICS was performed on the co-culture of T cells with spleen derived DCs Non stimulated DCs (utd) used as internal control, DCs from mice which had not received any vancomycin (ctr) , DCs from mice which received vancomycin orally (oral) and DCs from mice which received vancomycin intravenously (iv)

Representative results of two experiments.

Chapter 4

Discussion

Antibiotics treatments can alternate the composition of the gut microbiome and, indirectly, SCFAs and other metabolites produced by certain bacteria. The exact mechanisms and pathways by which the modulations of the gut microbiome could regulate host immunity are not clear yet. This thesis examines that changes in the concentrations of specific SCFAs can induce inhibition on the antigen presentation.

In our experiments we decided to expose bone marrow derived DCs to the most well studied and impactful SCFAs *in vitro*. DCs were stimulated either with OVA protein or OVA peptide and co-cultured with OT-1 CD8+ T cells. IFNγ ELISPOT assays were implemented after the co-culture to estimate the frequency of antigen specific CD8+ T cells.

IFNγ is produced by activated CD8+ T cells and holds a key role on immune responses by regulating macrophage activation, antigen presentation, Th1/Th2 balance and control cell proliferation and apoptosis (Tau & Rothman, 1999).

In the first experiments we treated DCs with butyrate and propionate for either 24 hours or 8 days, to determine the duration of our subsequent treatments. According to the literature, in studies relevant to this thesis, exposure of DCs to SCFAs for 24hours (the day of their maturation) is common practice (Nastasi et al., 2015). We observed that the 24 hours treatments could cause stronger inhibition of IFNγ secretion in most conditions. Despite those, we tested and eventually selected the 8 days exposure in order to observe the long-term effect of the treatment, in all stages of differentiation and maturation of the DCs.

Concentrations of 0.01mM, 0.1mM and 0.5mM of butyrate, propionate, or combination of both were tested. The 0.5Mm was eventually excluded as toxic because of the observed reduction in the cell numbers in culture and the damaging impact on the phenotype of the cells. By measuring the relative expression of IFNγ after the co-culture with the T cells, we were measuring the frequency of antigen specific CD8+ T cells. In an *in vivo* system it would be challenging to conclude if the observed impact was strictly related to antigen presentation, or it was due to the effects SCFAs could have on cytokines and other factors involved in T cells activation. In an *in vitro* system like this though, there are no circulating cytokines or other components, so we can claim that our treatments affect indeed the antigen presentation. Without further evaluation, it is unclear whether the SCFAs affect the antigen- presenting ability of the DCs or the recognition of the antigen by the T cells. In our studies, propionate was shown to inhibit antigen presentation and there was no synergistic or antagonistic effect after combining propionate and butyrate (Figure 3.2). Even though the mechanisms still need to be investigated, recent data have shown that pentanoate is able to modulate the immune

response. VPA (valproic acid) reprograms the metabolic activity of lymphocytes (Soria-Castro et al., 2019). It seems to be able to induce IL-10 production from regulatory B cells while reducing the Th17 cells and the production of IL-17A from CD 4+ T cells, all protective modulations against autoimmune diseases. At the same time, VPA seems to be a promising epigenetic anticancer drug (Duenas-Gonzalez et al., 2008). No statistically significant impact was observed in our in vitro studies. Differences in the impact of each treatment were observed when the stimulatory factor was changing from ovalbumin to OVA peptide. In general, the exposure of the DCs to each SCFA showed a distinct effect (Figure 3.2). It is noticeable that the inhibition is more profound in the DCs which have been stimulated with OVA protein when they have been treated with propionate or butyrate, but the exact opposite effect is observed with the pentatonate. These dissimilarities could be attributed to many mechanisms. Each SCFA might have different affinity leading to different responses. The molarity variation could be another explanation. When comparing the OVA peptide to ovalbumin, we compare one specific peptide to multiple ones since the protein can be degraded in numerous peptides after being processed by APCs. The more profound effect on the protein groups might also be suggesting a mechanism related to the antigen processing.

