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IMPACT OF MATERNAL OBESITY ON EARLY-LIFE MICROBIOTA-INDUCED IMMUNE SYSTEM PRIMING

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirement of Master of Science degree in Applied Microbiology

by

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December 2022

À minha mãe e à minha família, por todo o amor e apoio dado nesta jornada.

"You do not just wake up and become the butterfly, growth is a process" – Rupi Kaur

Resumo

A microbiota intestinal na infância é altamente modificável, estando sujeita à modulação por fatores externos, como a microbiota materna, o tipo de parto e a estratégia de alimentação. Nos primeiros 1000 dias de vida estabelece-se uma importante "janela de oportunidade", em que a microbiota intestinal do bebé influencia o desenvolvimento e maturação do sistema imune. Desta forma, qualquer perturbação à microbiota intestinal durante este período de desenvolvimento terá consequências na saúde futura da criança. A mãe representa uma das principais fontes de microrganismos para o bebé, sendo que, através do mecanismo de transmissão vertical de microbiota, poderá transmitir uma microbiota desregulada em casos de disbiose intestinal materna. Considerando o importante papel da mãe no estabelecimento da microbiota intestinal da criança, o objetivo deste trabalho consistiu na determinação do impacto da obesidade materna na capacidade imunomoduladora da microbiota intestinal infantil.

Para responder ao objetivo proposto foi necessário a otimização de protocolos experimentais, nomeadamente, i) a separação de microbiota intestinal de amostras fecais infantis e inativação com radiação UV, e ii) a estimulação de células dendríticas e células T com a microbiota previamente extraída. Após otimização dos protocolos, avaliou-se um conjunto de fezes provenientes de crianças nascidas de mães saudáveis (n=3) e obesas (n=3), recolhidas ao longo do primeiro ano de vida (1, 6 e 12 meses de vida).

Os resultados preliminares obtidos através da estimulação de células imunes com um número limitado de amostras de microbiota intestinal de bebés de mães obesas e saudáveis revelou que, apesar da microbiota intestinal ter levado à ativação das células imunes, não se observaram diferenças significativas entre as crianças de mães obesas ou de mães saudáveis relativamente à expressão de marcadores de ativação de células imunes.

Em conclusão, este trabalho permitiu a otimização do protocolo experimental para avaliação da capacidade imunomoduladora da microbiota intestinal infantil. Apenas com os resultados preliminares será precoce assumir que a obesidade materna não parece influenciar a modulação do sistema imunitário no início de vida, pois será necessário realizar mais experiências com um número maior de amostras para tirar conclusões robustas.

Palavras-chave: Microbiota Intestinal, Infância, Obesidade, Disbiose, Imunidade

Abstract

Early-life gut microbiota is highly modifiable, and subject to modulation by external factors such as maternal microbiota, mode of delivery, and feeding strategies. And important "window of opportunity" is established in the first 1000 days of life, in which the infant's gut microbiota influences the development and maturation of the immune system. Thus, any disturbances to the gut microbiota during this period of development will have consequences for the child's future health. The mother represents one of the main sources of microorganisms for the infant, and through the mechanism of vertical transmission of microbiota, it may transmit a dysregulated gut microbiota in cases of maternal intestinal dysbiosis. Considering the important role of the mother in establishing the infant's gut microbiota, this study aimed to determine the impact of maternal obesity on the immunomodulatory ability of the infant's gut microbiota.

To achieve our aim, it was necessary to optimize the experimental protocols, namely, i) the separation of gut microbiota from infant stool samples and inactivation with UV radiation, and ii) the stimulation of monocyte-derived dendritic cells and T-cells with the extracted microbiota. Following protocol optimization, a set of fecal samples from infants of healthy (n=3) and obese mothers (n=3), collected over the first year of life (1, 6, and 12 months), were assessed.

The preliminary results obtained through the stimulation of immune cells with a limited sample number of gut microbiota from infants of obese and lean mothers revealed that, although it led to immune cell activation, there were no significant differences between children from obese or lean mothers observed regarding to expression levels of activation markers of immune cells.

In conclusion, this work allowed the optimization of an experimental protocol for evaluating the impact of maternal obesity on the immunomodulatory ability of the infant's gut microbiota. Presenting only preliminary results it would be premature to assume that maternal obesity does not seem to influence the modulation of the immune system in early life, as it will be necessary to carry out more experiments with a larger number of samples to draw robust conclusions.

Keywords: Gut microbiota, Infancy, Obesity, Dysbiosis, Immunity

Acknowledgments

To my supervisor, Professora Doutora Benedita Sampaio-Maia, who always had time to help in whatever was necessary, and for the words of encouragement given in the times I needed most.

To my co-supervisor, Ângela Costa, for the knowledge transmitted and the support provided during my first days at the cell culture lab and the flow cytometer.

To my friends from the Nephrology and Infectious Diseases R&D group, for welcoming me with open arms, the hilarious moments while working at the lab, and the productive conversations about astrology. Mercury retrograde is no challenge when you have such a special group of people on your side.

To the amazing people from the Tumor and Microenvironment group, for the helpful insight in all things in Immunology and flow cytometry (who knows how this thesis would look without your help), and for the most enthusiastic (and longest) lab meetings I have ever attended to.

To the technicians Catarina Meireles and Emília Cardoso from the Translational Cytometry Platform at i3S, for the essential aid given during my first times working in the flow cytometer (and preventing me from having a meltdown while working), and for the help during data analysis.

To the Cell Culture technician Dalila Pedro at i3S, for all the valuable training and help when I was initiating in the cell cultures labs.

To my Master's colleagues, particularly those that have been with me since the first day of the bachelors in Microbiology, for making classes a little more bearable, the bonding moments during exam season, and the long-lasting memories that I will treasure forever. We were a tiny class, but the best one I could ask for.

To my mother, for encouraging me to do my best and believing in my potential (even when I did not), and for the relentless support and advice when I was overwhelmed with anxiety and burnout.

To my friends and family, who have been by me side through all the process, encouraging me and remembering of what I'm capable of.

To Escola Superior de Biotecnologia, for having me as a Master's student and for the all the support given during my academic journey.

And finally, to Instituto de Investigação e Inovação em Saúde (i3S), for giving me access to all its facilities and equipment, and for providing the best get togethers I ever been to.

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List of Abbreviations

AMPs – Antimicrobial peptides

APC – Antigen presenting cells

BCR – B cell receptor

BHA – Brain-Heart Agar

BMI – Body Mass Index

CARD - Caspase activation and recruitment domains

DCs – Dendritic cells

GALT – Gut associated lymphoid tissue

GI tract – Gastrointestinal tract

GLP - Glucagon-like peptide

GPCRs - G protein-coupled receptors

HDACs - Histone deacetylases

HMO - Human milk oligosaccharides

IBD – Inflammatory Bowel Disease

IBS - Irritable bowel syndrome

IECs - Intestinal epithelial cells

IFN-gamma – Interferon gamma

IgA - Immunoglobulin A

IL - Interleukin

ILC - Innate lymphoid cells

ILFs - Isolated lymphoid tissues

iNOS – Inducible nitric oxide

iTregs – Inducible regulatory T cells

LPS – Lipopolysaccharides

MAMPs - Microbe-associated molecular patterns

MFI – Median Fluorescence Intensity

MLNs – Mesenteric Lymph Nodes

MOI – Multiplicity of Infection

NF-kB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NICU – Neonatal Intensive Care Unit

NK cells – Natural killer cells

NLR - Nod-like receptor

NOD - Nucleotide-binding oligomerization domain

OTUs – Operational taxonomic units

PBS – Phosphate-buffered saline

PMA – Phorbol 12-myristate 13-acetate

PRRs - Pattern-recognition receptors

PSA - Polysaccharide A

RA – Retinoic acid

RegIIIγ - Regenerating islet-derived protein III-gamma

SCFA - Short-chain fatty acids

sIgA – Secretory immunoglobulin A

Tfh – Follicular T helper cell

TGF-β - Transforming growth factor beta

Th - T helper cells

Th – T helper cells

TLR - Toll-like receptors

TNF – Tumor necrosis factor

Treg - Regulatory T cells

AMPs – Antimicrobial peptides

1. Introduction

1.1. Gut Microbiota and Early-Life Development

Humans are heavily colonized by different microbial communities, which constitute the human microbiota. These collections of microorganisms can be encountered in different locations of the human body, namely the skin, nasal cavity, urogenital tract, oral cavity, and the gut, where microbial density and diversity are higher (Amabebe *et al*., 2020). The gut microbiota is composed of commensal bacteria, fungi, viruses, and protozoa, which establish a symbiotic relationship with the host, as well as opportunistic microorganisms that may become pathogenic if a disruption in the intestinal microbial community occurs (Milani e*t al*., 2017). The intestinal microbiome is dynamic, suffering alterations due to several host-related factors, such as age, health status, diet, antibiotics intake, and probiotics/prebiotics consumption (Singh *et al*., 2021). The gut microbiota exerts a crucial role in the maintenance of the overall body balance, being involved in numerous physiological processes, such as 1) breakdown of unabsorbed carbohydrates from digestion; 2) synthesis of beneficial microbial metabolites and vitamins as short-chain fatty acids (SCFA) and vitamin K; 3) mood regulation through the gut-brain-axis, as by the production of tryptophan, a serotonin precursor; 4) protection against external pathogens by the competition with adhesion sites and/or release of antimicrobials; and 5) immunoregulation (Ruan *et al.*, 2020; Singh *et al*., 2021).

One of the most important functions of the gut microbiota is the stimulation and modulation of the immune system. The gut microbiota and the immune system are closely related, and any disturbance to the delicate balance of intestinal microbial communities may lead to the development of diseases such as allergies, metabolic disorders, and gastrointestinal (GI) diseases like inflammatory bowel disease. This intimate link between gut microbes and immunity begins early in life, with the initial colonization of the infant's GI tract. It is stated that the GI tract is colonized at birth, during the passage through the birth canal, which goes by the "sterile womb" theory (Escherich, 1988). However, this topic remains controversial, since some studies have detected microbial particles, through 16S rRNA sequencing, in the umbilical cord blood, placenta, and meconium from healthy pregnancies (without reporting any infections) (Senn *et al*., 2020). Nevertheless, both theories highlight the important role the mother plays as the main source of microorganisms for the offspring in early life, influencing the initial microbial colonization patterns (through the vertical transmission of microbiota).

At birth, the infant's gut is rapidly colonized by a wide array of microbes, mainly by facultative anaerobes, such as *Staphylococcus* spp., *Streptococcus* spp., *Enterobacter* spp., and other

members of the *Enterobacteriaceae* family (Lin *et al*., 2022; Martin *et al*., 2016). These anaerobes gradually deplete intestinal oxygen and reduce intestinal oxidation-reduction potential within 48 h of birth, which facilitates the colonization of absolute anaerobic bacteria such as *Bifidobacterium*, *Clostridia*, and *Bacteroides* (Lin *et al*., 2022). Notwithstanding, it is believed that microbial colonization patterns are heavily influenced by mode of delivery. For instance, vaginally delivered infants will be exposed to the mother's vaginal and intestinal microbiota, essentially composed of *Lactobacillus* and *Prevotella*. Infants born through this route, normally, exhibit a more diverse gut microbiota (Martin *et al*., 2016; Milani *et al*., 2017). Concerning children born by C-section, the microorganisms from the mother's skin and hospital environment promote the initial exposure, presenting different colonization patterns in comparison to vaginally delivered infants. In this case, the infant's gut microbiota will be colonized by *Staphylococcus* and *Clostridium*, and it is characterized by low diversity and richness (Martin *et al*., 2016; Milani *et al*., 2017). Furthermore, cesarean-delivered infants experienced a delay in colonization by *Bifidobacterium* (Isolauri, 2012).

Due to its plastic and dynamic characteristics, early life microbiota can be altered or modulated by external factors, namely early life diet (breastfeeding or formula), gestational age at birth, if were preterm or term newborns, and maternal health or habits.

Early-life gut microbiota can differ between term and preterm infants. Compared to term infants, preterm newborns exhibit lower microbial diversity, and experienced a delay in the establishment of a healthy microbiota, due to the overgrowth of certain bacterial species, such as *Enterococcus* (commonly associated with nosocomial infections, and consistent with a hospital stay) (Korpela, Blakstad, *et al.*, 2018; Rougé *et al*., 2010). It is also known that preterm babies are at higher risk of developing complications, often requiring invasive medical interventions, antibiotic administration, and a stay at the Neonatal Intensive Care Unit (NICU) (Henderickx *et al*., 2019). A recent study by Chi *et al* (2019) (Chi *et al*., 2019) found that the gut microbiota of preterm infants was enriched *Klebsiella*, a microorganism often implicated in hospital infections, that may be transmitted through medical procedures.

Maternal habits during pregnancy, namely the use of antibiotics or probiotics, can also impact the infant's intestinal microbiota. A study in an animal model demonstrated that antibiotic treatment during gestation leads to intestinal microbiota disruption (imbalance between bacterial phyla) and intestinal damage in neonatal mice (Chen *et al.*, 2021). Recent studies in human babies revealed similar conclusions. Turta *et al* (2022) (Turta *et al*., 2022) demonstrated that intrapartum administration of antibiotics (during gestation) resulted in short-term disruption of infant gut microbiota, with a reduction in microbial richness and an increased in

the relative abundances of *Clostridiaceae* and *Erysipelotrichaeceae*. On the opposite end, maternal probiotic treatment seems to have a restorative effect on early-life gut microbiota. Korpela *et al* (2018) (Korpela, Salonen, *et al*., 2018) evaluated the impact of probiotics, when taken during gestation and after birth, on infant gut microbiota, and found that the treatment increased levels of *Bifidobacteria* and decreased levels of potential pathogens (*Clostridia* and *Pseudomonadota*), although this modulation of the gut microbiota was heavily dependent on the infant's diet.

Early life feeding strategies may impact microbial colonization of the gut of the infant (Robertson *et al*., 2019). As such, an exclusive breastfeeding strategy constitutes an important source of lactic acid-producing bacteria and human milk oligosaccharides (HMO), creating an environment beneficial for the proliferation of *Lactobacillus* and *Bifidobacterium*, known probiotic genera that can be metabolized by certain bacterial strains (Ventura *et al*., 2019). In comparison, formula-fed infants exhibited a more diverse microbiota (higher number of bacterial genera) than breastfed counterparts, possibly because formula milk contains a different array of nutrients that modulate the gut microbiota (Milani *et al*., 2017). To demonstrate the impact of an early-life diet on the gut microbiota, Brink *et al* (2020) *(Brink et al.*, 2020) compared fecal microbiota and metabolites in breastfed and formula-fed infants (with dairy-based and soy formula milk), during the first year of life. Soy-based infant formula differs from the common dairy-based formula, due to the presence of soy protein isolates instead of bovine protein isolates (Byrne *et al*., 2021). The different diet regimens lead to differences in overall microbial diversity, with breastfed infants displaying lower diversity and higher levels of *Bifidobacterium*. In a cross-sectional study, Ma *et al* (2020) compared the microbial composition and diversity of the gut microbiota of infants fed with breastmilk or formula. It was found that despite *Bifidobacterium* being the most abundant genera in both groups, the relative abundance of potentially pathogenic bacteria (such as *Streptococcus*, *Clostridium*, and *Enterococcus*) was higher in formula-fed babies (Ma *et al.*, 2020). To further highlight the beneficial effects of breastfeeding, Liu *et al* (2019) evaluated the restorative effects of breastmilk in cesarean-delivered infants. The study has shown that in infants born by C-section and exclusively breastfed, not only their gut microbiota closely resembles the one of vaginally delivered infants, but that breastmilk was able to restore the presence of beneficial bacteria (such as *Faecalibacterium*) and reduce the abundance of bacterial genera typically associated with this mode of delivery (such as *Enterococcus* and *Veillonella*) (Liu *et al.*, 2019).

After 6 months, with the introduction of solid foods (weaning period), there is a significant increase in α-diversity[1] , shifting from Pseudomonadota (previously Proteobacteria) and Actinomycetota (previously Actinobacteria) to Bacillota (previously Firmicutes) and Bacteroidota (previously Bacteroidetes) (Milani *et al*., 2017). Also, in the transition from an exclusively-breastmilk regimen to solid foods, a significant decrease in bifidobacteria was reported (Zhuang *et al*., 2019). During this period, the child's gut microbiota changes, leading to a more adult-like composition, which reaches a stable composition at the age of 3. The adult microbiota is dominated by bacterial strains capable of producing SCFA, as well as degrading glycans and complex carbohydrates. Hence, proper nutrition during the weaning period is crucial for the establishment and maintenance of mature microbiota. Malnourishment and/or a high-fat diet, prevalent in Western societies, may lead to gut dysbiosis and growth impairment (Singh *et al.*, 2020). This topic will be further discussed in the last chapters, regarding gut dysbiosis and obesity.

Other factors that may influence the composition and colonization patterns of infant gut microbiota include geographical location (closely related to cultural and dietary practices), family lifestyle (close relatives, siblings, and pets), and host genetics (Zhuang *et al*., 2019). The contribution of the host's genetics to the composition of gut microbiota remains unclear, as most conclusions are drawn from studies on twins. While some studies demonstrate that monozygotic twins exhibit a very similar microbiota, other studies debunked those theories, demonstrating no significant differences were found between identical twins (RodrÍguez *et al*., 2015). Therefore, more studies are necessary.

Interaction with household members such as siblings and pets can modulate the child's gut microbiota. Studies have found that living with older siblings was associated with increased microbial diversity (increased alpha and beta diversity) and richness and that the number of siblings was positively correlated with microbial diversity (Christensen *et al.*, 2022; Laursen *et al*., 2015). Furthermore, the existence of siblings could offer protection against allergies(Christensen *et al*., 2022). Similarly, living with pets is related to increased microbial diversity and richness in the intestinal microbiota of infants, with an increased abundance of certain bacterial species, such as *Ruminococcus* and *Oscilospirra* (Tun *et al*., 2017; Zoratti *et al*., 2020). Furthermore, it was found that prenatal exposure might be associated with a decreased streptococcal colonization and, thus, may lead to decreased risk of developing

[1] Alpha-diversity (α-diversity) is a diversity parameter used to evaluate the richness and evenness of a given sample, in a certain environment. Beta-diversity (β-diversity) is applied to analyze microbial diversity between different samples in a certain environment (Walters, K. E., & Martiny, J. B. H. (2020)).

metabolic diseases and atopy (Tun *et a*l., 2017). However, additional studies are necessary to further comprehend the link between pet-infant gut microbiota and its impact on the child's health.

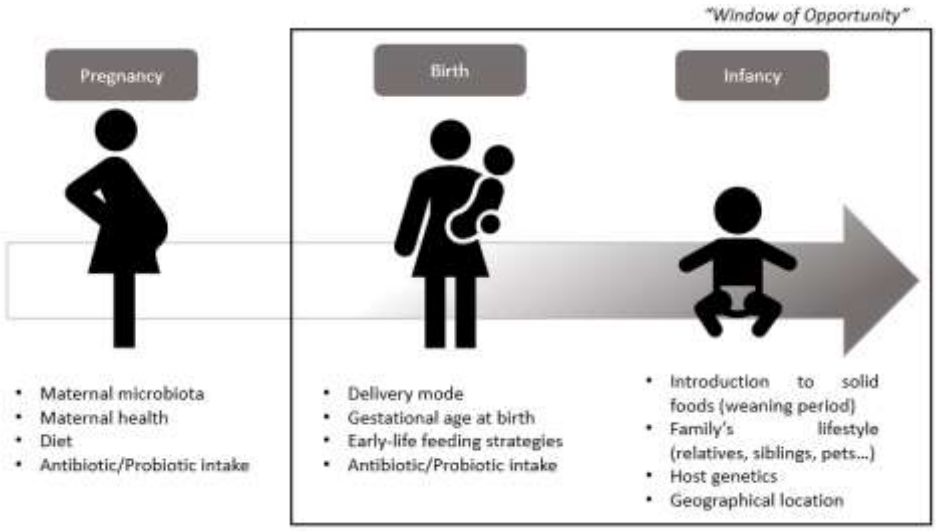


Figure 1.1 – Factors that influence microbiota composition in early-life.

In summary, the infant gut microbiota is dominated by specific bacterial genera, depending on the development stage. After birth, the early-life gut microbiota is mainly composed of *Bifidobacterium* and *Lactobacillus*, due to maternal transmission of microbiota and breastfeeding (as these bacterial genera thrive in the presence of breastmilk HMO). Other bacterial genera that may be present in infant gut microbiota include *Clostridium* (despite being a pathogenic organism, it can remain asymptomatic in newborns), *Bacteroides*, *Enterobacteriaceae*, *Streptococcus*, and other genera found in lower relative abundances, such as *Akkermansia* (Moore & Townsend, 2019; Yao *et al*., 2021).

It is postulated that, after the first 1000 days of life, the microbiome is already established and maintained by the host. During this period, the microbiota and its metabolites modulate the development of the infant's immune system, offering the antigenic stimulus necessary for its maturation, through the establishment of the tolerance system that differentiates commensal microbiota from pathogenic microorganisms (Zhuang *et al*., 2019). Therefore, throughout the first days of life, an important "window of opportunity" is established, in which the interactions between gut microbiota and the host are crucial for the development of a balanced immune system (Zhuang *et al*., 2019).