Since mature DCs can no longer process antigens, we explored if our treatments could promote DCs maturation and thus regulate the immune response. The maturation profile of DCs was analyzed with flow cytometry, studying the expression of CD11b, CD11c, CD40, CD80, MHC II and PD-L1 molecules (Figure 3.3). Despite the fact that all DCs share features that distinguish them from other leukocytes, they do not all belong to one lineage. Different groups have been identified with the plasmacytoid (pDCs) and the classical DCs (cDCs) being the two major subsets. pDCs are a small subset of DCs located mainly in blood and lymphoid tissues, while cDCs refer to all other DCs and populate most lymphoid and non-lymphoid tissues. All DCs express constitutively CD11c, CD45 and MHC II which are hematopoietic markers, but depending on the subset and the location DCs can be CD11b positive or negative (Merad et al., 2013). CD11b are predominant markers of macrophages and microglia. CD40 is the co-stimulatory molecule on APCs and binds on the CD40L on T cells. It serves on identifying mature and immature DCs. Similarly, CD80, a member of the B7 family, is another molecule present on APCs which also interacts with T cell molecules and specifically the CD28. PD-L1, another B7 family ligand, acts as an immune brake because its binding partner, PD-1, is an inhibitory checkpoint molecule on T cell response. Both CD80 and PD-L1 are very important as checkpoint proteins downregulating T cell

immune activation. All, CD40, CD80 and PD-L1 are expressed not only on DCs, but on macrophages as well. The expression of the maturation markers was not negatively affected by the exposure to SCFAs (Figure 3.3). On the contrary, butyrate was found to increase the expression of MHC II, PD-L1 and CD80, while propionate was found to increase the expression of CD40+ compared to the control groups. The upregulation of PD-L1 could be preventing the T cell activation since it is an inhibitory molecule. These findings could imply that SCFAs might actually induce further maturation of the DCs than the LPS and IFNγ alone. The differences between the markers could suggest stimulation of different pathways.

DCs uptake and process antigens, form MHC-peptide complexes, migrate to tissues and interact with Natural Killer (NK) cells and T cells, orchestrating innate and adaptive immune responses and inducing immune tolerance (Théry & Amigorena, 2001; Y. Wang et al., 2020). To investigate the impact of SCFAs on genes related to those processes, we looked into IFN*α, IFNβ, Batf3* and *MHC II.* More specifically, type 1 interferons (IFN α and β) are key cytokines for innate and adaptive immune responses. They are produced massively by activated DCs, IFNα especially by plasmacytoid DCs. IFN I can also induce maturation of DCs and modulate their immune abilities. This way they can affect the activation and cytokine secretion by T cells. Batf3 –dependent DCs prime CD8+ T cells and are also required to present cell-associated antigen to CD4+ T cells (Theisen et al., 2019). The expression of the highly polymorphic MHC molecules in cells determines whether an antigen in those cells will be recognized by T cells. Among several other features, the expression of MHC II molecules can be regulated by cytokines and other signals. The principal cytokine which enhances the MHC II expression on APCs is IFNγ produced by NK cells and antigen-activated T cells. TLR signaling induces DC maturation resulting from increased expression of peptide -MHC II molecules. (A. K., Abbas et al., 2022) Maturation of DCs leads to the termination of MHC II synthesis, limiting the generation of peptide–MHC II molecules (Walseng et al., 2010). According to our results, *IFNα* expression was upregulated after propionate administration, while *MHC II* expression is enhanced in the presence of butyrate (Figure 3.4). These results implicate possible involvement of SCFAs in dendritic cells regulation. More specifically, propionate might be enhancing the activation of DCs while butyrate their antigen-presenting ability.