1.2. Gut Microbiota and Immune System Interactions in Early-Life

1.2.1. Early-Life Immune System Development

Under normal conditions, the dynamic interactions between the gut microbiota and the host's immune system aid in the maintenance of the overall body balance. Along with intestinal and immune cells, the gut microbiota constitutes another barrier of protection, competing with pathogens for nutrients and adhesion sites in the intestinal mucosa and preventing the colonization and replication of external pathogens (Ji Youn Yoo *et al*., 2020). Moreover, the gut microbiota can regulate both local and systemic immune responses either directly, by interacting with adaptive and innate immune cells, or indirectly through the production of microbial metabolites (Ji Youn Yoo *et al*., 2020). As the infant's gut is colonized by different microbial populations, it triggers the immature immune system and induces tolerance towards the commensal microbiota. Observations from germ-free mice demonstrated that gut microbiota colonization in early life is essential for optimal immune development, as these animals presented underdeveloped mucosal immunity, reduced number of immune cells as IgA-producing plasma cells, CD4+ and CD8+ T-cells, and reduced capacity of resisting to pathogenic bacteria (Sommer & Bäckhed, 2013). Therefore, early-life gut colonization is a sequential process that influences the maturation and development of the child's immunity, such as through the production of SCFA (small organic monocarboxylic acids, composed of a chain with less than 6 carbon atoms, primarily comprised of acetate, propionate, and butyrate, with beneficial properties to the host's health (Y. P. Silva *et al*., 2020; Yang et al., 2020)) or the control of proliferation and differentiation of T and B cells (Tanaka & Nakayama, 2017).

The immune system development starts in utero, as soon as 8 weeks of development when the thymus is colonized by hematopoietic cells and at 12 weeks of gestation the lymph nodes of the fetus are already evident. T-cell development in the thymus begins around 14 to 17 weeks of gestation and, around this time, these cells are already detected at the lymph nodes as well. On the other hand, B cells are already present in the primary follicles of lymph nodes by 17 weeks of gestation. Conversely, dendritic cells (DCs) and circulating monocytes are only detected in the second trimester of pregnancy, increasing until the end of pregnancy. As for neutrophils, these are not present until the third trimester of pregnancy but are the predominant population of cells at birth (Wilson & Messaoudi, 2015). Although throughout this period the exposure of the fetus to live bacteria remains controversial, the presentation to diverse microbial metabolites and fragments may happen via the placenta (Jain, 2020). Specific gut commensal-specific IgG antibodies may be transferred to the fetus, regulating its mucosal CD4+ T-cell response to

microbial antigens at birth. Interestingly, in a mouse model, maternal antibodies that only were present during pregnancy improved the transmission of microbial molecules (during pregnancy), which primed their pup's immune system and suppress allergic airway diseases by increasing Treg cells number and functions later in life (Alison N. Thorburn et al., 2015).

During delivery, infants receive "a bacterial boost" mainly from the mother, exposing the immature immune system of the newborns to a significant microbial load (Abenavoli *et al*., 2019). A recent study by Wampach *et al* (2018) assessed the immunostimulatory potential of maternal microbiota transferred to infants born through vaginal or caesarean delivery. This study reported that faecal LPS isolated 3-days after vaginally-delivered neonates were born induced higher levels of cytokines in monocyte-derived dendritic cells, compared with faecal LPS from caesarean-delivered neonates (L. Wampach *et a*l., 2018). Since LPS is recognized by toll-like receptor 4 (TLR-4) on the membranes of intestinal epithelial cells and it stimulates components of the immune system associated with the prevention of mucosal damage and infection, these results suggest that differences in the initial microbiota inherited by type of delivery may impact the immune system stimulation and predispose to a vulnerability against infection (Jašarević & Bale, 2019). Moreover, a recent study in mice observed that vaginally-delivered offspring acquired immune tolerance via spontaneous activation of the intestinal epithelial cells and acquired resistance to LPS shortly after delivery, whereas C-section-born pups and TLR4-deficient mice did not (Lotz *et al*., 2006).

After delivery, the immune system of the new-born is still functionally immature and is markedly modulated to avoid excessive inflammatory responses. For this reason, it is observed a dampening of the immune system mechanisms, characterized by a decrease in the complement function, neutrophils amount and functions, production of pro-inflammatory cytokines (IL-1β, tumor necrosis factor (TNF)-α, and IL-12p70) by antigen-presenting cells (APC) and an increased expression of inhibitory receptors by NK cells, such as NKG2A/CD94 (Esteve-Solé et al., 2018; Wang *et al*., 2007). Simultaneously, CD8 T cells present low activity and monocytes, and macrophages still show immaturity (Méndez et al., 2021). Still, *in utero*, the fetal immune system has a Th2- T-cell phenotype to prevent alloimmune responses against the mother (Abenavoli *et al*., 2019). This phenotype remains after delivery, with the infant's immune system still biased towards a Th2 phenotype, presenting low IL-2 and IFNγ (Wilson & Messaoudi, 2015). The low production of such cytokines can be due to functional differences in dendritic cells which may prevent the differentiation of naïve CD4 T cells towards Th1 (Wilson & Messaoudi, 2015). However, after multiple pathogenic encounters and in a time- and age-dependent manner, the infant switches towards a Th1 polarization, promoting a pro-

inflammatory profile with negative consequences in the child's long-term health (Abenavoli *et al*., 2019).

Likewise, B cells are mostly naïve at birth and their ability to respond is limited possibly due to their immaturity, poor B cell repertoire, or reduced strength of B cell receptor (BCR) signaling (Basha *et al*., 2014; Wilson & Messaoudi, 2015). Additionally, neonates have underdeveloped germinal centers in lymph nodes and spleen and low expression of B-cell receptors, resulting in low levels of primary IgG responses to infections and vaccines (Basha *et al*., 2014). Therefore, neonates exhibit an increased susceptibility to infections because of the immaturity of their lymphocytes, low numbers of effector-memory T-cells, low Th1 cytokine secretion (Th2 polarization), and reduced strength of BCR signaling.

Since the newborn adaptive immune system is exclusively constituted by naïve T and B cells, during the first 6 months of life, maternal antibodies acquired *in utero* and breastmilk, provide additional protection against pathogens in the GI and respiratory tract (Wilson & Messaoudi, 2015). Indeed, the feeding strategy also may impact early-life immunity by modulation of the production of microbial metabolites, with breastfed infants displaying higher concentrations of fecal butyric acid (derived from the metabolism of SCFA and able to increase regulatory T cell production), d-sphingosine (involved in immune cell trafficking) and betaine (inhibits inflammatory responses) (Brink *et al*., 2020). Furthermore, breastmilk also provides IgA, the main immunoglobulin in mucosal protection, enabling a tolerant and balanced immune system (Milani *et a*l., 2017). The secretory IgA (sIgA) present in the breastmilk is produced by plasma cells in the mammary gland but originally from the gut of the mother. Therefore, the specificity of the IgA is modulated by the maternal exposure to her enteric bacteria, both commensals, and pathogens, therefore protecting the expansion of the latter while the child's immune system is maturing (van den Elsen *et al*., 2019). Furthermore, IgA also seems to shape the gut microbiota of the child with a long-lasting effect, which is fundamental to prevent excessive expansion of pro-inflammatory microbial taxa, and in the modulation of microbial gene expression and metabolic function (van den Elsen *et al.*, 2019).

The concentration of immune cells varies in different age groups (Méndez *et al*., 2021; Valiathan *et al*., 2016). Lymphocytes and B cells appear to decrease significantly with age, whereas neutrophils and CD8+ T cells increase in adulthood, and NK cells increase in adolescence. Throughout infancy and early childhood, the Th2 phenotype changes to a Th1 phenotype, potentiating macrophage activation and cellular immunity (Wilson & Messaoudi, 2015). At the age of 2, dendritic cells start producing the cytokine IL-12p70, which then stimulates the Th1 response. Furthermore, with further antigen exposure, T-cell response

increases, and cytotoxic and helper T-cells develop memory towards the antigen (Méndez *et al*., 2021).

In sum, the first 1000 days of life are fundamental not only to the microbiome establishment, but also to the concomitant development and maturation of the immune system and, consequently, to the interplay between both of them. Hence, this period is considered to represent a microbiological and immune 'window of opportunity' during which any event will have a pivotal impact on the metabolic, immunological, and microbiological priming, which may later affect human health (Selma-Royo *et al*., 2019).

1.2.2. Gut Microbiota and Immune System Interactions

Concerning innate immunity in the gut, it begins with the direct exposure of intestinal epithelium and commensal bacteria that compose the gut microbiota. The intestinal epithelium, besides constituting a physical barrier separating the commensal microbiota from the underlying tissues, also has physiological functions such as the secretion of antimicrobial compounds, cytokines, and sIgA (C. M. Maranduba *et al*., 2015; Wu & Wu, 2012).

The complex balance between host immunity and gut microbiota is established when the commensal microorganisms interact with IECs, through pattern-recognition receptors (PRRs). PRRs, which can include TLR and nucleotide-binding oligomerization domain (NOD)-like receptors, when activated by microbe-associated molecular patterns (MAMPs), induces the production of antimicrobial peptides, such as RegIIIγ, and immunological mediators such as IL-18, IL-33, IL-25, and tumor growth factor-β (TGF-β) which, consequently, promote the production and proliferation of tolerogenic macrophages (producers of high levels of IL-10) and dendritic cells (DCs), which stimulate the production of regulatory T cells (Treg) (C. M. Maranduba *et al*., 2015; Thaiss *et al*., 2016). In the specific case of TLR, these receptors can be activated by a wide variety of microbial antigens (including LPS, peptidoglycan, and flagellin) and trigger a signaling cascade that results in the activation of the NF-kB transcription factor, which regulates the expression of cytokines, chemokines, and other immunological mediators. A known example of the role of TLR in gut eubiosis (balanced gut microbiota) is the model of polysaccharide A (PSA) of *Bacteroides fragilis*. PSA can be detected by TLR2 and TLR1 couples with Dectin (a C-type lectin PRR), promoting the activation of anti-inflammatory genes. Furthermore, Dectin-1 can control gut immunity by altering the microbiota pattern, leading to Treg differentiation (Al-Rashidi, 2022). Moreover, PRRs can also contribute to the elimination of pathogenic microorganisms, through the expression of the inflammasome-

forming NLR family CARD-domain containing protein 4 (NLRC4) which triggers the detachment of the infected epithelial cells when it detects to be infected by a pathogen (Thaiss *et al*., 2016).

The gut microbiota can also stimulate different sets of innate lymphoid cells (ILC), cells derived from the common lymphoid progenitor in the bone marrow, and divided into different groups, according to their cytokine profiles and transcriptomes: group 1 ILC, group 2 ILC, and group 3 ILC (Ganal-Vonarburg & Duerr, 2020) (Al-Rashidi, 2022). Each group of ILCs acts as the "innate counterpart" of T helper cells, Th1, Th2 and Th17, since they share a similar cytokine expression profile. However, despite some similarities, ILCs can respond faster to a pathogen, compared to T helper cells. In the intestine, ILCs, particularly ILCs from group 3, can be found in the small intestine lamina propria, representing the first line of defense towards external invaders (namely, by presenting antigens to Th cells and promoting their differentiation and activation) and acting as essential players in the maintenance of intestinal barrier integrity (upon damage to the epithelium, ILCs are activated to restore barrier function) (Zheng & Zhu, 2022). ILCs can also interact with the intestinal microbiota. The commensal microbiota induces the secretion of intestinal cytokines, and in turn, ILCs can react to the gut microbiota by altering their structure, with consequences on gut immunity (Han *et al*., 2019).

Concerning cell types, M cells are one of the most important cell types present in the intestinal epithelium, since they directly interact with the local immune system, sampling antigens from the lumen and presenting them to antigen-presenting cells located under the epithelium. Enteroendocrine cells act in the local immune system through the production of glucagon-like peptide 2 (GLP-2), involved in the regulation of the innate immune response, by controlling the expression of AMPs (C. M. Maranduba *et al*., 2015). Lastly, the third layer contains elements of the local immune system, namely, gut-associated lymphoid tissues (GALT). Additionally, in the lamina propria, mature isolated lymphoid tissues (ILFs) can be found, where B and T cells are located. The immune response begins with the recognition of MAMPs by PRRs (pattern recognition receptors), located on IECs or dendritic cells, which can then recruit activated B and T cells. Payer patches, located under IECs, also contain plasma cells that produce IgA, implicated in mucosal immunity (C. M. Maranduba *et al*., 2015).

Intestinal dendritic cells are pivotal to the initiation of the primary immune response, limiting reactivity towards the commensal gut microbiota and recognizing potential pathogens (D'Amelio & Sassi, 2018). These immune cells are typically found in gut-associated lymphoid tissues, such as Peyer's Patches, and under the epithelial monolayer, where they can recognize

foreign antigens, including antigens from commensal microbiota, and present them to cells from the adaptive immune system (Owen & Mohamadzadeh, 2013).

Intestinal DCs are continuously exposed to luminal antigens and thus, in order to distinguish between innocuous microbiota and pathogenic microbes, establishing a tolerance system, they sample the luminal content (Stagg, 2018). Intestinal DCs have distinct ways of antigen recognition and capture: they can extend dendrites across the epithelial layer and capture translocated IgA immune complexes, or sample the luminal space through M cells (present in the epithelium of Peyer's Patches), which internalize microorganisms and deliver to DCs (Stagg, 2018; Swiatczak & Rescigno, 2012). After capturing the microbial antigen, gut DCs' activation is facilitated by PRRs, such as TLRs. Concerning DC interaction with gut microbiota, the typical response is tolerance to prevent inflammation – a mechanism known as oral tolerance, a state of non-response towards antigens from food (Sun *et al*., 2020). The main location for this process is within the mesenteric lymph nodes (MLNs), in which DCs are strong promotors of tolerance. Certain subsets of DCs, such as DCs expressing CD103, are capable of mediating the tolerogenic process by capturing the commensal antigen and migrating into the MLNs, in which they induce the *de novo* differentiation of naïve T cells into $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs), via Tumour Growth Factor-β (TGF-β), and retinoic acid (RA) dependant mechanism (Rescigno *et al*., 2008). RA is particularly important as it is involved in the induction of gut-homing specificity in activated T cells, inducible Tregs (iTregs), and suppression of differentiation of Th17 (Owen & Mohamadzadeh, 2013). Additionally, intestinal DCs can promote IgA class switching in B cells, further enhancing the maintenance of gut homeostasis (Tezuka & Ohteki, 2019). For instance, bacterial products, and certain species of probiotics (beneficial microorganisms) have been shown to promote tolerance in DCs, simply by attaching to their surface. A few examples include the interaction between intestinal DCs and *Lactobacillus acidophilus* NCFM (a food supplement), in which the bacteria was able to promote the secretion of interleukin-10 (IL-10), an anti-inflammatory cytokine (Swiatczak & Rescigno, 2012). Besides live bacteria, bacterial products can also induce a tolerogenic profile in intestinal DCs. Wang *et al.* (2006) shown that, when exposed to the bacterial product polysaccharide A, derived from *Bacteroides fragilis*, intestinal DCs promote inducible nitric oxide synthase (iNOS), to produce nitric oxide, which has a protective role in the gut (Tezuka & Ohteki, 2019; Wang *et al*., 2006).

The adaptive immune system is composed of B cells and T cells. B cells are responsible for antibody secretion, which then coat pathogens, targeting them for opsonization, activation of the complement system, and neutralization. This antibody production, affinity maturation, and

class switch recombination require complex interaction between DCs-B cells in the Peyer patches, dependent on the secretion of TGF-β by ILCs. One important immunoglobulin secreted is IgA, which is secreted by plasma cells in the lamina propria (Kurachi, 2019). Bacterial-loaded DCs induce the differentiation of B cells into IgA+ plasma cells, IgA secretion, and IgA coating of the pathogen for elimination. This stimulation can be done either by T-cell-dependent or independent mechanisms (Tezuka & Ohteki, 2019). Regarding the interaction between B cells and the gut microbiota, as the production of sIgA is low after birth, the absence of sIgA is newborns is compensated by the presence of these antibodies in the mother's breastmilk. In this way, breastmilk IgA plays an important role in modulating the gut microbiota in early life by driving microbial colonization and immune system maturation and priming (A. N. Thorburn et al., 2015). As the neonate begins to be progressively colonized by different species, the immune system responds by producing endogenous sIgA, which will then modulate the composition of the microbiota (Thorburn *et al*., 2015). The mechanisms behind the control and proliferation of commensal bacteria are still to unveil, but it is hypothesized that it might be related to the sIgA binding promoting changes in bacteria itself via regulation of gene expression, which will influence their metabolic processes and the biogeography of the gut (Kato *et al*., 2014).

Regarding T cells, there are two main subsets: CD4+ T helper (Th) cells and CD8+ cytotoxic T cells. The main function of CD8+ T cells is clearing intracellular pathogens and tumors and the effect of gut microbiota in the CD8+ cytotoxic T cell functions is still poorly characterized (Kurachi, 2019; Wang *et al*., 2019). CD4+ T cells can be subdivided into Th1, Th2, Th17, Treg, and follicular helper (Tfh) cells. All these subsets have distinct tasks: Th1 produce cytokines such as IL-2 and IFNγ, responding promptly to virus and bacteria; Th2 direct their response to parasites, through the production of IL-4 and IL-10; Th17 have a crucial role in the response to extracellular bacteria and fungi; Treg are negative regulators of the immune response and protect against auto-immunity; and finally, Tfh main role is to regulate B cell immunity in the germinal centers of the lymph nodes (Wilson & Messaoudi, 2015). T cells are activated and differentiated after contact with APCs, such as macrophages or dendritic cells, by the presentation of pathogen-derived peptides. After activation, CD4+ T cells differentiate into different subsets, from which the most studied ones are Th17 and Treg. In the case of Th17, in response to stimuli (such as segmented filamentous bacteria – SFB), naïve CD4+ T cells can migrate to the intestinal lamina propria and differentiate into IL-17A-producing Th17 cells. Consequently, cytokines produced by these cells (IL-17A, IL-17F, IL-22) stimulate IECs to produce AMPs, helping to preserve a non-inflammatory state of the intestinal barrier(Wang *et al*., 2019). On the other hand, Treg cells located in the intestinal lamina propria have a

fundamental role regarding tolerance towards commensal microbiota. For instance, RORγt+ Tregs population located in the colon seems to be expressed in a microbiota-dependent manner. In germ-free mice, this population seems to decrease and, during weaning, the generation of RORγt+ Tregs is associated with a decreased susceptibility to allergic inflammation later in life. Moreover, the induction of peripheral Treg cells can suppress abnormal inflammatory responses (Wiertsema *et al.*, 2021). Overall, intestinal homeostasis is maintained by a balance between the effector T cells and Treg cells, which mediate immune responses and confine excessive immune activation, respectively (Wang *et al*., 2019). Likewise, some probiotic *Lactobacillus* strains improve intestinal inflammation by modulating the ratio between Treg and Th17 cells (Wang *et al*., 2017).

The production and release of gut microbial metabolites, such as bile acids, branched-chain amino acids and trimethylamine N-oxide, can also greatly impact the host's immune response, influencing inflammatory signaling and interactions with immune cells (Agus *et al*., 2021). Certain gut bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis* (known probiotics), can generate microbial metabolites through the anaerobic fermentation of complex carbohydrates (dietary fibers and resistant starch), namely SCFA (Silva *et al.*, 2020). Shortly after its production, SCFA are absorbed by colonocytes, through H^+-dependent or Na^+-dependent monocarboxylate transporters, and then transferred to the liver, where they are used as an energy supply for hepatocytes, with the exception of acetate, which is oxidized. A small fraction of SCFA can also be carried through the systemic circulation to other tissues (Y. P. Silva *et al*., 2020). Being the most abundant microbial-derived metabolites, SCFA influences gut health through several mechanisms, specifically, maintenance of gut barrier integrity, mucus production, and protection against inflammation and pathogens. These mechanisms occur due to signaling triggered by SCFA, which can bind to G protein-coupled receptors (GPCRs), expressed in several cell types, or through the inhibition of histone deacetylases (HDACs), a mechanism often linked to immune tolerance and anti-inflammatory phenotype, aiding in the maintenance of immune homeostasis (Ygor Parladore Silva *et al.*, 2020; J. Y. Yoo *et al*., 2020). Nevertheless, the activation of these receptors will result in different outcomes, depending on the type of cell. For example, SCFA, particularly butyrate, influences systemic immune response through the control of the differentiation process of T cells, thus, influencing the adaptive immune response. These microbial metabolites can impact the differentiation of T cells either in T helper cells (Th) Th1, Th2, and Th17 cells, or into T regulatory cells (Treg). Moreover, SCFA can reduce inflammation, regulating the expression of pro-inflammatory cytokines (such as IL-6, IL-12, and tumor necrosis factor alfa (TNF-)α) by activating

macrophages and dendritic cells (J. Y. Yoo *et al*., 2020). SCFA can also lead to the induction of regulatory B cells, inhibiting the generation of Th17 cells, which may play a role in preventing autoimmune disorders or gastrointestinal diseases such as inflammatory bowel disease (IBD) (Wiertsema *et al*., 2021). Furthermore, the commensal bacteria can impact the innate immune response, for example, through lymphoid stimulation in the spleen, activation of macrophages, and stimulation of the maturation process of NK cells (Wiertsema *et al*., 2021). Overall, the interactions between IECs, DCs, ILCs, T and B cells contribute to the tolerogenic state of the intestinal environment (Wang *et al*., 2019). Under physiological conditions, the gut microbiota contributes to intestinal tolerance via sIgA and Treg production, and tolerogenic DCs and macrophages contribute further to their production, via IL-10 secretion. Gut microbiota, SCFAs, and IL-21 secreted from Tfh cells contribute to the secretion of specific sIgA, which will then coat gut commensal bacteria to control their proliferation and toxin production. Commensal bacteria components induce further Treg and B cell production and promote IFNγ secretion from CD8+ cells. On the other hand, sIgA and tolerogenic DCs negatively regulate the stimulation of Th17 cells, inhibiting a strong pro-inflammatory response against commensal bacteria. Tregs also downregulate DCs and Th17 cells by producing TGF-β (Wang *et al*., 2019). In this scenario, the intestinal balance is well maintained.

1.3. Obesity-Associated Gut Dysbiosis in Early-Life and Immune System Interactions

During early life, the infant's intestinal microbiota is highly plastic and shaped by several external factors, including the maternal microbiota. During this period of development, the mother plays a crucial role in the establishment of early-life gut microbiota, since mothers vertically transmit their microbiome and their metabolites to the offspring maybe *in utero*, but certainly during delivery and breastfeeding (Soderborg *et al.*, 2016). Therefore, the mother can impact the composition of the infant's gut microbiota, as well as its immunity. The composition of the maternal microbiota can vary depending on different factors such as diet, exposure to antibiotics, systemic health status, and diseases such as obesity, which in turn, affect the infant's gut microbiota (Arrieta *et al.*, 2015; Neff *et al*., 2018).

Obesity has become a worldwide problem, with over 500 million people suffering from this condition, often associated with other comorbidities like diabetes and heart diseases. High-calorie diets and sedentarism might have contributed to the steady rise of these numbers. Still, obesity is a complex disease, influenced not only by the environment, but by the host's genetic makeup (genetic polymorphisms) and, as unveiled more recently, by the gut microbiota. Altered

gut microbiota can contribute to obesity by increasing energy intake, inflammation, and alterations in metabolic pathways (Kincaid *et al*., 2020). It is estimated that maternal obesity affects two of every five pregnancies, representing a serious health issue since maternal obesity can influence the infant's health, which is already vulnerable to external threats in early life (Di Gesù *et al.*, 2021).

The link between obesity and gut microbiota has been studied in animal models and human patients. Menni *et al*. (2017) observed that patients with a more diverse gut microbiota were less prone to develop long-term weight gain, suggesting a preventive effect of the gut microbiota (Menni *et al.*, 2017). Other studies focused on the differences observed in Bacillota/Bacteroidota ratio between obese and lean individuals (Magne *et al*., 2020). In a study to evaluate differences between obese and lean Japanese individuals, Kasai *et al.* (Kasai *et al*., 2015) noted that obese patients had a high Bacillota/Bacteroidota ratio, compared to their healthy counterparts. However, there is some controversy in using the Bacillota/Bacteroidota ratio as a marker for obesity since discrepancies were reported in other studies in which obese patients showed decreased Bacillota/Bacteroidota ratio (Magne *et al*., 2020). Another factor that contributes to these inconsistencies is related to the metabolic endotoxemia hypothesis, which proposes that increased adiposity and development of low-grade inflammation could be associated with the dissemination of lipopolysaccharide (LPS) – the theory is at odds with decreased levels of Bacteroidota observed in obese patients since this phylum is mainly composed of Gram-negative bacteria (rich in LPS) (Magne *et al.*, 2020). These divergences could also be related to the experimental procedure applied in the studies, such as the number of patients and the methods used, or even the heterogeneity between the studies' participants (Magne *et al*., 2020). Additionally, scientific evidence suggests that another putative mechanism for this causal relationship between the gut microbiota and obesity may be a higher energy extraction from the diet by the gut microorganisms. The gut microbiome metabolizes certain compounds from the diet that would otherwise not be digested, such as some carbohydrates and fibers. These are then converted by the intestinal microbiota into SCFA, which, in turn, correspond to 10% of the human daily energy intake and are essential for colon cells and the liver. A study in germ-free mice reported that these animals weigh less and lose 2 times more calories in feces than conventional mice on the same diet (Lee *et al*., 2020). In humans, it has been found that when lean and obese adults eat a diet with excess calorie content, lean subjects lose more energy through stool than obese subjects (Jumpertz *et al*., 2011). But a core theme in the link between obesity-microbiota it's that diet has a deep impact on the intestinal microbiota and, by consequence, on the host's health. A Westernized diet, rich in

saturated fats and poor in fiber, can cause a shift in the individual's gut microbiota, leading to reduction in the abundance of beneficial microorganisms and increasing inflammation, a profile often associated with the development of metabolic disorders such as obesity (Kim *et al*., 2019). In an animal model, Zhang *et al* (2012) demonstrated that mice fed with a high-fat diet displayed reduced alpha-diversity, an increase in potentially pathogenic microorganisms and decrease in gut barrier-protective bacteria. However, the effects of the caloric diet were reversed once the mice were placed in typical mouse diet, demonstrating the resilient characteristic of the gut microbiota, and further highlighting the effect of diet on the gut microbiota (Zhang *et al.*, 2012).

Despite the gaps in the literature concerning this matter, the transmission of microbiota between mother and child with the potential to promote obesity has been suggested as a possible route of intergenerational transmission of obesity, with maternal weight being the factor unrelated to the child that most influences the development of obesity in childhood and throughout the years of life (Skrypnik *et al.*, 2019). In an animal model, the microbiome analysis of colon section from pups born to dams fed a western diet showed an increased Bacillota/Bacteroidota ratio that corresponded to increased colonic inflammation (Gibson *et al.*, 2015). Moreover, it was also observed that a high-fat diet during pregnancy is linked to gut dysbiosis in infants, characterized by reduced diversity at 1 year of age even after switching to a healthy diet at the time of weaning (Ma *et al.*, 2014). Therefore, it seems that the effect of maternal diet exposure during gestation and breastfeeding can modulate the composition of the microbial community, with long-lasting effects.

In humans, Tun et al. (Tun *et al.*, 2018) also verified that, in a cohort of 935 mother-child pairs (aged 1 and 3 years), birth mode and infant's gut microbiota (Bacillota richness, especially of the *Lachnospiraceae* family) sequentially mediated the association between maternal pre-pregnancy overweight and childhood overweight at ages 1 and 3 years. However, the genera of *Lachnospiraceae* differed between infants delivered vaginally and those delivered via cesarean birth. Moreover, Stanislawski *et al.* (2018) followed up a cohort of 165 children 4, 10, 30, 120, 365, and 730 days after delivery and at 12 years of age. The authors observed that, in early life, taxa within the gut microbiota that best predicted later childhood BMI, substantially overlapped with the maternal taxa most strongly associated with overweight and obesity, namely *F. prausnitzii* and *Ruminococcacae*. In other words, certain bacterial taxa associated with BMI of children, later in life, might derive from bacterial taxa found in the mother's gut microbiota, and that is often associated with obesity. The results show an association between the infant gut microbiota and later BMI, and they offer preliminary evidence that the infant gut microbiota,

particularly at 2 years of age, may have the potential to help identify children at risk for obesity (Stanislawski *et al.*, 2018). Considering the birth mode in the analysis modified associations of pre-pregnancy BMI with several genera, including the most abundant genus, *Bacteroides*. In the vaginal-delivery group, maternal overweight or obesity was associated with higher infant gut microbiome diversity and higher relative abundance of 15 operational taxonomic units (OTUs), including the overrepresentation of *Bacteroides fragilis*, *Escherichia coli*, *Veillonella dispar*, and OTUs in the genera *Staphylococcus* and *Enterococcus* (Singh *et al.*, 2020). Another recent study reported that high maternal BMI is associated with a lower abundance of butyrate-producing bacteria (such as *Ruminococcus*, *Turicibacter*, and *Roseburia*) in their children 1 month after delivery and that higher microbial diversity at this stage may predict higher adiposity later in life (Gilley *et al.*, 2022). Additionally, at 6 months, infants from obese mothers had lower abundance of family *Lachnospiraceae* and, at 12 months, these infants had lower abundance of family *Desulfovibrionaceae*, *Porphyromonadaceae*, and an increased abundance of *Enterobacteriaceae* (Gilley *et al.*, 2022).

Since early life represents a critical window for immune stimulation, maternal obesity may reprogram the neonatal immune system (Neff *et al*., 2018). For instance, a study that assessed umbilical cord blood samples from babies born from lean mothers versus babies of obese mothers reported that the latter had fewer eosinophils and CD4 T helper cells, reduced monocyte and dendritic cell responses to TLR ligands, and increased levels of IFN-α2 and IL-6 (Wilson *et al.*, 2015). Another study in a human cohort verified that maternal obesity was associated with a higher level of plasma superoxide-dismutase activity, IL-6, and IL-7 in neonates (Hernández-Trejo *et al.*, 2017). Moreover, a study by Enninga *et al.* (2021) reported that newborn from women with high BMI presented significant increased levels of CD4+ T cells and decreased myeloid cell populations, as well as increased concentrations of IL-12p40 and macrophage-derived chemokine (Enninga *et al*., 2021). However, it remains to clarify if these changes in immune and inflammatory profiles persist during child development.

The importance of the initial seeding of early-life gut microbiota with maternal microbes in the development of the immune system of the child may be the reason why maternally transmitted strains are more likely to adapt and persist in the infant's gut than non-maternally acquired strains (Azevedo *et al*., 2020; McDonald & McCoy, 2019; Tamburini *et al.*, 2016). In the case of obese mothers, it is possible to hypothesize that, if the maternal microbiota is vertically transmitted to the child and if this microbiota is already dysbiotic, combined with the fact that the child's immune system is influenced by the mother in early life (e.g. *in utero* transmission of immune cells, immunoglobulins, and cytokines stimulated with maternal gut microbiota; IgA

transmitted via breastfeeding stimulated with the maternal gut microbiota), this may predispose the colonization and selection of specific obesity-associated microorganisms. This microbiota would remain in the gut of the child and would perpetuate an obesogenic-microbiome-associated phenotype in early life. The child may therefore be "primed" to be colonized by a dysbiotic maternally-transmitted gut microbiota and impairing the immune tolerance profile and overall obesogenic phenotype of the child.

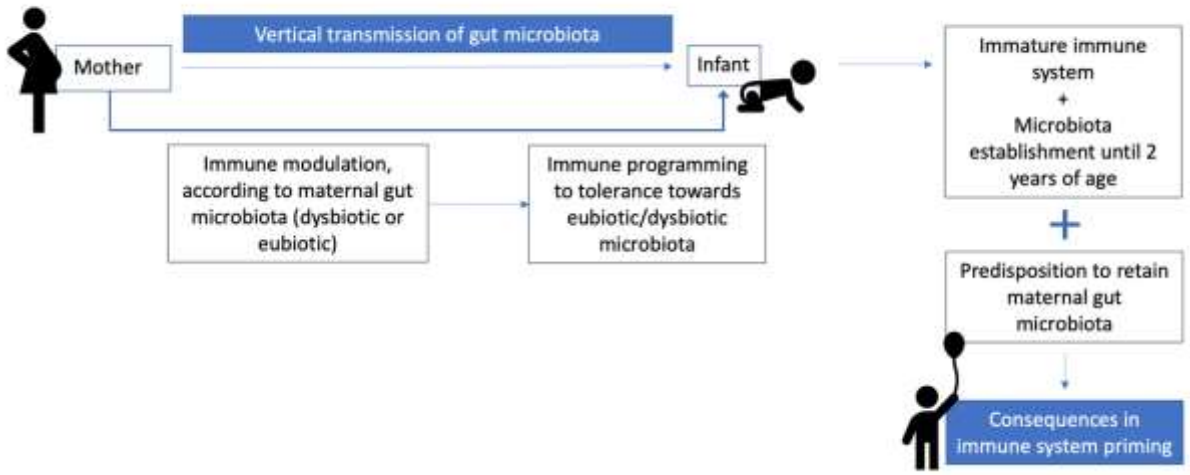


Figure 1.2 – Transmission of maternal gut microbiota and impact on the child's immunity.

1.4. Aim of the study

Considering the important link gut-immune system and the mother's role in the modulation of early-life gut microbiota, this study aimed to understand the impact of maternal obesity on the child's gut microbiota ability of immunomodulation.

2. Materials and Methods

The experimental protocol was divided into two tasks. The first task consisted in infant gut microbiota isolation and inactivation. The second task involved stimulation of immune cells, with infant gut microbiota. A simplified scheme of the experimental procedure is displayed in Figure 2.1.

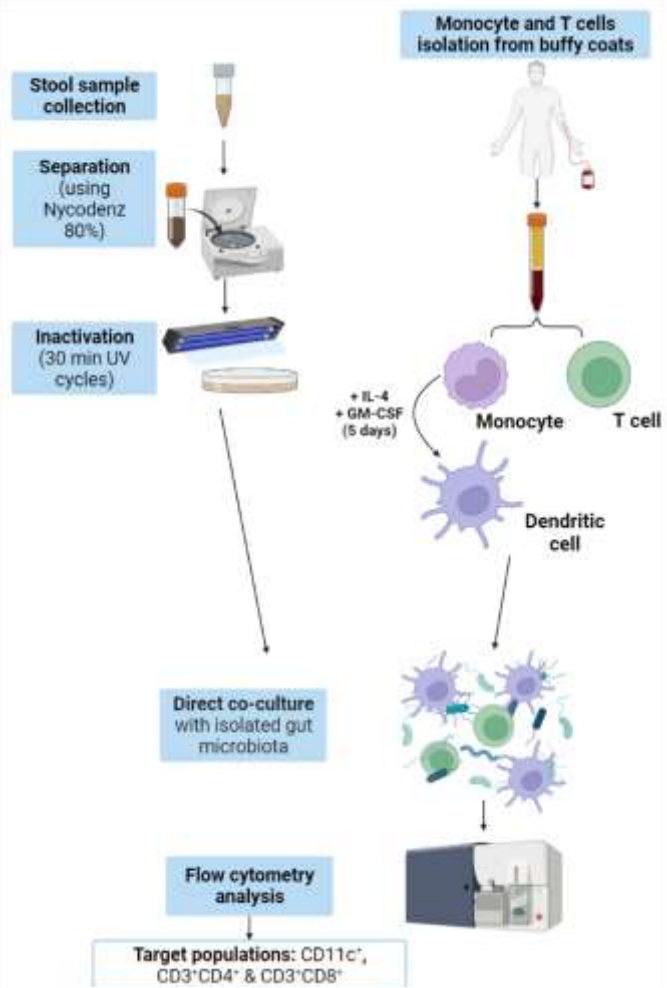


Figure 2.1 – Flowchart of experimental protocol (created with BioRender).

2.1. Participant selection and sample collection

Samples were selected from the OralBioBorn cohort of infants and respective mothers recruited at Centro Universitário Hospitalar São João or Hospital Pedro Hispano. This cohort follows mother-child pairs up to 1 year after childbirth, collecting stool samples of the mothers at the 3rd trimester of pregnancy, and from the mother-child dyad at 1 month, 6 months and 1-year post-partum. The stool samples were collected from the dipper brought by the mothers from home, in the morning before coming to the Hospital or up to two days before. In this last case, samples were frozen until transported to the Hospital. In either case, stools were transported

refrigerated. After receiving the samples, the research team transferred them to 1.5 ml microtubes and stored them at -80ºC until analysis.

The OralBioBorn cohort included 232 participants, dividing lean and obese mothers according to Body Mass Index: Lean group, BMI ≥ 18.5 and < 25 and Obese group, BMI ≥ 30. Considering the main aim of the study, adult participants with BMI ≥ 25 and < 30 (classified as "overweight") were excluded since they did not fit the obese criteria (BMI ≥ 30).

Relevant data concerning the participants (mothers and their children) were collected, as displayed in Table 2.1.

Table 2.1 – Relevant collected data from the participants.

To whom	What data
Mother	• Mode of delivery • Post-partum complications • Weight and Length • Breastfeeding/Formula feeding • Antibiotic intake
Infant	• Weight and Length • Post-partum complications • Antibiotic intake • Probiotic intake

2.2. Gut microbiota isolation and inactivation

To evaluate the impact of the child microbiota on the immune system priming, there was a need to isolate the intestinal microbiota from the infant's stool samples and consequent inactivation. With that purpose, we adapted protocols from the literature and optimized them, namely from López *et al* (2016) (López *et al*., 2016) and Wampach *et al* (2018) (Wampach *et al*., 2018).

Stool samples were weighed (approximately 2 grams) and homogenized in 9 ml of saline solution (NaCl 0.9%), using gentleMACS™ Dissociator (#130-095-937) for one minute (program "m_spleen_4.01", 3000xg), in tubes suitable for the equipment (C tubes used specifically for automated tissue dissociation). The homogenized samples were transferred to 15 ml tubes, containing 3.5 ml of Nycodenz 80% (w/v), and centrifuged (10 000xg, for 40 minutes, and at 4ºC), creating a density gradient with distinct layers as shown in Figure 2.2.

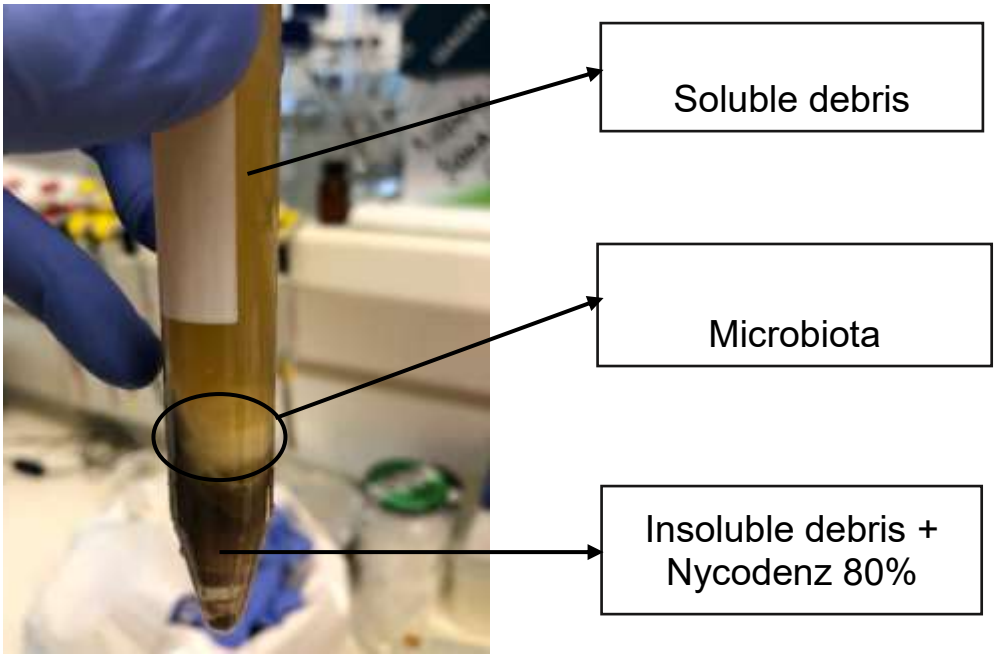


Figure 2.2 – Gradient formation after centrifugation of fecal sample.

The layer corresponding to the gut microbiota (white layer) (López *et al*., 2016) was transferred to 1.5 ml microtubes and placed on ice, for five minutes. The samples were washed twice, with 1 ml of PBS 1x (saline solution), in five minutes centrifugation cycles.

For microbiota inactivation, the microbiota solution (after isolation and washing steps), was transferred to empty Petri dishes and inactivated by exposure to UV radiation, in three (or more, if necessary) inactivation cycles. The inactivation cycles consisted of 30 minutes with the plates open and the UV lamp (V-TAC UV-C Germicidal Lamp with ozone; wavelength: 185-253 nm) turned on, and 30 minutes with the plates closed and the UV lamp turned off, inside a closed box (Figure 2.3). Afterward, the microbiota solution was suspended in 1 ml of PBS 1x solution, because a significant part dried out and this volume allowed us to achieve the desired final concentration of microbiota (~10^8 bacterial cells/ml).

Figure 2.3 – Plates with gut microbiota solution during inactivation procedure.

To confirm the inactivation, the absence of growth, 50 µl of the microbiota solution was inoculated in plates containing BHI (Brain-Heart Agar) media, using the spreading technique, and placed in an incubator (37ºC, for 24h). Once the absence of growth was confirmed, quantification with a Neubauer chamber, at an objective of 40x, with 1:100 dilution in PBS 1x, was performed to determine the sample's concentration. The final solution was stored at -80ºC.

2.3. Immune cell isolation and differentiation

Human monocytes were isolated from buffy coats from healthy blood donors, as previously optimized in our lab (Cardoso *et al.*, 2014). Information from each blood donor was annotated for future reference (namely blood type and donor's code). The blood samples were labeled, carefully mixed, and transferred to 50 ml tubes. The samples were centrifuged (1200xg, speed 5, brake 0, for 20 minutes and at room temperature), creating a gradient with distinct layers, as depicted in Figure 2.4. The donors with the biggest and clearest PBMCs (human Peripheral Blood Mononuclear Cells) ring were selected for the next step. The ring was collected and transferred to 15 ml tubes, and the final volume of PBMCs was divided between two 15 ml tubes (for monocytes and T cells, respectively).