The prevailing cytokine profile of an immune response can elucidate the type of response and the prognosis of a disease or treatment. TNFα and IFNγ are two cytokines which can act in synergy and are able to stimulate immune cells differentiation and migration, among many other functions (Vila et al., 2012) . TNFα can also be selectively cytotoxic for transformed

cells, especially when combined with IFNα (Psarras et al., 2021). In the second series of our experiments we analyzed the production of IFNγ and TNFα by CD8+ T cells, after intracellular staining. IFNγ is secreted from cytotoxic T cells and regulates the immune response, while TNFα mediates in inflammatory and immune functions. TNFα is able to promote the activation and proliferation of naïve and effector T cells and it can also induce apoptosis of the highly activated effector T cells, maintaining the balance between pathogenic and protective T cell pool (Mehta et al., 2018). In our experiments we observed that both propionate and butyrate were inhibiting the antigen- specific CD8+ T cell activation. While the results are in line with prior studies, there are recent studies with contradictory conclusions. A study about tumor responsiveness to chemo- and immunotherapies in mice and human proved recently that gut metabolites and especially butyrate are able to promote CD8+ T cell response (He et al., 2021) . It is unclear why there is such contradiction between these results. Gut microbiome regulates the immune responses in a very delicate way and there are multiple factors that could affect the outcome of a study, such as variations in experimental design (e.g. *in vitro* versus *in vivo* models), combination of therapies and the disease model itself.

SCFAs concentration in the human body and particularly in the gut can be regulated by antibiotics. Antibiotics have been shown to induce dysbiosis, change the bacterial diversity and abundances of taxa and thus, the concentrations of SCFAs (Ramirez et al., 2020). Gas chromatography on faecal samples of healthy subjects who received antibiotics orally showed that SCFAs faecal excretion was seriously affected after the treatments. Vancomycin reduces median total concentration of SCFAs from 69.3 mmol/kg to 19.4 mmol/kg, ampicillin from 62.4 mmol/kg to 47.8 mmol/kg ($p < 0.05$), whereas metronidazole does not change the SCFAs concentrations significantly. All SCFAs returned to normal levels several weeks after the termination of the treatments (Høverstad, Carlstedt-Duke, Lingaas, Midtvedt, et al., 1986; Høverstad, Carlstedt-Duke, Lingaas, Norin, et al., 1986). It is known that broad spectrum antibiotics can cause neutropenia as side effect (Solis & Dehority, 2019). It is also known that some antibiotics can deplete bone marrow Tregs and inhibit GM-CSF secretion by T cells (Lenhoff & Olofsson, 1996) . Vancomycin, which targets Gram-positive bacteria and disrupts the gut microbiota, reduces Tregs to similar levels as observed in GF (germ-free) mice. However, when SPF (specific pathogen-free) mice were treated with a combination of vancomycin and SCFA, the reduction in Tregs was completely restored.(Smith et al., 2013) Collectively, these results suggest that SCFA, play a role in T reg homeostasis (Hansen et al.,

2012). Tregs regulate intestinal homeostasis and control inflammation by limiting proliferation of effector CD4+ T cells. SCFA via Ffar2 may affect T regs through HDAC inhibition (Smith et al., 2013) .When it comes to CD8+ cytotoxic T cells and antibiotics, there are no robust data on possible direct impact of the latter on the former.