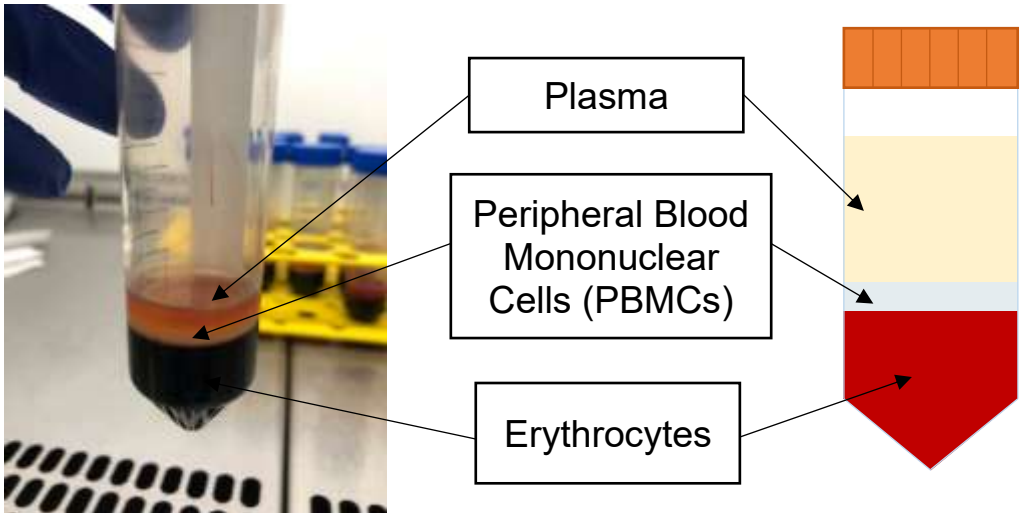


Figure 2.4 – Gradient after centrifugation of blood samples.

For the isolation of the target cells, RosetteSep (STEMCELL Technologies) was used. Distinct volumes of the reagent were used, according to the cell type to be isolated and the obtained PBMCs volume: 67 µl of RosetteSep™ Human Monocyte Enrichment Cocktail per ml of PBMCs for monocyte isolation, and 50 µl of RosetteSep™ Human T Cell Enrichment Cocktail per ml of PBMCs for T cell isolation. Following the addition of the reagent, the tubes were incubated in a horizontal mixer for 20 minutes (the color changed to bright red). After, the suspension was diluted with an equal volume of PBS + 2% FBS (equal volume of PBMCs before adding RosetteSep).

In a new 15 ml tube, an equal volume of Ficoll-Histopaque (equal volume of PBMCs before adding RosetteSep) was added, directly to the bottom of the tube. In this new tube, at a 45º angle, the PBMCs' suspension was slowly transferred over the Ficoll-Histopaque (to avoid mixing the two solutions). Afterward, the tube was centrifuged (1200xg, speed 2, brake 0, for 20 minutes and at room temperature) and the interface was collected and transferred to a new 50 ml tube, making up the volume with PBS 1x. The solution was centrifuged, once more.

The supernatant was discarded, and the remaining pellet was washed in PBS by centrifugating three times at 300g for 10 minutes, and once at 120g for 10 minutes. Both monocytes and T cells were resuspended in complete RPMI medium (Gibco) and counted. After, T cells were placed in freezing medium (90 % FBS (Biowest) and 10% dimethyl sulfoxide (DMSO, Sigma)), and stored at -80ºC. For dendritic cell differentiation, monocytes were plated at a density of $2x10^6$ cells per well, in 6-well culture plates, in complete RPMI medium, supplemented with interleukin-4 (IL-4) (50 ng/ml; Immunotools) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (50 ng/ml; Cell Signaling), according to our previous studies

(Castro *et al.*, 2017). The plates were maintained in an incubator for 5 days, at 37ºC and 5% CO_2 humidified atmosphere.

2.4. Immune cells activation with child microbiota

To understand how child microbiota stimulated the immune cells, a co-culture with monocyte-derived DCs (dendritic cells) and T cells and inactivated microbiota was performed. This protocol was based on previous scientific reports (López *et al.*, 2016), however, optimization was needed. Five days after monocyte and T cell isolation, infant gut microbiota at a Multiplicity of Infection (MOI) of 10, was added to the DC cultures for 3 hours. After that time, T cells were thawed and centrifuged at 1200 rpm for 5 minutes, counted and added to the culture. $1x10^6$ T cells were added to the DC with the microbiota. As positive control for DC and T cells activation, LPS (25 ng/ml), and PMA (25 ng/ml) plus Ionomycin (1 µg/ml), respectively, were used. Co-cultures were maintained in complete RPMI medium for 24 hours, after which biological material was collected. Additionally, a control sample of infant gut microbiota was used during protocol optimization. The control sample belonged to an infant, over 1 year of age, that was not included in the study.

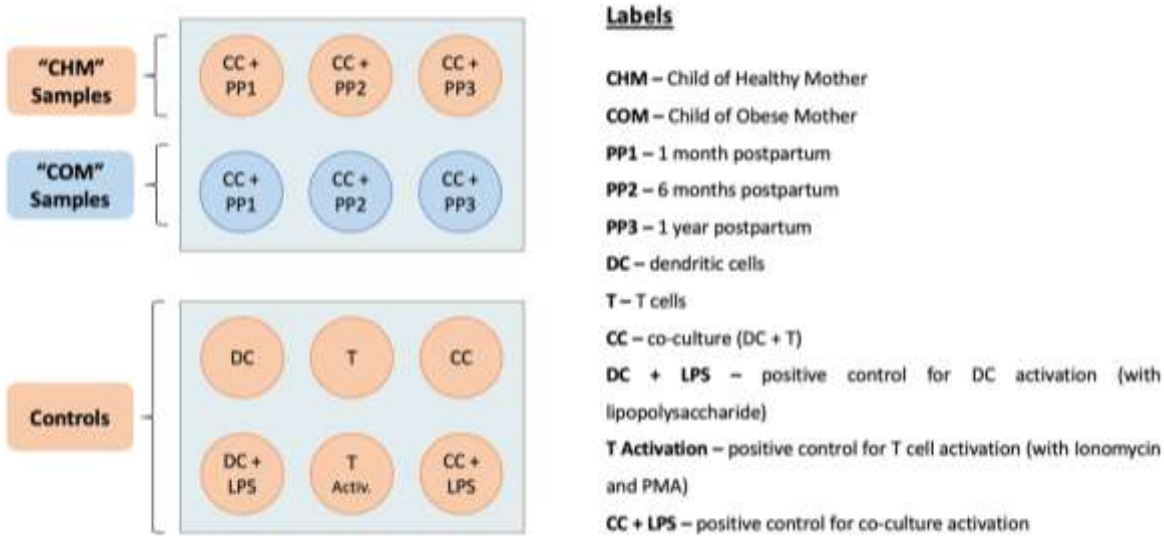


Figure 2.5 – General scheme of the experimental conditions.

2.5. Flow Cytometry Analysis

Immune cell activation was assessed by flow cytometry, using specific DC and T cell markers. At the end of co-culture, the cells were collected to 15 ml Falcon tubes and centrifuged (1200 RPM, for 5 minutes), and the pellet was suspended in 5 ml of cold PBS 1x and placed on ice.

A staining protocol was performed in which specific antibodies/markers for DC and T cells were used (Tables 2.2 and 2.3). Briefly, cell pellets were resuspended in flow cytometry buffer (PBS, 2% BSA, 0.01% sodium azide).

After centrifugation, the wells were marked with the antibodies, depending on if it was for DC or T cell analysis, and the plate was incubated (for 40 minutes, at 4ºC, in the dark). Afterward, the plate was subjected to a series of washings with flow cytometry buffer and centrifugation cycles (1500 RPM, for 5 minutes, at 4ºC).

Before fixing the cells, the Live/Dead protocol was performed in which the wells were stained with a viability die (Live/Dead). The plate was incubated for 30 minutes, at 4ºC, in the dark. Then, the plate was washed twice, with 150 µl of flow cytometry buffer and centrifugation cycles (1500 RPM, for 5 minutes, at 4ºC).

To fixate the cells, 150 µl of PFA 2% were added to each well, and the plate was incubated for 15 minutes, at room temperature. Afterward, the plate was washed twice with flow cytometry buffer and centrifugation cycles (1500 RPM, for 5 minutes, at 4ºC). For the final steps of the staining protocol, the plate content was filtered, using nylon mesh filters, and transferred to flow cytometry tubes.

The samples were analyzed with a flow cytometer (FACS Canto II, BD Biosciences) and an acquisition software BD FACSDiva. Analysis and presentation of results were performed using computer software FlowJo and GraphPad Pris.

Table 2.2 - Antibodies/immune markers used in the flow cytometry staining, for dendritic cells.

Function	Antibody	Fluorochrome	Clone	Catalog Nº	Company
Lineage marker	CD11c	APC	BU15	21487116	ImmunoTools
Activation marker	CD40	PE	HI40a	21270404	ImmunoTools
	CD86	FITC-A	BU63	21480863X2	ImmunoTools
	HLA-DR	Pacific Blue	L243	48-9952-42	Invitrogen
Viability marker	Live/Dead	APC-Cy-7	-	65-0865-14	Invitrogen

Table 2.3 - Antibodies/immune markers used in the flow cytometry staining, for T cells.

Function	Antibody	Fluorochrome	Clone	Catalog Nº	Company
Lineage marker	CD3	FITC-A	HIT3b	21810033X2	ImmunoTools
	CD4	Pacific Blue	RPA-T4	300521	BioLegend
	CD8	PerCP-Cy7	HIT8a	21810085X2	ImmunoTools
Activation marker	CD69	APC	FN50	21620696X2	ImmunoTools
Viability marker	Live/Dead	APC-Cy-7	-	65-0865-14	Invitrogen

2.6. Ethics statement

Human samples obtained and procedures were performed in agreement with the principles of the Declaration of Helsinki. Regarding stool samples collection ethics approval and written informed consent of each participant or legal tutor was previously obtained within OralBioBorn (Centro Hospitalar Universitário São João Ethics Committee Protocol Nº294/2018). Monocytes and T cells were isolated from buffy coats of healthy donors, kindly provided by the Immunohemotherapy Department of Centro Hospitalar Universitário de São João, Porto, Portugal. This is covered by the ethical approval of the service, under which blood donors give informed written consent for the byproducts of their blood collections to be used for research purposes (Protocol reference Nº 90/2019).

2.7. Statistical analysis

Participants' clinical data were expressed through relative frequencies (%) for categorical variables and as the mean with Standard Deviation (SD) for continuous variables. The appropriate Chi-square independence test was applied to analyze hypotheses regarding the categorical variables and Mann–Whitney U test concerning continuous variables. Statistical Package for Social Sciences (IBM SPSS version 28.0) was used and a level of 0.05 was considered significant.

For the immunomodulation studies, data were expressed as the mean with Standard Deviation (SD) and collected from at least three independent experiments with immune cells from different donors. For comparison between two independent groups, t-test (Wilcoxon, and Mann-Whitney) was used. For comparison among four independent groups, one-way ANOVA

(RM one-way ANOVA, Friedman, and Kruskal-Wallis), with Dunn's multiple comparison was used. Statistical tests were performed in GraphPad Prism 8. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Participants' characteristics

Participants (mothers and children) were divided into two groups, considering the mothers BMI: Healthy and Obese groups. Due to the need of performing several optimizations in different experimental steps, we only had time to include a sample of 3 children for each group. All infants were delivered vaginally. Infants were mainly breastfed in the first month of life, while a combination of breastfeeding and solid food was observed at 6 and 12 months postpartum. Concerning postpartum complications, 1 infant from a healthy mother developed infant jaundice and a respiratory infection, at 1 month postpartum; 1 infant from a healthy mother experienced an ear infection and weight loss at 6 months postpartum; at 12 months postpartum, the most common complications were infections (ear, respiratory, skin and/or gastrointestinal). Regarding biotic intake (antibiotic and probiotic) by the infants, only 1 healthy mother reported the usage of probiotics, while both healthy and obese mothers reported the use of antibiotics (particularly at 12 months postpartum). Table 3.1 presents the participants' characteristics. No significant difference was found between both groups, since the number of participants is low.

Table 3.1 – Participants' characteristics.

	Healthy mothers (n=3)	**Obese mothers (n=3)**
Maternal Body Mass Index (BMI)	21.34±2.70	34.05±4.09
Gestational age at birth	38.67±0.56	38.67±1.16
Mode of delivery		
Vaginal delivery	100%	100%
Maternal postpartum complications	0.0%	0.0%
Maternal antibiotic intake		
During pregnancy	0.0%	0.0%
Postpartum	33.3%	0.0%
Children weight (kg)		
A**t birth**	2.85±0.43	3.24±0.24
PP1	3.86±0.55	4.55±0.69
PP2	7.47±0.90	7.72±0.74

PP3	9.61±1.12	9.87±0.86
Children length (cm)		
At birth	47.83±1.89	49.17±1.89
PP1	51.06±4.01	55.50±2.10
PP2	66.50±4.09	66.83±1.04
PP3	74.13±3.81	75.00±2.65
Feeding Habits (PP1)		
Breastfeeding	33.3%	66.7%
Both	66.7%	33.3%
Feeding Habits (PP2)		
Breastfeeding and solid food	33.3%	33.3%
Formula and solid food	33.3%	33.3%
Breastfeeding, formula, and solid food	33.3%	33.3%
Feeding Habits (PP3)		
Breastfeeding and solid food	33.3%	66.7%
Solid food	33.3%	33.3%
Breastfeeding, formula, and solid food	33.3%	0.0%
Children postpartum complications		
PP1	66.7%	0.0%
Infant jaundice	33.3%	0.0%
Respiratory infection	33.3%	0.0%
PP2	66.7%	0.0%
Ear infection	33.3%	0.0%
Weight loss	33.3%	0.0%
PP3	100%	66.7%
Ear infection	25.0%	33.3%
Gastrointestinal infection	25.0%	33.%
Respiratory infection	25.0%	0.0%
Throat infection	0.0%	33.3%
Skin infection	25.0%	0.0%

Antibiotic intake		
PP1	0.0%	0.0%
PP2	33.3%	0.0%
PP3	33.3%	33.3%
Probiotic intake		
PP1	33.3%	0.0%
PP2	0.0%	0.0%
PP3	33.3%	0.0%

*Results are shown in prevalence (%) or mean ± standard deviation.
PP1 – 1 month postpartum; **PP2** – 6 months postpartum; **PP3** – 12 months postpartum.

3.2. Protocol Optimization

3.2.1. Separation of infant gut microbiota from stool samples

The first task of the experimental protocol consisted of the separation of gut microbiota from the participants' stool samples and consequent inactivation with a UV lamp. There was the need to optimize the protocol, and, therefore, certain steps in the original protocol were revised.
In the separation phase, one of the issues observed was the initial quantity of fecal samples from 1 month postpartum, since these samples are collected directly from the baby's diaper that is highly absorbent, resulting in smaller fecal samples. To overcome this problem, samples from the first timepoint that had low quantities (that did not reach the postulated 2 grams), were suspended in 1ml of PBS 1x and vortexed (prior to homogenization). The sample's weight was registered using the difference between a microtube containing the sample and an empty microtube. It was confirmed that fecal amount less than 2 grams also reached the desired number of microbial cells at the end of the protocol.

In the homogenization step, two devices were examined: Stomacher (a homogenizer, that relies on the action of two paddles to homogenize the sample) and gentleMACS^{TM} Dissociator (an automated tissue dissociator, that uses a rotor to homogenize the sample). After testing both types of equipment, it was verified that the gentleMACS^{TM} was, not only superior in homogenizing the sample, but the ratio of water to sample mass is reduced (less water is needed for the same sample's weight), and thus, less sample is wasted. The reduction of the volume of saline solution (NaCl 0.9%) to half (18ml to 9ml) was also tested. The reduction in NaCl volume

resulted in a larger microbiota layer (after centrifugation with Nycodenz 80%). This alteration was performed to not lose sample in the next step (centrifugation) since the tube was only 15

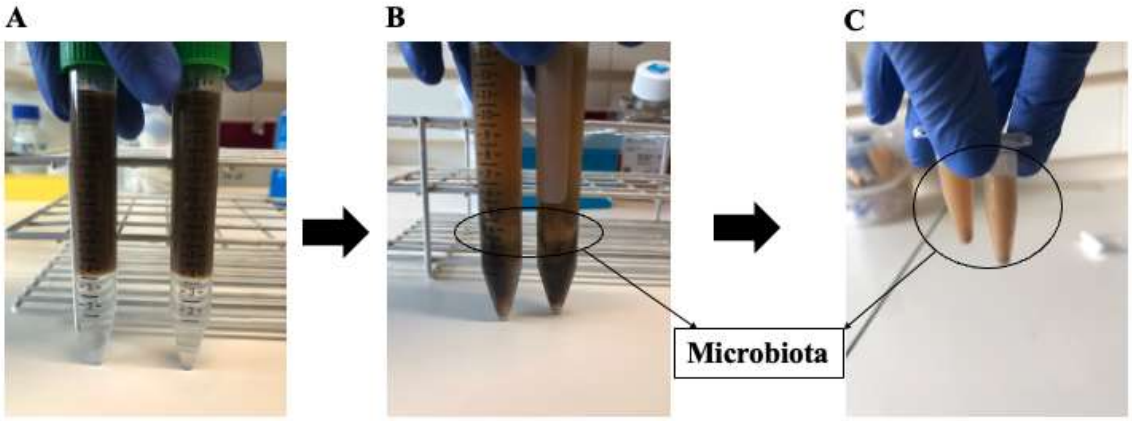


Figure 3.1 – Samples before centrifugation (A), after centrifugation with Nycodenz 80% (B) and the extracted microbiota solution (C).

ml.

3.2.2. Inactivation of gut microbiota

The inactivation protocol was subjected to several optimization steps, namely in what respects to sample volume and concentration post UV (due to sample evaporation), microbial growth post UV (that required multiple UV rounds) and the cell counting method (due to difficulties in counting with 100x objective). The main issue found the in the inactivation step was sample evaporation during UV cycles, which greatly affected the sample's volume and concentration, post-UV treatment. Two alternatives were evaluated to prevent sample evaporation during UV exposure: using Petri dishes with a smaller diameter and washing the plates with 1 ml of PBS 1x, after UV exposure (to recover the sample that had dried on the plate). Although the first option enabled the recovery of a greater volume of microbiota solution, it still did not prevent the drying of the sample and led to the need for numerous UV cycles, to limit microbial growth. Thus, the second option was chosen, allowing recovery of most of the sample, without the need for three or more cycles.

Furthermore, another issue was found in the inactivation step. Following the protocol of Lopéz *et al* (López *et al*., 2016), three cycles of 30 minutes of UV exposure was enough. However, we found necessary to increase the number of UV cycles to completely restrict microbial growth. It was not possible to overcome this issue since only with more than two inactivation rounds it was possible to confirm the absence of growth in the BHI media plates, as can be

noted in Figure 3.2. Nevertheless, this did not interfere with the sample's concentration, due to the plate's washing with saline solution described earlier.

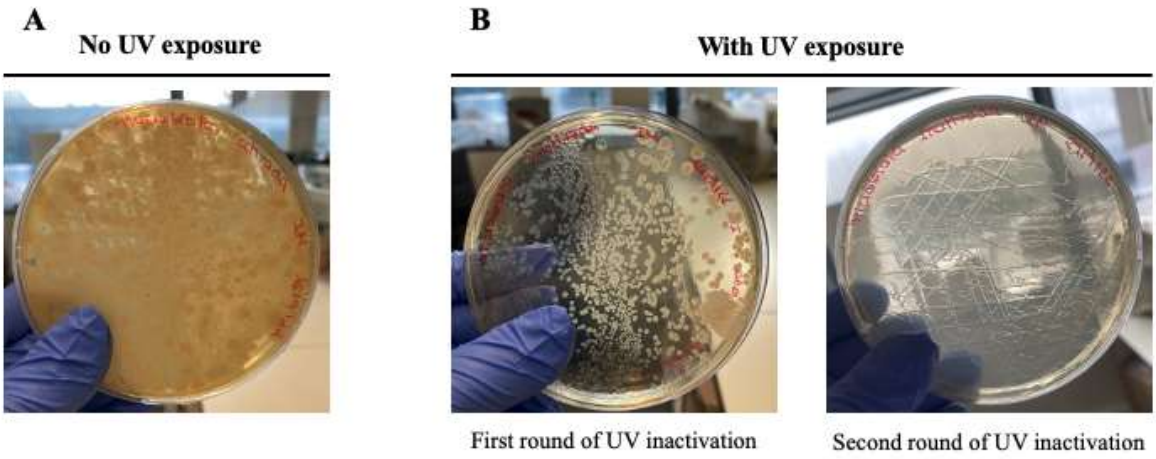


Figure 3.2 – BHI media plates of (A) activated microbiota and (B) inactivated microbiota.

The last issue tackled in the inactivation protocol was the cell counting method. In the original protocol, the authors performed cell counting with the Neubauer chamber using an objective of 100x (López *et al*., 2016). However, as several issues were encountered while trying to replicate this step (namely the inability to count at 100x objective, without risking breaking the coverslip on top of Neubauer chamber, or the chamber itself), two alternatives were tested: using a different microscope (which allowed for field visualization in a computer monitor) or using a lower objective (40x). Due to technical difficulties observed in the first option (namely, the impossibility of visualizing and counting in multiple layers), the second option was chosen.

3.2.3. Immune cell isolation and DC and T cell activation

The second stage of the experimental procedure consisted of the stimulation of immune cells isolated from the blood of healthy donors with the gut microbiota. Before stimulating with the gut microbiota, certain aspects of the immune cell isolation protocol were revised and optimized.

3.2.3.1. Dendritic cell culture optimization

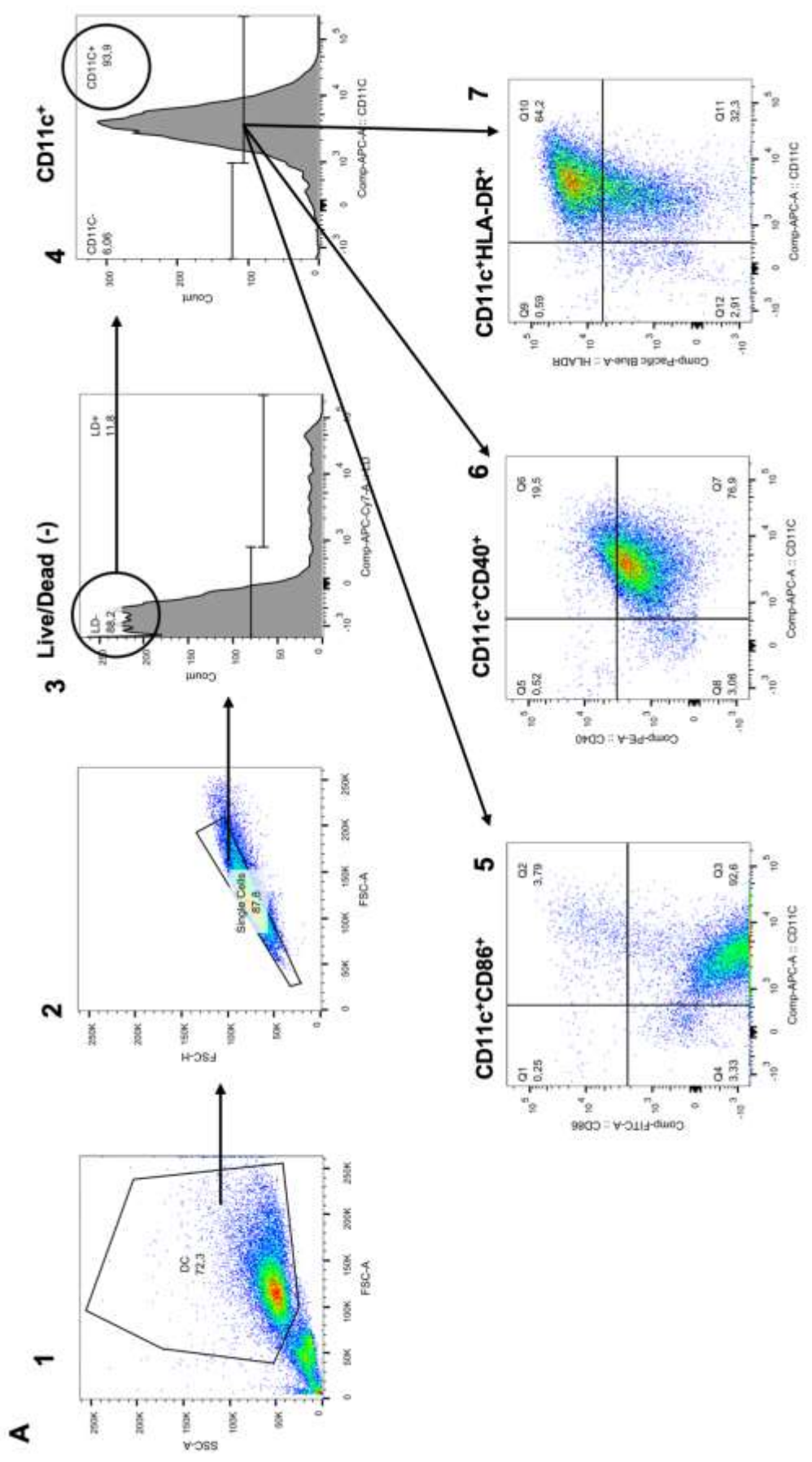
One of the main issues tackled was the selection of the most efficient co-culture protocol. For this purpose, two co-culture protocols were tested: the first one was extracted from a research article (labeled as "A" protocol) (López *et al.*, 2016) and the second was a protocol used in the

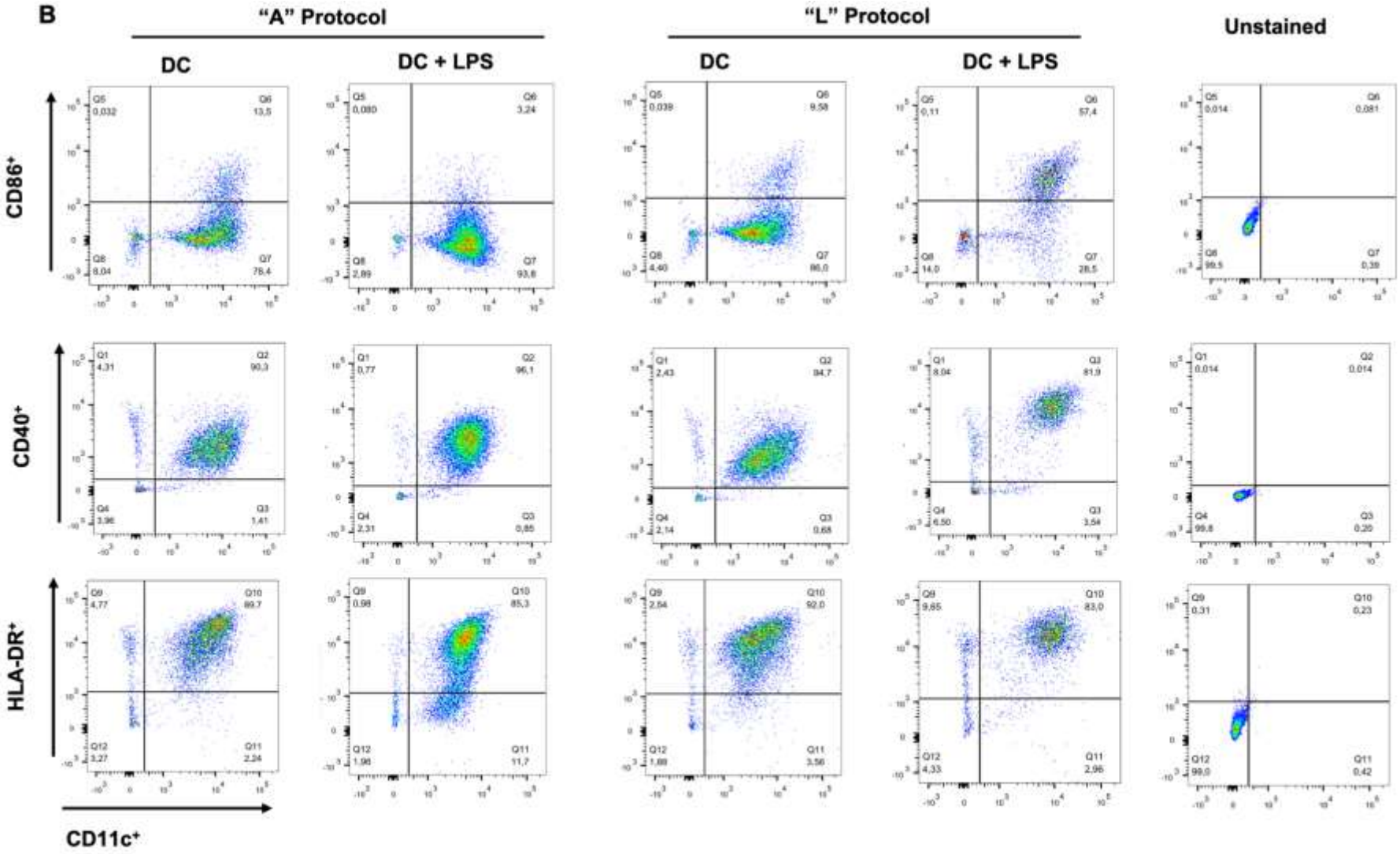
lab for macrophages and T cells co-cultures (labeled as “L” protocol). Both protocols differ in the timing of the immune cells’ stimulation. While the “A” protocol establishes that the stimulating factor is added before DC maturation/differentiation (at day 0), in the “L” protocol the stimulus is added after the dendritic cell differentiation is completed (day 5). Further, different brands of GM-CSF and IL-4 for DC differentiation were also assessed during the protocol optimization.

Since we want to analyze the effect of microbiota on the innate and adaptative cells, the protocol was optimized both for the stimulation of DC alone, and for the stimulation of co-cultures of DC and T cells. At this stage of protocol optimization, immune cell stimulation was only performed with LPS. As expected, dendritic cells that were not stimulated presented the lowest percentages of expression of the activation markers analyzed (CD86, CD40 and HLA-DR), compared to LPS-treated dendritic cells (as exhibited in dot plots B and graphs C from Figure 3.3). The results seem to demonstrate that the “A” protocol was not able to trigger DC activation effectively, as there is a decreased in CD86 expression observed in DC stimulated with LPS (Graphs C, Figure 3.3), both in terms of percentage of double positive cells and Median Fluorescence values (MFI, characterized by level of protein expression per cell). Concerning the markers CD40 and HLA-DR, it does not seem to be any significant changes in the quantity of positive cells, but an apparent increased in MFI values in the LPS-treated DCs (graphs C, bottom panel, Figure 3.3). The “L” protocol was seemingly able to generate expression of the analyzed activation markers in the stimulated condition.

The differences observed in the different co-culture protocols, in terms of DC activation, can be explained by the different timings of addition of the stimulus: for instance, in the “A” protocol, the cells stayed for 5 days in contact with the stimulus, which might have interfered in the differentiation process of monocytes in dendritic cells, leading to lower activation levels. While in the “L” protocol, the cells were allowed to properly differentiate, before coming in contact with the LPS and become maturated/activated.

Thus, considering the obtained results, the “L” co-culture protocol was chosen for further experiments.


A
1
SSC-A
FSC-A
DC
72.3
2
FSC-H
FSC-A
Single Cells
87.8
3
Live/Dead (-)
Count
Comp-APC-Cy7-A :: LD
LD-
88.2
LD+
11.8
4
CD11c+
Count
Comp-APC-A :: CD11C
CD11C-
6.06
CD11C+
93.9
5
CD11c+CD86+
Comp-FITC-A :: CD86
Comp-APC-A :: CD11C
Q1
0.25
Q2
3.79
Q3
92.6
Q4
3.33
6
CD11c+CD40+
Comp-PE-A :: CD40
Comp-APC-A :: CD11C
Q5
0.52
Q6
19.5
Q7
76.9
Q8
3.06
7
CD11c+HLA-DR+
Comp-Pacific Blue-A :: HLADR
Comp-APC-A :: CD11C
Q9
0.59
Q10
64.2
Q11
32.3
Q12
2.91



Labels:
DC – Dendritic cells (unstimulated)
DC + LPS – DC stimulated with lipopolysaccharide (positive control for DC activation)

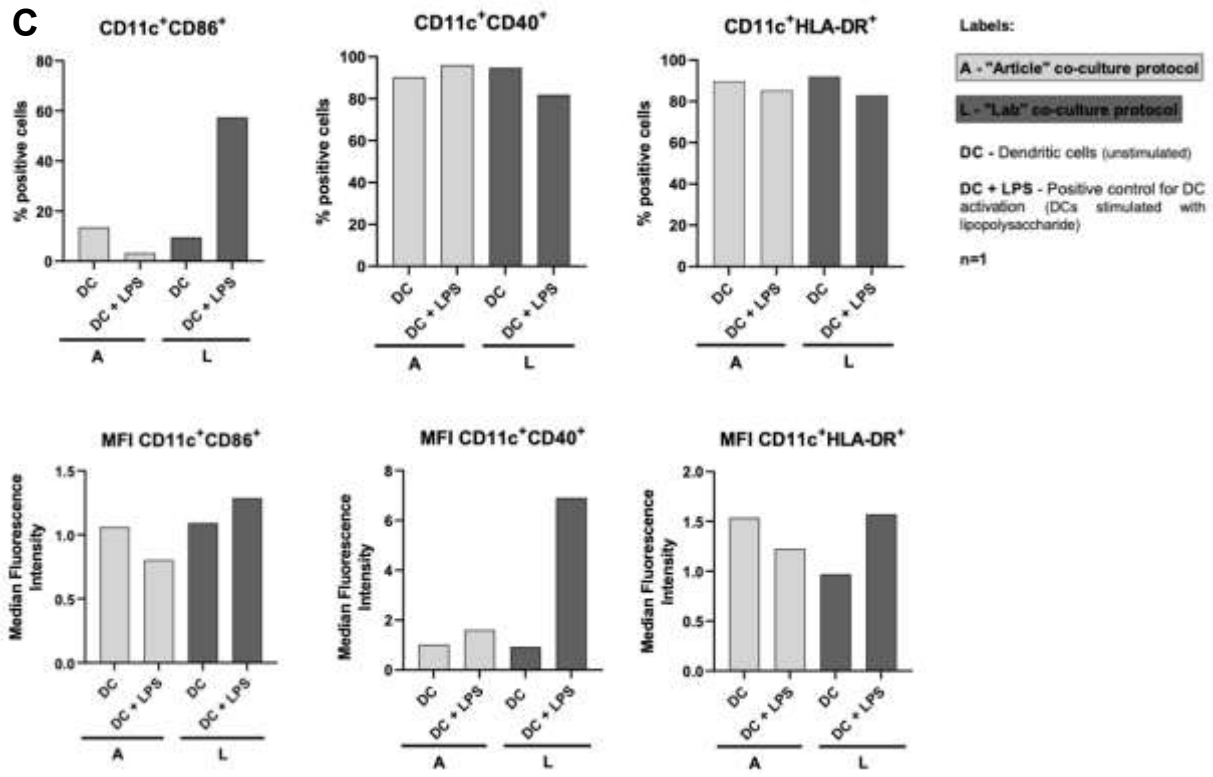


Figure 3.3 – **Expression of activation markers in DCs by flow cytometry** – CD86, CD40 and HLA-DR expression was determined by flow cytometry in DCs stimulated with LPS.

(A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. 1: FSC-A/SSC-A – represents single cells in the light scatter based on cell size and granularity, respectively; 2: FSC-A/FSC-H – represents single cells; 3: Comp-APC-Cy-7-A represents live cells; 4: Comp-FITC-A represents CD11c+ cells; 5: Comp-FITC-A/Comp-APC-A represents double positive cells $CD11c^{+}CD86^{+}$; 6: Comp-PE-A/Comp-APC-A represents double positive cells $CD11c^{+}CD40^{+}$; 7: Comp-Pacific Blue-A/Comp-APC-A represents double positive cells $CD11c^{+}HLA\text{-}DR^{+}$. Gating strategy performed in a sample of unstimulated DCs (monoculture), with positive expression for CD40 and HLA-DR markers. (B) Pseudo color plots representing expression of activation markers, in different co-culture protocols, in stimulated and unstimulated DCs. (C) Graphs with representation of percentage of double positive cells (top panel) and intensity of median fluorescence (bottom panel) in stimulated and unstimulated DCs, using different co-cultures protocols (N=1).

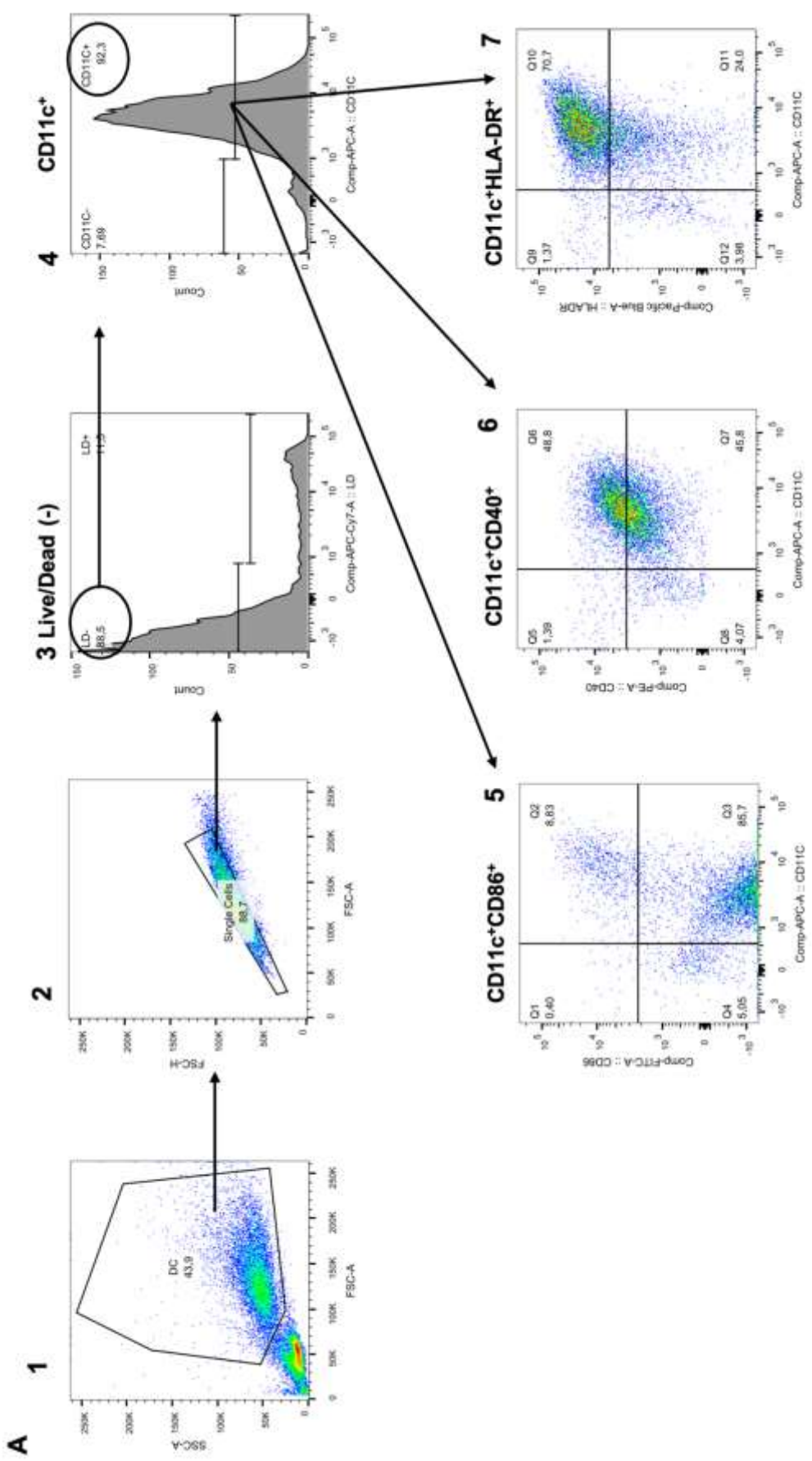
3.2.3.2. Co-culture protocol optimization

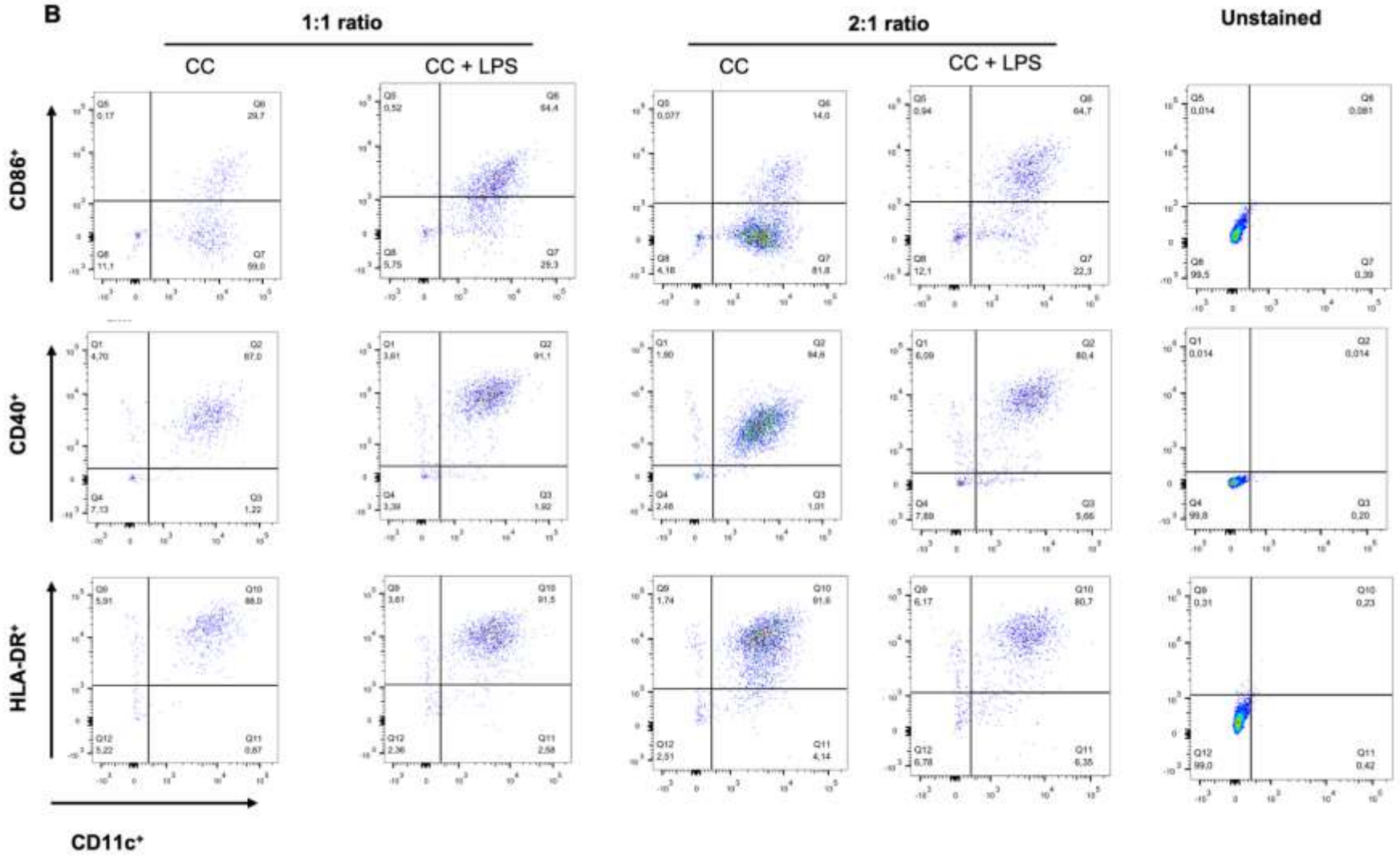
After selecting the ideal co-culture protocol, and since one of our aims is to study the effect of infant gut microbiota in the activation of acquired immunity, the next step in the optimization process was to evaluate which DC:T cells ratio to apply in co-culture. For that purpose, two DC:T cells ratios were tested (1:1 and 2:1), in DCs in co-culture with T cells, unstimulated and stimulated with LPS. The results are represented in Figure 3.4 and 3.5.