We decided to explore the impact antibiotics could have on antigen presentation and CD8+ T cells activation. We began by exploring the specificity of SCFAs treatments on the regulation of the immune responses. More specifically, we tried to define if the observed phenomena were due to the direct effects of antibiotics as chemical compounds on the immune cells, or the indirect regulation of SCFAs levels by antibiotics. Co-cultures of DCs and OT-1 T cells were exposed to antibiotics *in vitro* and the impact on antigen presentation was subsequently assessed by ICS. The antibiotics were selected in order to target different bacteria species and especially bacteria that produce SCFAs. The concentrations of the treatments were selected according to the mean and peak serum concentrations described in the literature. More specifically, vancomycin is a glycopeptide antibiotic active mainly against Gram- positive bacteria. The recommended serum concentration is 15-20mg/L (0.015-0.02 mg/ml). Higher levels of those would be toxic, while for effective treatments a sustaining minimum concentration is necessary for the duration of the therapy. Metronidazole is a broad-spectrum antibiotic targeting mostly Gram- negative bacteria. Its peak serum concentration is 12-40 ug/ml. Ampicillin is a beta-lactam antibiotic. It belongs to the third generation penicillins and is classified as aminopenicillin. Aminopenicillins were created by joining penicillin to an amino group or side chain and aiming to high effectiveness against both Gram- positive and Gram- negative organisms. Ampicillin has very variable minimum inhibitory concentrations in order to achieve therapeutic efficacy while at the same time it is believed that its efficacy depends on a minimum serum concentration for a minimum duration. A concentration higher than the MIC has to be maintained for at least half of the time of the interdose interval (Giachetto et al., 2004). Ampicillin MICs can vary from 0.00003mg/ml to 0.004 mg/ml, while the mean serum concentration can be measured in a range of 0-58mg/ml. According to our observations, antibiotics can be toxic in high concentrations and cause direct cell death, but besides that antigen presentation and T cell activation are not at all affected by non-toxic concentrations of antibiotics, at least *in vitro*. While working with non-toxic concentrations, the percentage of the alive cells was preserved in all conditions and no inhibition was observed with any of the antibiotics. These results show that antibiotics have no direct impact

on antigen presentation and CD8+ T cell activation. This suggests that the observed effects of SCFAs on immune responses can be attributed specifically to them.

In order to examine the immune regulation *in vivo* we decided to use vancomycin, because of its already known role on immune system regulations (Nazzal et al., 2021; Uribe-Herranz et al., 2020; Yang et al., 2021). Oral vancomycin is poorly absorbed, but it has systemic effects, through the robust modulation of the gut microbiome. Because of the above-mentioned characteristics of vancomycin, two routes of administration were selected for our experiments, the oral and the intravenous in order to achieve immediate systemic distribution. Spleen and bone marrow derived DCs were collected from all mice in order to explore plausible phenotypical alterations but also investigate the impact of the antibiotic on antigen presentation. Expression of the markers CD80, PD-L1, CD103, XCR1, and GR1 were analyzed with flow cytometry on CD45+CD11c+CD11b+/- cells. CD103+ DCs protect against infections through cross-presentation to CD8+ T cells and modulate tolerance by inducing T regs (Cao et al., 2016) $XCR1^{+}$ DCs hold a key role in successful adaptive immune responses because they are responsible for processing innate signals to induce specific CD8+ T cell responses, while they participate in the maintenance of peripheral tolerance (Audsley et al., 2020). GR1 is a marker of myeloid differentiation since it increases when myeloid cells mature to granulocytes. Its expression allows distinguishing between macrophages, dendritic cells and other myeloid derived cells and subsets. GR1+ DCs are also known to participate in the inflammatory response and stimulate Th1 responses (Hammond et al., 2012) (Figure 3.7). We did not observe significant impact of the vancomycin treatments in the maturation of the DCs. As for the T cell activation, CD8+ IFNγ and TNFα secretion were evaluated after intracellular staining on co-cultures of the DCs with OT-1 T cells. Vancomycin had no impact on the CD8+T cell activation in any of the two different routes of drug administration. The maturation process of DCs as well as the activation of T cells does not seem to be affected by the route of administration of vancomycin. Mature and immature DCs were evaluated in order to distinguish if the involved mechanisms could be correlated with the antigen processing or the presentation of the antigens. Indeed, we observed that IFNγ secretion was reduced only in the immature bmDCs sourced from mice treated orally with vancomycin. The obvious explanation would be that the immature DCs cannot present the antigens they have processed to T cells, but that doesn't happen with the iv treated mice. These results could suggest that systemic administration affects maturation even in the bone marrow, but further investigation is needed before making such an assumption.