Regarding DC analysis, it was verified that a higher ratio of DC:T cells seemed to induce higher expression of the activation markers analyzed, as evidenced by graphs C from Figure 3.4. However, there seemed to be no significant differences in the expression of CD40 and HLA-DR between both ratios, in terms of percentage of positive cells. In terms of MFI, a higher ration appears to induce higher MFI values in all analyzed markers (graphs C, bottom panel, Figure 3.4). In other words, the 2:1 DC:T cells ratio seems to induce the expression of a higher number of activation markers per DC, even if there are no distinguishable differences in the quantity of double positive cells for a specific marker.

In T cells analysis, the results seem to reveal a similar tendency to the one observed in DC, with a 2:1 DC:T cells ratio enabling an increased expression in the T cell activation marker CD69 (graphs C, Figure 3.5). However, it was also noted that unstimulated T cells condition seemed to exhibit similar MFI values to conditions in which the cells were stimulated LPS. It should be noted that, in this particular optimization process, the immune marker CD4 was not functioning properly. Therefore, the results display the percentage of positive cells and MFI values for the subset $CD3^{+}CD69^{+}$.(For sake of simplicity the gate strategy showed is for an experiment in which all the antibodies were functioning).

Considering the obtained results, the ratio 2:1 DC:T cells was chosen for further experiments.


A
1
DC
43.9
SSC-A
FSC-A
2
Single Cells
88.7
FSC-H
FSC-A
3 Live/Dead (-)
LD-
88.5
LD+
11.5
Count
Comp-APC-Cy7-A :: LD
4
CD11c+
CD11C+
92.3
CD11C-
7.69
Count
Comp-APC-A :: CD11C
5
CD11c+CD86+
Q1
0.40
Q2
8.83
Q3
85.7
Q4
5.05
Comp-FITC-A :: CD86
Comp-APC-A :: CD11C
6
CD11c+CD40+
Q5
1.39
Q6
48.8
Q7
45.8
Q8
4.07
Comp-PE-A :: CD40
Comp-APC-A :: CD11C
7
CD11c+HLA-DR+
Q9
1.37
Q10
70.7
Q11
24.0
Q12
3.98
Comp-Pacific Blue-A :: HLADR
Comp-APC-A :: CD11C



Labels:
CC – Dendritic cells in co-culture with T cells (unstimulated)
CC + LPS – DC in co-culture with T cells, stimulated with lipopolysaccharide

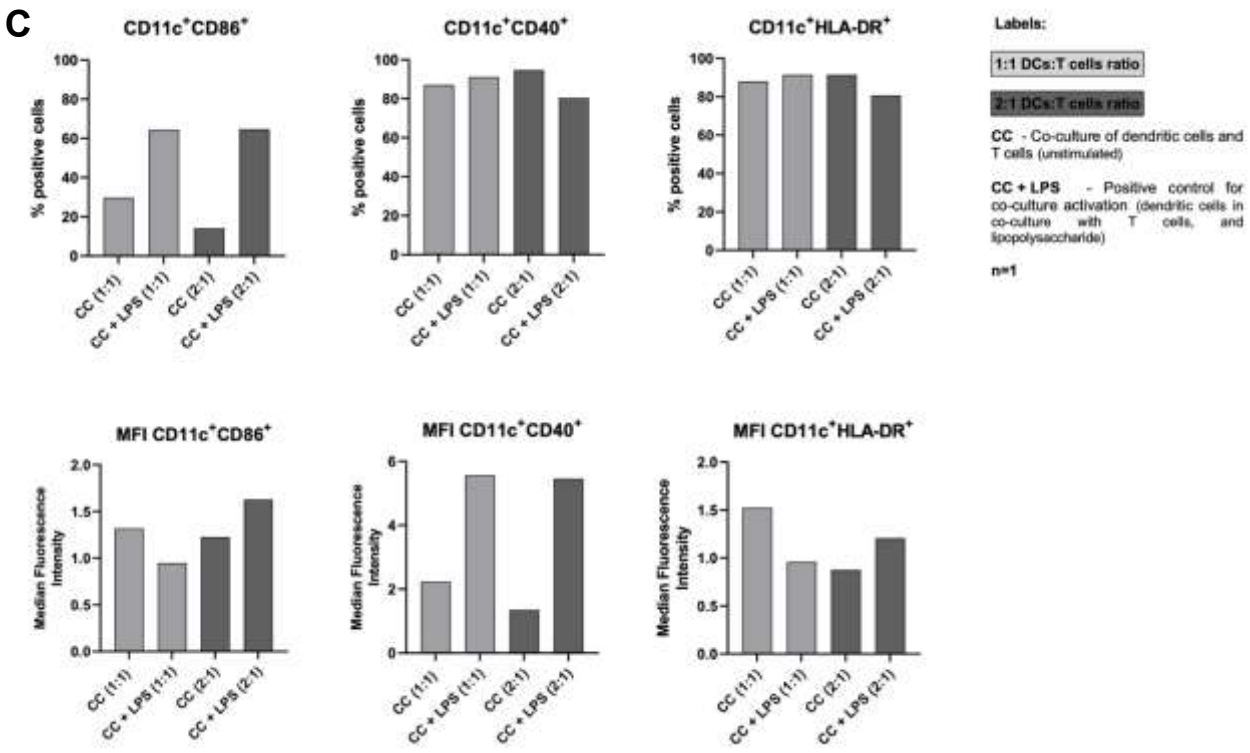
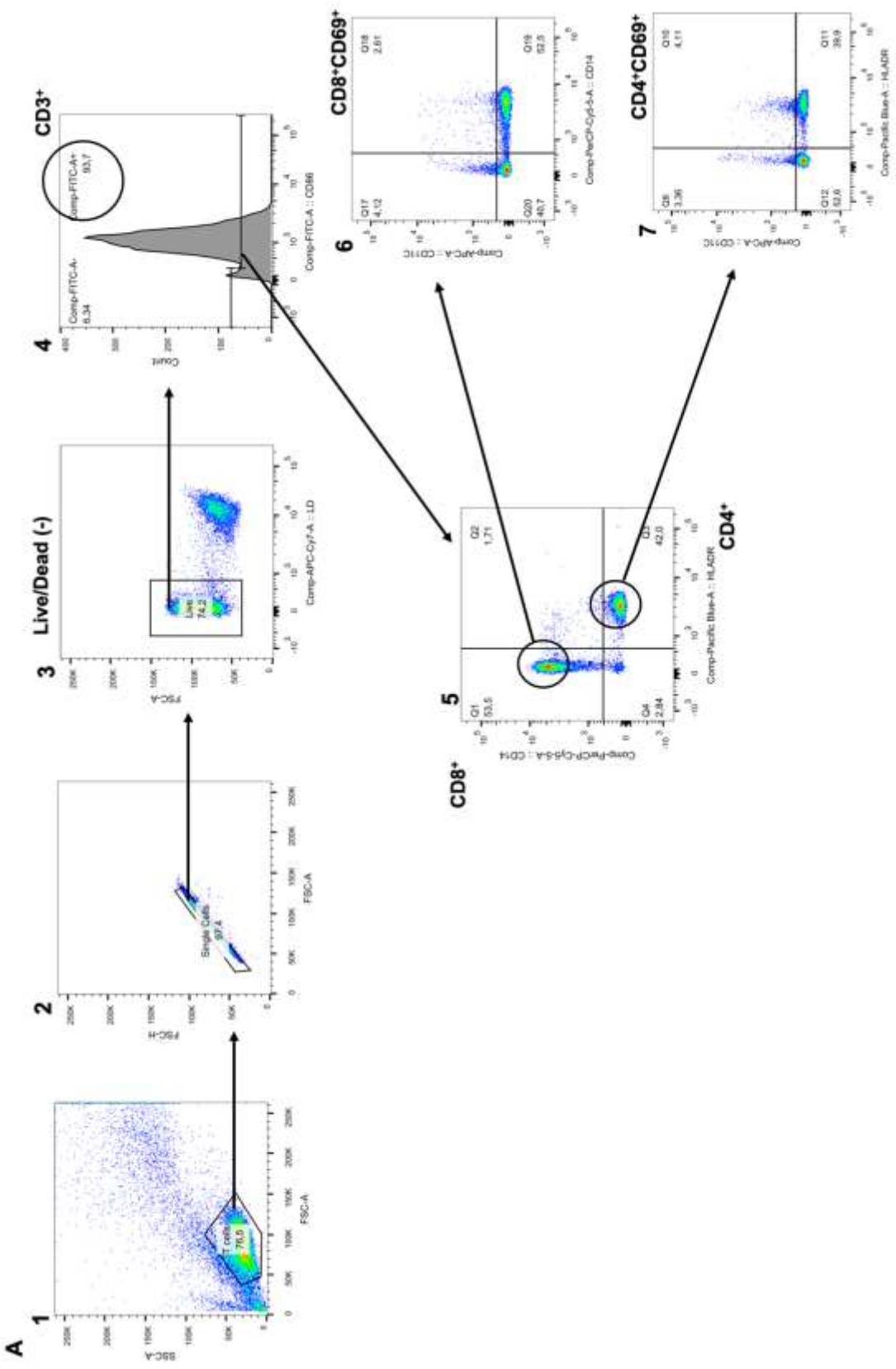


Figure 3.4 – Expression of activation markers in DC by flow cytometry – CD86, CD40 and HLA-DR expression was determined by flow cytometry in DC in co-culture with T cells (in different ratios DC:T cells) and stimulated with LPS. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. 1: FSC-A/SSC-A – represents single cells in the light scatter based on cell size and granularity, respectively; 2: FSC-A/FSC-H – represents single cells; 3: Comp-APC-Cy7-A represents live cells.; 4: Comp-FITC-A represents CD11c+ cells; 5: Comp-FITC-A/Comp-APC-A represents double positive cells $CD11c^{+}CD86^{+}$; 6: Comp-PE-A/Comp-APC-A represents double positive cells $CD11c^{+}CD40^{+}$; 7: Comp-Pacific Blue-A/Comp-APC-A represents double positive cells $CD11c^{+}HLA\text{-}DR^{+}$. Gating strategy was performed on a sample of unstimulated DCs in co-culture with T cells, with positive expression for CD86, CD40 and HLA-DR markers. (B) Pseudo color plots representing expression of activation markers, in different co-culture protocols, in stimulated and unstimulated DCs in co-culture with T cells. (C) Graphs with representation of percentage of double positive cells (top panel) and intensity of median fluorescence (bottom panel) in stimulated and unstimulated DCs in co-culture with T cells, using different DC:T cells ratios (N=1).


A
1
SSC-A
FSC-A
T cells
70,5
2
FSC-H
FSC-A
Single Cells
97,4
3
Live/Dead (-)
FSC-A
Comp-APC-Cy7-A :: LD
Live
74,2
4
CD3+
Count
Comp-FITC-A+
93,7
Comp-FITC-A-
6,34
Comp-FITC-A :: CD66
5
CD8+
Comp-PerCP-Cy5-5-A :: CD14
Comp-Pacific Blue-A :: HLADR
Q1
53,5
Q2
1,71
Q3
42,0
Q4
2,84
CD4+
6
CD8+CD69+
Comp-APC-A :: CD11c
Comp-PerCP-Cy5-5-A :: CD14
Q17
4,12
Q18
2,61
Q19
52,5
Q20
40,7
7
CD4+CD69+
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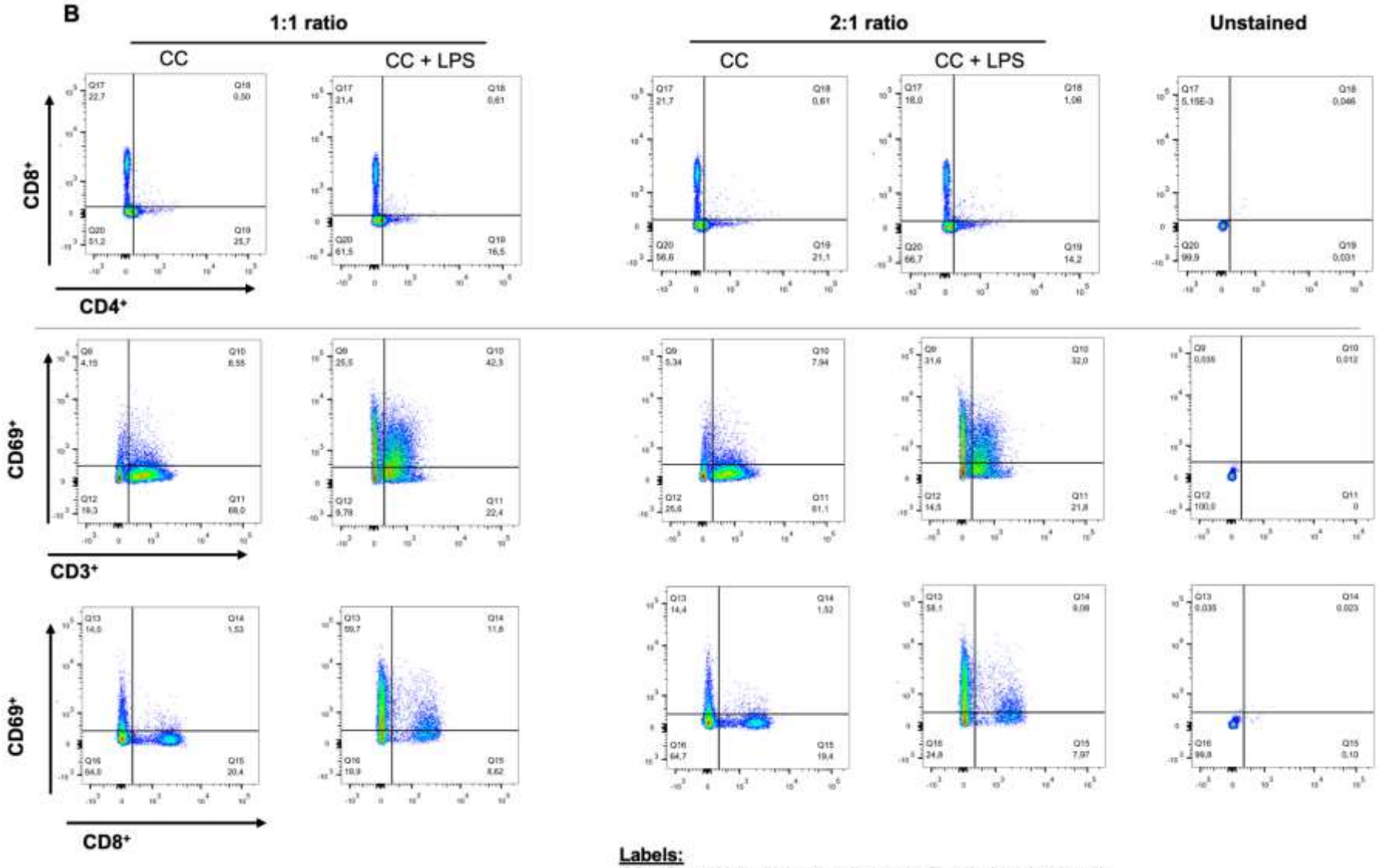


Labels:
CC – Dendritic cells in co-culture with T cells (unstimulated)
CC + LPS – DC in co-culture with T cells, stimulated with lipopolysaccharide

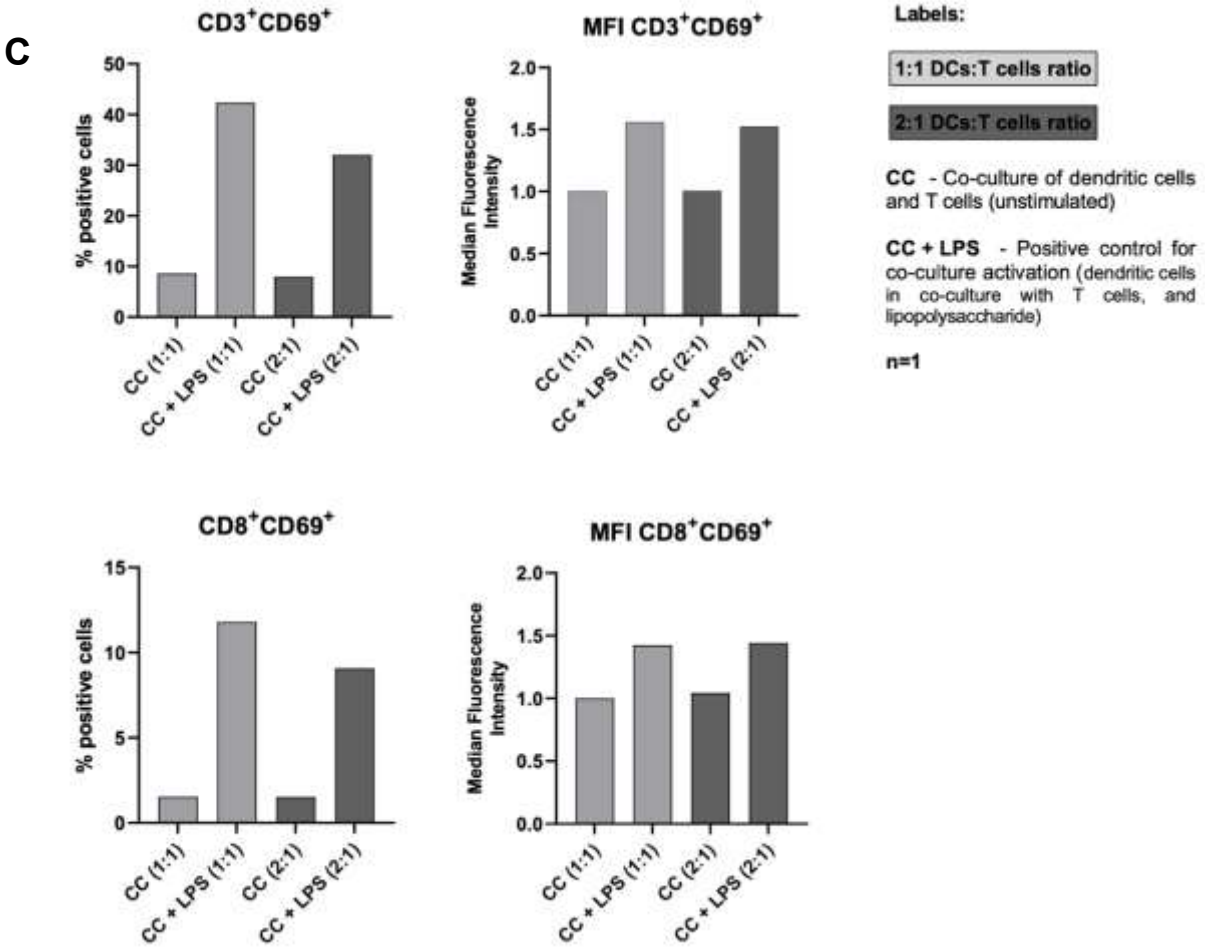


Figure 3.5 – Expression of activation markers in T cells by flow cytometry – CD4/CD8 and CD69 expression was determined by flow cytometry in T cells and DC in co-culture with T cells (in different ratios DC:T cells) and stimulated with LPS. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. 1: FSC-A/SSC-A – represents single cells in the light scatter based on cell size and granularity, respectively; 2: FSC-A/FSC-H – represents single cells; 3: FSC-A/Comp-APC-Cy7-A represents live cells; 4: Comp-FITC-A represents the $CD3^+$ T cells; 5: Comp-PerCP-Cy5-5-A/Comp-Pacific blue-A represents $CD4^+$ and $CD8^+$ cells; 6: Comp-APC-A/Comp-PerCP-Cy5-5-A representing double positive cells CD8+CD69+ cells; 7: Comp-APC-A/Comp-Pacific Blue-A representing double positive cells CD4+CD69+ cells. Gating strategy was performed in a sample of unstimulated DCs in co-culture with T cells, with positive expression for CD69 marker. In the presented gating strategy, pseudo color plots corresponding to CD4/CD8 distribution were displayed to show that the antibody CD4 was not functioning properly. (B) Pseudo color plots representing expression of activation markers, in different DC:T cells ratios, in stimulated and unstimulated DCs in co-culture with T cells. (C) Graphs representing percentage of double positive cells and intensity of

median fluorescence in stimulated and unstimulated DCs in co-culture with T cells, using different DC:T cells ratios (N=1).

3.2.3.3. Optimization of stimulation assay: MOI 5 vs. MOI 10

The next step in the optimization process was to determine the adequate MOI to use in future experiments. MOI is defined as the ratio of infectious particles to cells in cell culture. In the study, it would be the ratio of bacteria to dendritic cells.

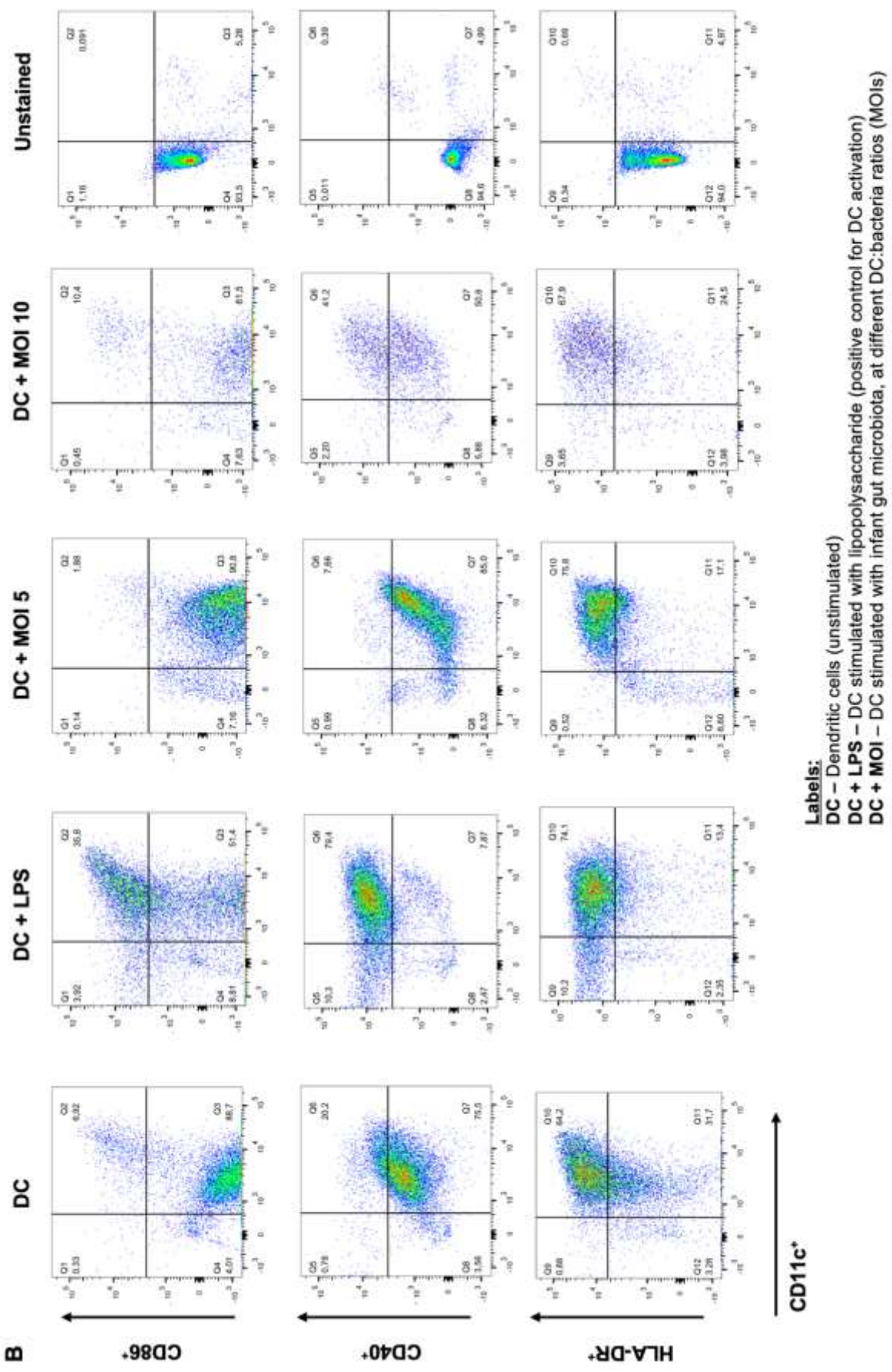
In this experiment, two MOI were tested: MOI of 5 and MOI of 10, using the microbiota control as stimulus. The results are presented in Figures 3.6 and 3.7. The results indicate a dose-dependent response, in which DCs and T cells stimulated with a higher MOI (MOI of 10), seem to display higher expression of the activation markers.

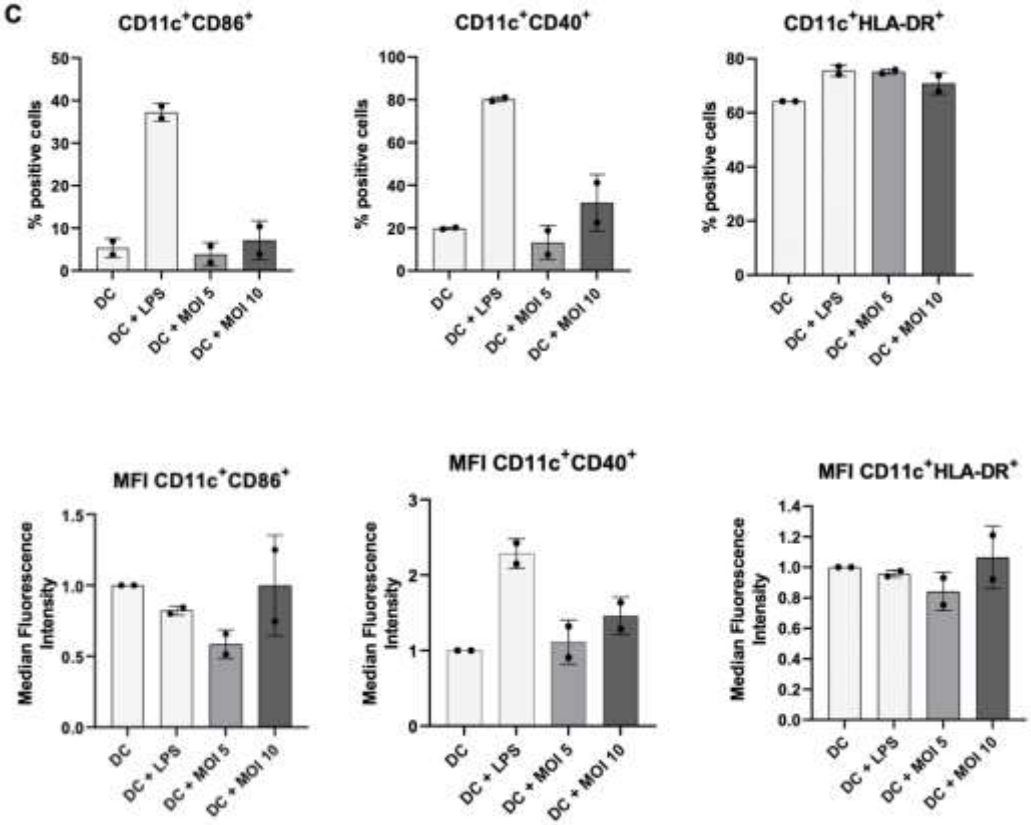
Concerning DC analysis, and first focusing on the impact of the different MOIs on the activation of DCs alone, it seems that a higher ratio of DC:bacteria induces higher expression of the activation markers CD86 and CD40, both in terms of percentage of double positive cells and MFI values (graphs C, top panel, Figure 3.6). However, regarding the expression of the marker HLA-DR, the tendency seems to reverse, with the lowest MOI (MOI of 5) resulting in higher expression of the activation marker, in terms of percentage of double positive cells. The same was not verified in the MFI graphs, which showed a similar tendency to CD86 and CD40 markers. Nevertheless, the DCs stimulated with gut microbiota, regardless of the MOI used, seem to not express as much activation markers, compared to the positive control of DCs stimulated with LPS. That might be due to fact that in the positive control, it was used a pure commercial LPS, which induced a more potent response. While in the test conditions, DCs were stimulated with the gut microbiota solution, which might contain other components that might interfere with DC activation, potentially dampening the expression of the activation markers and resulting in lower values.

In the analysis of DCs in co-culture with T cells, the results appear to exhibit a similar tendency to the previous analysis in DCs alone, with a MOI of 10 leading to higher expression of the activation markers (graphs F, Figure 3.6).

Regarding T cell analysis, the results displayed in Figure 3.7 seem to indicate that, in the majority of the test conditions, a MOI of 10 resulted in higher expression of CD4+ T cells, and a higher expression of the activation marker CD69, in terms of percentage of positive cells and MFI values (graphs C, Figure 3.7).

Therefore, the MOI of 10 was chosen for further experiments.


B
DC
DC + LPS
DC + MOI 5
DC + MOI 10
Unstained
CD86+
CD40+
HLA-DR+
CD11c+
Labels:
DC – Dendritic cells (unstimulated)
DC + LPS – DC stimulated with lipopolysaccharide (positive control for DC activation)
DC + MOI – DC stimulated with infant gut microbiota, at different DC:bacteria ratios (MOIs)



Labels:

DC - Dendritic cells (unstimulated)

DC + LPS - Positive control for DC activation (DCs stimulated with lipopolysaccharide)

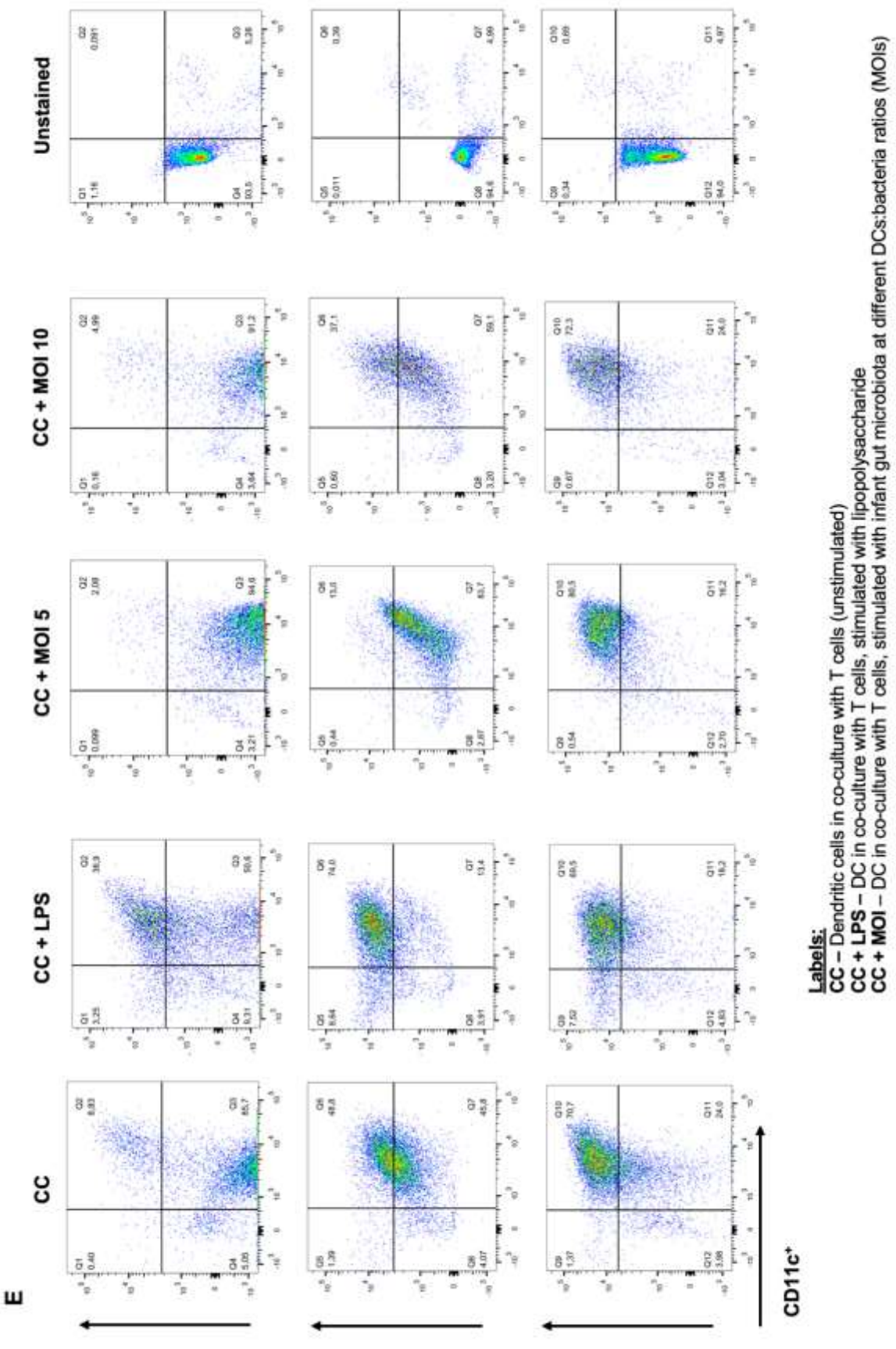
DC + MOI - DCs stimulated with infant gut microbiota (at different ratios DCs:Bacteria)

DCs = $2x10^6$ cells/well

MOI 5 → 5 x ($2x10^6$) = $10x10^6$ cells

MOI 10 → 10 x ($2x10^6$) = $20x10^6$ cells

n=2



Labels:
CC – Dendritic cells in co-culture with T cells (unstimulated)
CC + LPS – DC in co-culture with T cells, stimulated with lipopolysaccharide
CC + MOI – DC in co-culture with T cells, stimulated with infant gut microbiota at different DCs:bacteria ratios (MOIs)

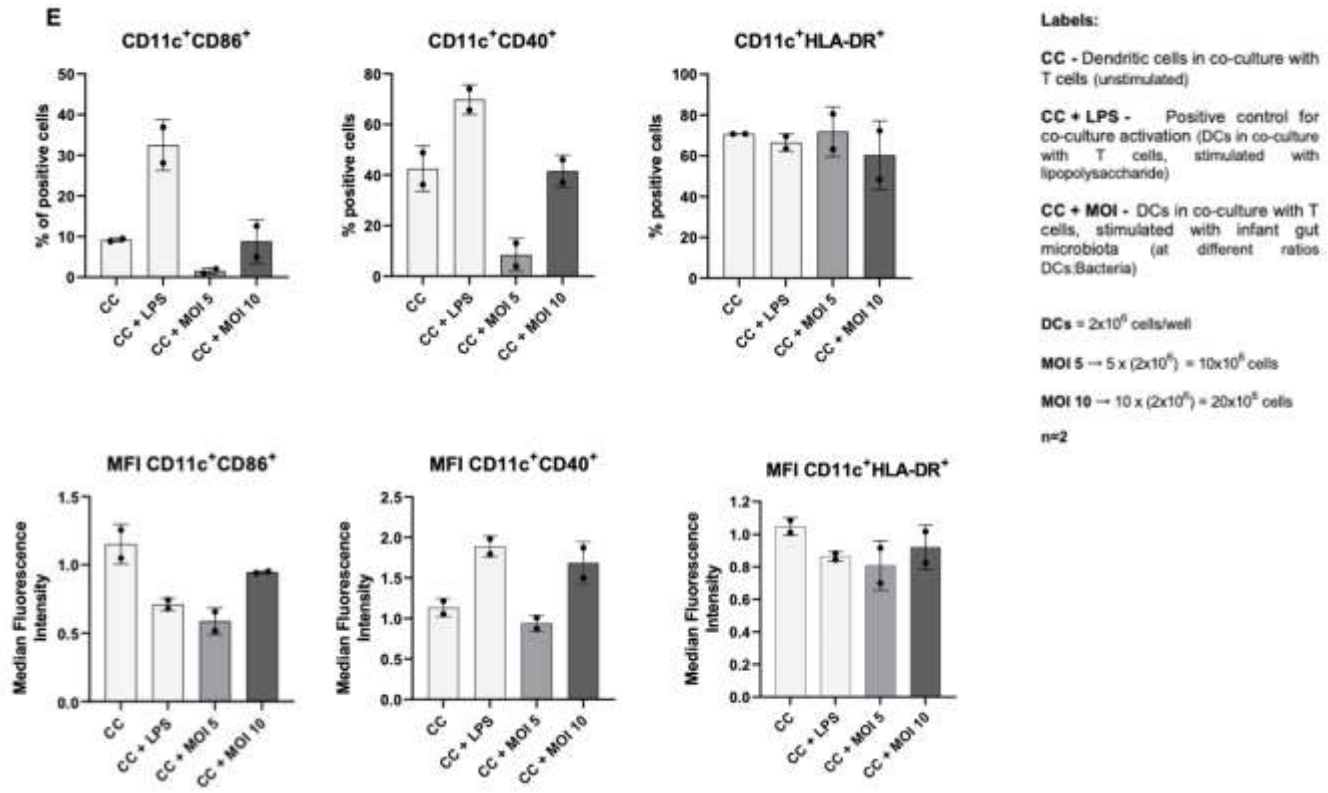
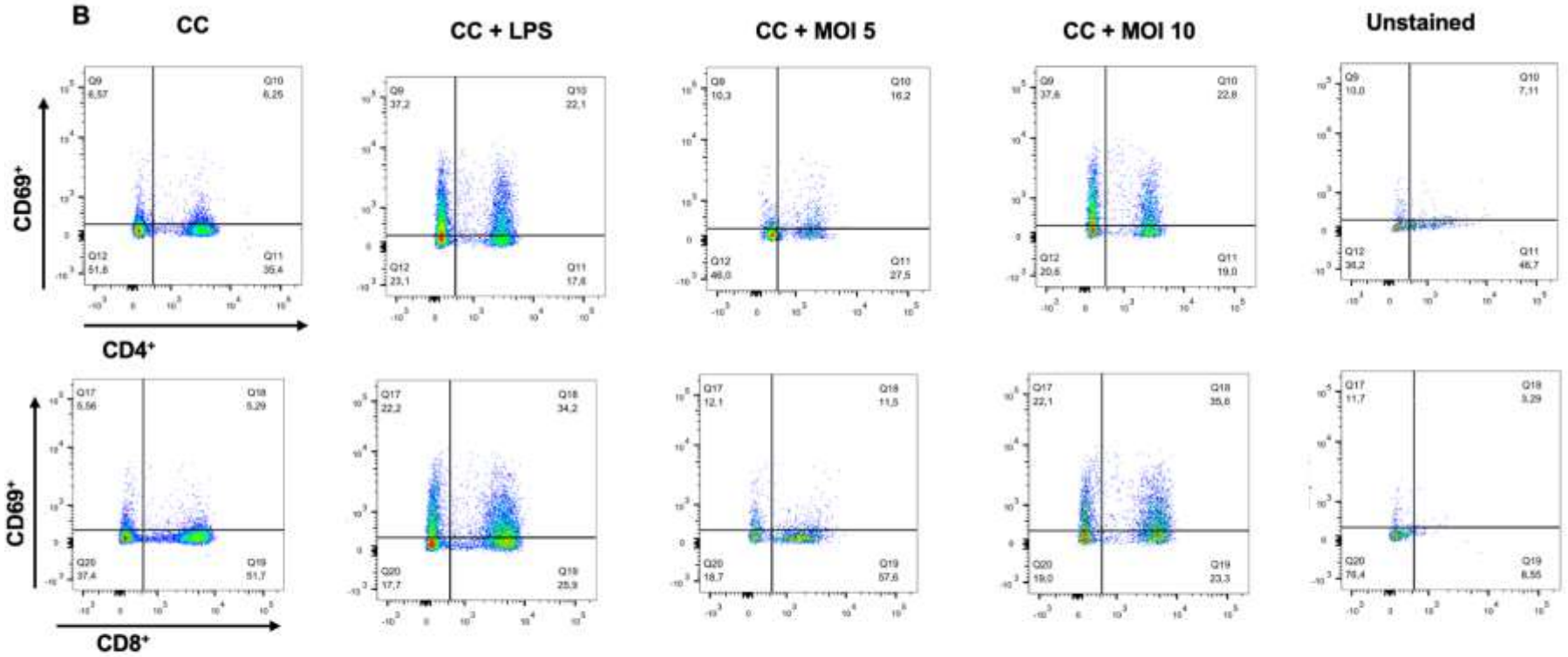


Figure 3.6 – Expression of activation markers in DCs by flow cytometry – CD86, CD40 and HLA-DR expression was determined by flow cytometry in DCs and DC co-culture with T cells and stimulated with LPS and infant gut microbiota, using different ratios DC:bacteria.