Overall, antibiotics have no direct impact on antigen presentation and CD8+ T cells activation *in vitro* or *in vivo*, at least in a healthy microenvironment. Propionate though seems to cause inhibition of the immune responses *in vitro* and it would be interesting to explore if similar results would be observed by regulating its concentration *in vivo*. Additionally, the mechanism of the inhibition is suggested to be related to antigen processing and DC maturation. In this study propionate was found to increase *IFNα* expression in DCs and decrease TNFα production in T cells. Given Psarras' recent study proving that TNFα can be selectively cytotoxic in the presence of IFNα (Psarras et al., 2021), further investigation in this interplay would be very valuable.

4.1 Limitations of study

Most of the experiments described in this thesis were performed in vitro. Whether our observations would also be validated in vivo and on top of the in vivo experiments conducted in mice, in humans, needs to be evaluated. We did use different concentrations in our in vitro experiments, but this is far from deciding the optimal doses of SCFAs that could be supplemented as regulators of the immune response. How other bacteria and metabolites impact each other and get involved in the immune responses after specific diets, antibiotic treatments and in any different disease, is not clear yet. Moreover, the ovalbumin system allowed us to examine only the CD8+ T cell response and not the CD4+.

4.2 Future directions

Overall, the experiments described here suggest that SCFAs regulate immune responses. Based on these findings and prior studies, the modulation of the gut microbiome could be further studied for its implication in the regulation of distant immune responses. Depending on the bacterial taxa that thrive and the metabolites they produce at each specific time point, the immune response can be boosted or inhibited. However, a series of additional experiments need to be performed in order to further elucidate the interplay between the bacterial-derived SCFAs or other factors and the immune response modulation. A large-scale metabolite screening in healthy subjects and patients who suffer from infectious or autoimmune diseases or cancer patients would set light in related future studies.

All metabolites need to be tested eventually in vivo, in correct concentrations and in different pathologies. Butyrate is probably able to enhance specific immunotherapies efficacy, Radiation Therapy (RT) efficacy and boost CD8+ T cells response, while propionate probably causes immunosuppression. Different durations and doses of treatment and combinations with other therapies need to be studied. All those factors could affect the functions of immune cells and/ or cancer cells, even more in an environment of infection, hypoxia or in the tumor microenvironment. Moreover, genetic immune cell– deficient mouse models could be used in order to validate hypothesized mechanisms involved in immune system regulations.

After responding to vital questions as the above, human studies should be carried out. A wide range of antibiotics needs to be studied, along with the characterization of the gut microbiota composition after treatments with antibiotics. The bacterial taxa that favor or suppress different immune responses need to be determined. Evaluation with metagenomics analyses and taxonomic profiling could provide valuable information, especially after modulation of the gut microbiome with antibiotics, SCFAs supplements, dietary approaches and faecal microbiome transfer (FMT). It would be extremely important all the above to be tested in different combinations with treatments like RT and immunotherapies in order to identify the less favorable microbiome for each modality and be able to modulate it in a favorable way.

Chapter 5

Conclusion

This research thesis has shown that delicate changes in the levels of certain metabolites are able to either boost or inhibit the immune responses. Propionate was shown to have inhibitory effects on antigen presentation and that the mechanism behind that is probably related to antigen processing and involves upregulation of the *IFNα* gene. Butyrate might be impacting the maturation and thus the antigen presenting ability of DCs through regulation of the *MHC II*. Data from FACS and qPCR analyses also suggest that the SCFAs induce maturation of the DCs and regulate the immune responses related to antigen processing. In this study we also proved that antibiotics have no direct impact on T cell activation *in vitro* or *in vivo*. In conclusion, this experimental study provides some insight on the complexity of the crosstalk of the gut microbiome and the immune system. According to our findings we suggest that different SCFAs have distinct effects, but that even the same SCFA can induce different responses if factors like the concentrations or duration of exposure are altered. Subsequent studies will give a better understanding of the underlying mechanisms and might give us the opportunity to modulate the gut microbiome in favorably to treatments and against modalities.

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