(A) and (D) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. The gating strategy applied was the same as indicated in previous figures. (B) and (E) Pseudo colors plots display expression of activation markers in DCs and DCs in co-culture with T cells, respectively, stimulated with LPS and infant gut microbiota, using different DC:bacteria ratios. (C) and (F) Graphs with representation of percentage of double positive cells (top panel) and intensity of median fluorescence (bottom panel) in DCs and DCs in co-culture with T cells, stimulated with LPS and infant gut microbiota, using different ratios DC:bacteria (N=2).



Labels:
CC – Dendritic cells in co-culture with T cells (unstimulated)
CC + LPS – DC in co-culture with T cells, stimulated with lipopolysaccharide
CC + MOI – DC in co-culture with T cells, stimulated with infant gut microbiota at different DCs:bacteria ratios (MOIs)

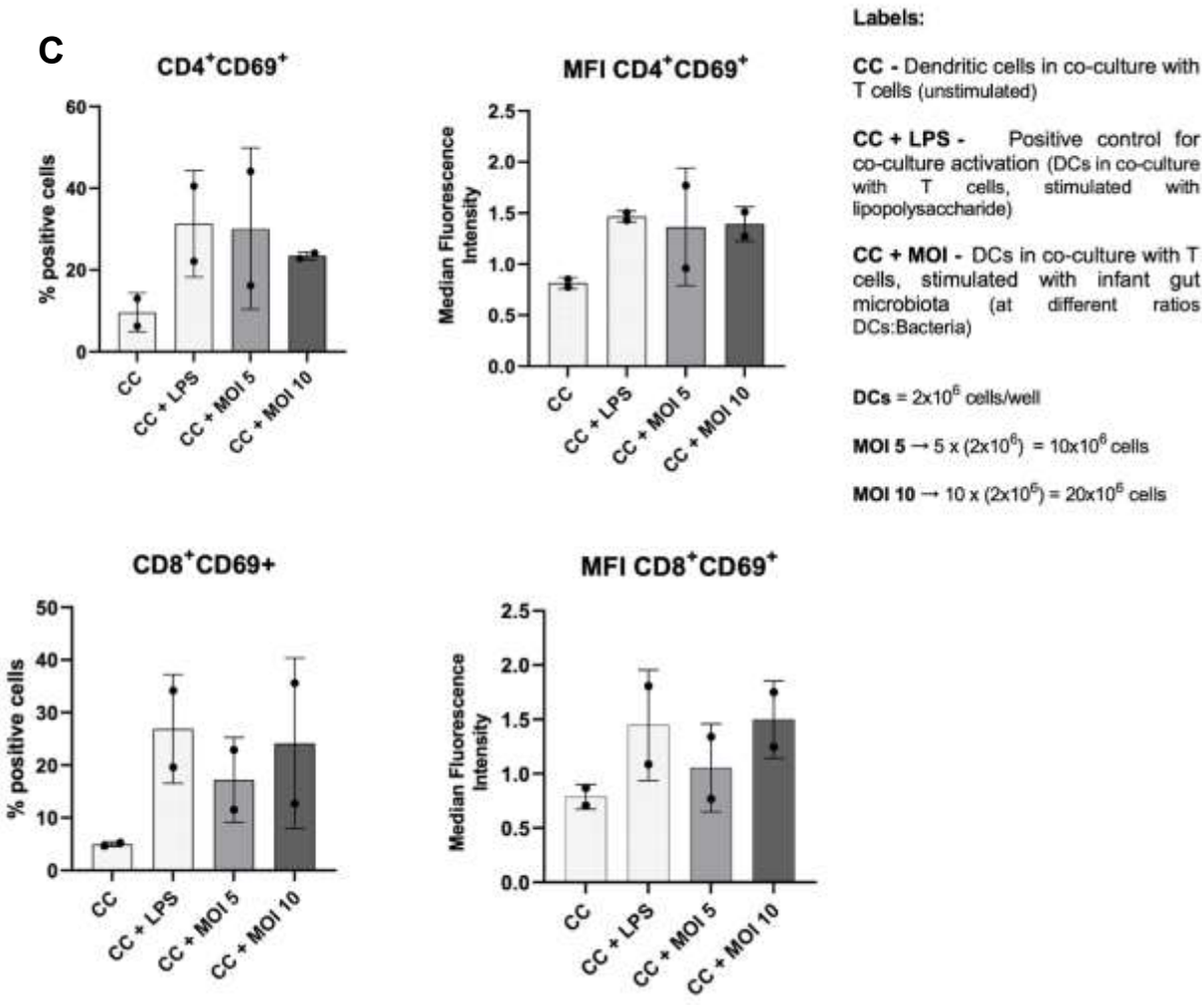


Figure 3.7 – Expression of activation markers in T cells by flow cytometry – CD4/CD8 and CD69 expression was determined by flow cytometry in T cells and DC co-culture with T cells and stimulated with LPS and infant gut microbiota, using different DC:bacteria ratios). (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. The gating strategy applied was the same as indicated in previous figures. (B) Pseudo color plots representing expression of activation markers, in DCs in co-culture with T cells, stimulated with LPS and infant gut microbiota, using different DC:bacteria ratios. (C) Graphs representing percentage of double positive cells and intensity of median fluorescence in DCs in co-culture with T cells, stimulated with LPS and infant gut microbiota, using different DC:bacteria ratios (N=2).

3.2.4. *Effect of activated or inactivated gut microbiota on dendritic cell stimulation*

For safety and logistic reasons, the process of protocol optimization was performed using inactivated microbiota. However, it was important also to assess whether there were significant differences regarding immune cell stimulation using inactivated or non-inactivated microbiota. For that, and with the protocol conditions optimized, immune cells were stimulated with control samples of infant gut microbiota from the same source both inactivated, as mentioned above, or non-inactivated (active), using LPS as control.

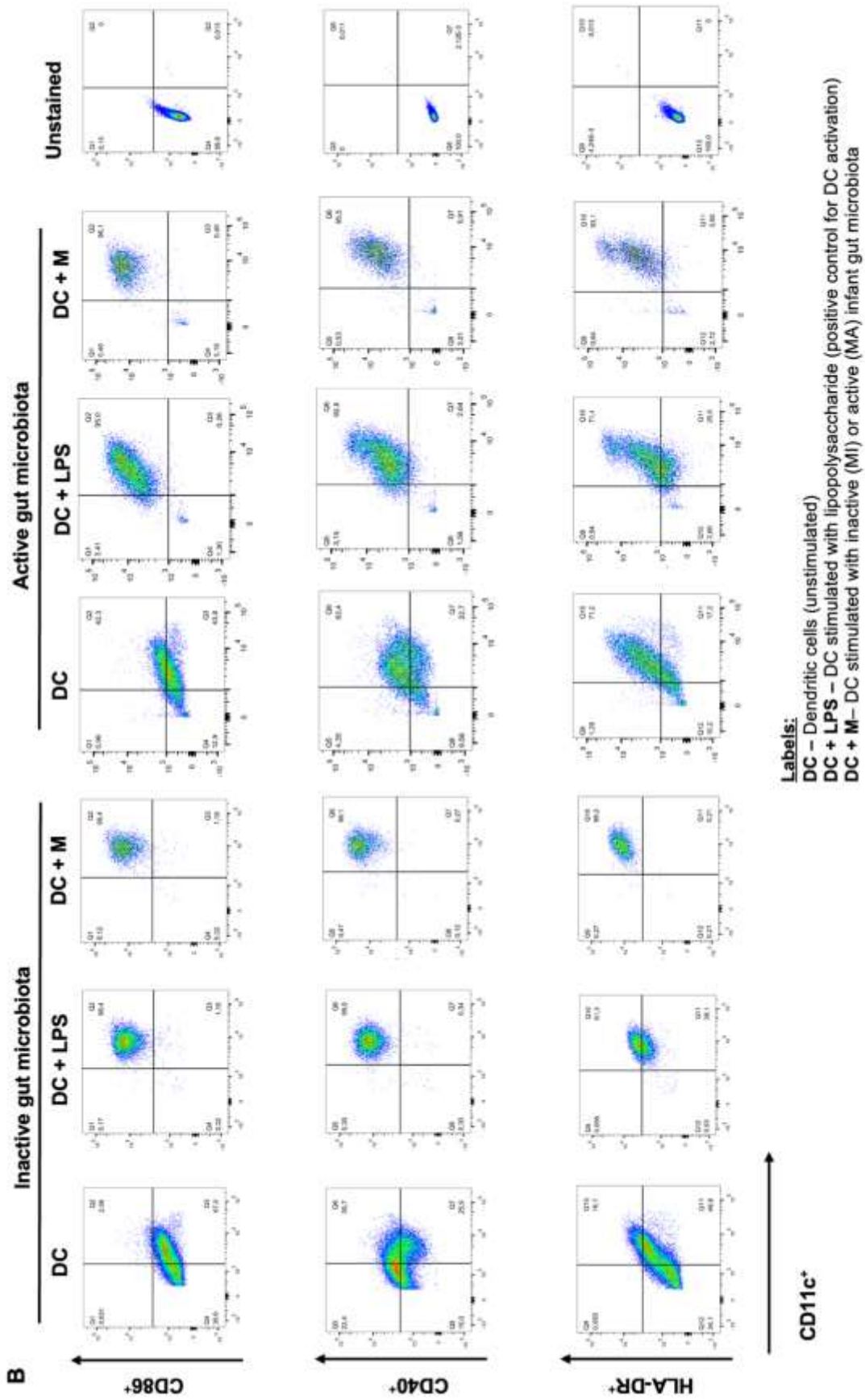
Results concerning dendritic cell stimulation with inactive or active gut microbiota or LPS are displayed in Figure 3.8. As expected, unstimulated dendritic cells presented lower expression of activation marker CD86.

Regarding stimulated DCs, when comparing the test conditions (inactive microbiota and active microbiota) with the positive control (LPS), no significant differences were found. In fact, the conditions in which DCs were stimulated with gut microbiota (whether active or inactive) seemed to follow a similar tendency to DCs stimulated with LPS. The explanation behind these results may lie in the composition of the stimulus used. The gut microbiota solutions used in this experiment differ in whether they were submitted to inactivation cycles with a UV lamp, or not. UV lamps are typically used in biological safety cabinets or to sterilize large surfaces and enable microbial inactivation through photochemical reactions to the microorganism's genetic material (e.g., DNA denaturation) and thus, interfering with its ability to replicate (Sharrer *et al.*, 2005). Therefore, through this mechanism, most UV lamps blocks bacterial growth without considerably damaging the cell wall. Therefore, LPS (as a component of the cell of Gram-Negative bacteria) is present in both groups (although differing in the degree of purity) and, thus, it would be expected to obtain similar results in terms of cellular activation. The fact that no significant differences were found between the two conditions indicate that stimulation of the immune cells should occur through the components of the extracellular membrane of the microbiota and are not dependent of the activation of any cellular machinery or signaling pathways that would occur in the presence of live microbiota.

The results also seem to indicate no significant differences in the percentage of positive cells between active microbiota and inactive microbiota stimulation. It could be expected that, by using an active gut microbiota to stimulate DCs, that the expression of the activation markers would be higher and lead to higher number of double positive cells, since there might be other microbial components that can interact with DCs receptors. However, it has been described that LPS present in the bacterial cell wall is a potent stimulus for DC maturation, and sufficient to trigger its activation and consequent immune response. The results also revealed that a tendency

for inactive gut microbiota to generate higher MFI values, compared with DCs stimulated with active gut microbiota (graphs C, bottom panel, Figure 3.8). It has been stated that UV damage to bacteria can induce the formation (6-4) photoproducts, and that could be an explanation for the increased MFI values observed, despite the fact that there is no description of photoproducts interaction with immune cells (Takada *et al.*, 2017). Other explanation may be linked to the released to the extracellular environment of bacterial immune-active molecules, including peptidoglycan, that activate pattern recognition receptors in host cells (Ragland & Criss, 2017). A possible explanation to this phenomenon could be related to the presence of lysozyme, an enzyme with antimicrobial activity, present in human blood (more precisely in white blood cells, such as monocytes and lymphocytes). Lysozyme can be found in human fluids (saliva, breastmilk and blood, and acts on bacteria by dissolving the beta-1,4 glycosidic bonds between adjacent peptidoglycan monomers, creating instability in the bacterial cell wall, and the release of peptidoglycan. The peptidoglycan can, then, interact with TLR-2 and NOD receptors on innate immune cells, triggering an immune response (Ragland & Criss, 2017; Mercado-Lubo & McCormick, 2010). Therefore, it is possible that the potential release of a potent inflammatory mediator, such as bacterial peptidoglycan, could also interact with the cells, resulting in the elevated levels of expression of DC activation markers obtained from stimulation with inactive microbiota.

Taking the obtained results into consideration, the remaining experiments were performed with inactive microbiota.


B
Inactive gut microbiota
Active gut microbiota
Unstained
DC
DC + LPS
DC + M
DC
DC + LPS
DC + M
CD86+
CD40+
HLA-DR+
CD11c+
Labels:
DC – Dendritic cells (unstimulated)
DC + LPS – DC stimulated with lipopolysaccharide (positive control for DC activation)
DC + M– DC stimulated with inactive (MI) or active (MA) infant gut microbiota

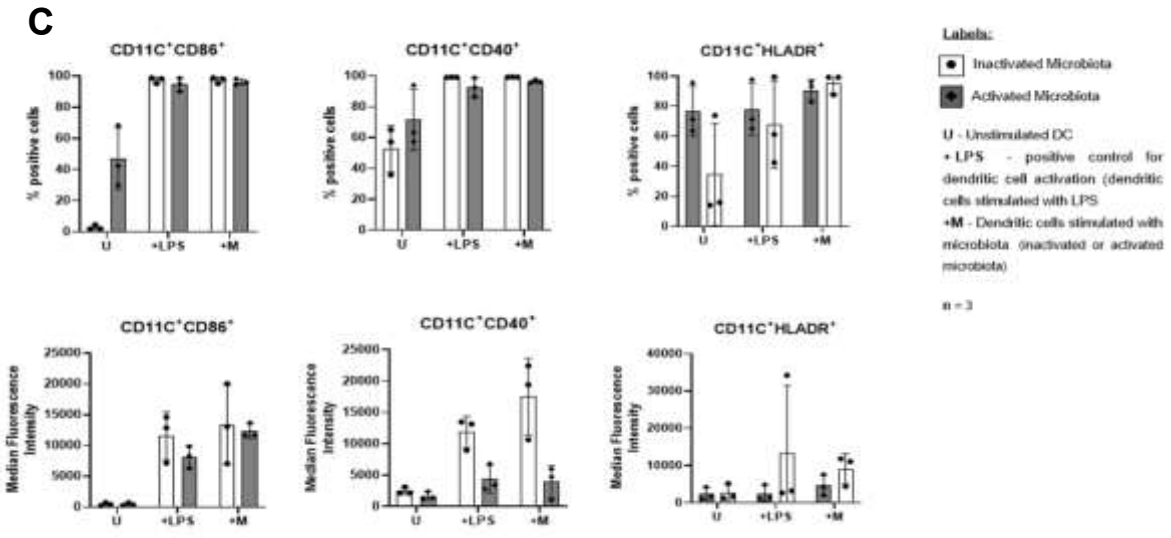


Figure 3.8 – Expression of activation markers in DCs by flow cytometry – CD86, CD40 and HLA-DR expression was determined by flow cytometry in DCs, stimulated with LPS and with inactive or active infant gut microbiota. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. The gating strategy applied was the same as indicated in previous figures. (B) Pseudo color plots representing the expression of the activation markers in DCs, stimulated with LPS and inactive or active infant gut microbiota. (C) Graphs with representation of double positive cells (top panel) and intensity of median fluorescence (bottom panel) in DCs, stimulated using LPS and active or inactive infant gut microbiota (N=3).

3.3. Interaction between gut microbiota and immune system – the impact of maternal obesity

Following optimization of the experimental protocol, the next step was to study the interaction of infant gut microbiota on the activation of dendritic cells, and consequently on T cells priming and activation. Further, we evaluated the impact of maternal gut microbiota on immune cell activation, depending if it's from an obese or lean mother. For that, gut microbiota from the participants was used to stimulate dendritic cells alone, and dendritic cells in co-culture with T cells, as displayed in Figure 3.9.

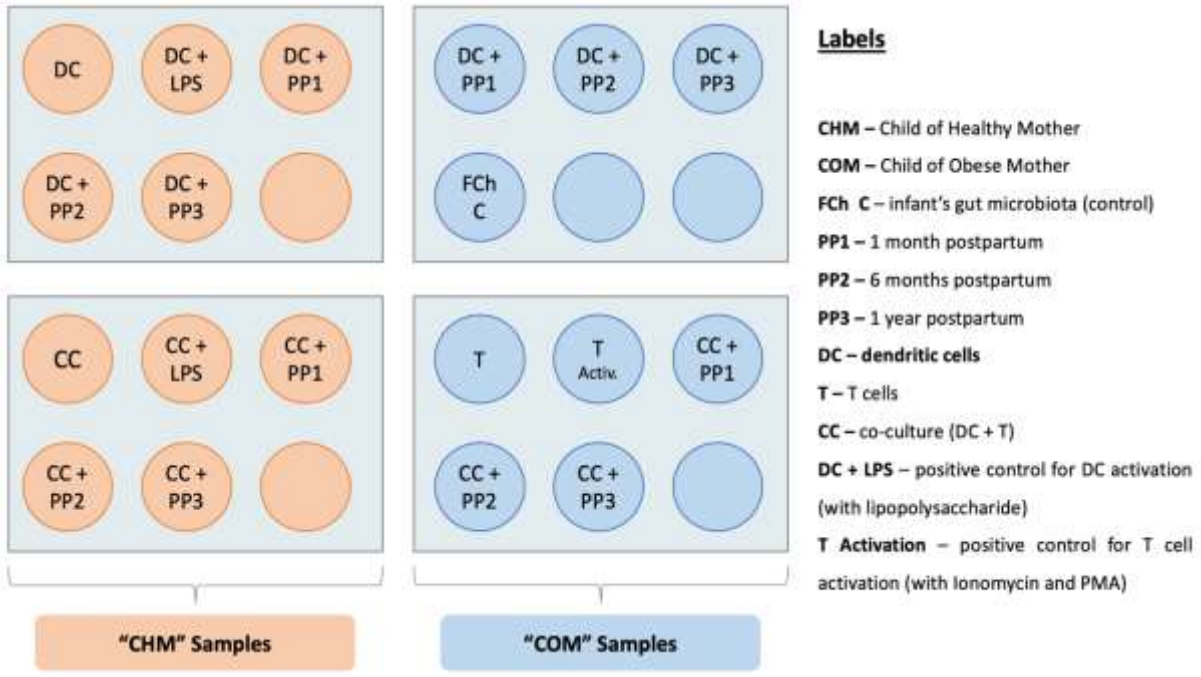


Figure 3.9 – General scheme of experimental conditions.

The study's preliminary results obtained from immune cell stimulation with gut microbiota collected from the study's participants are displayed in Figures 3.10 – 3.14.

Regarding DC and T cell analysis, the obtained results indicate that infant's gut microbiota enabled immune cell activation, regardless of the mother's metabolic status (Figures 3.10 – 3.14). No significant differences were found in immune cell activation levels of infants from obese or lean mothers, both in the percentage of positive cells and in the MFI values. In DC analysis, the results exhibited in Figure 3.12 seemed to indicate no significant differences in the activation levels of DCs stimulated with gut microbiota from infants of obese and lean mothers, both in DCs alone and in co-culture with T cells. A similar tendency was observed in T cell analysis, as the results displayed in Figure 3.14 seem to indicate no differences in the expression

of activation marker CD69 in cells treated with gut microbiota from infants of obese and lean mothers. Also, it was observed that $CD4^+$ T cells seemed to have higher expression compared to $CD8^+$ T cells. In the obtained results, it was also noted that unstimulated T cells exhibited higher activation levels than the positive control for T cell activation, in which PMA and ionomycin were used, namely in the graphs representing percentage of double positive cells $CD4^+CD69^+$ (Figure 3.14).

Additionally, the results also seemed to indicate that there were no significant differences between the three timepoints of microbiota collection (1, 6, and 12 months postpartum), and there were no differences in the three timepoints when comparing the "Healthy" and "Obese" group.

Despite the reduced literature regarding the impact of maternal gut microbiota on immune system priming (since the topic of maternal transfer of gut microbiota to offspring is still quite recent), the remaining information regarding immune cell interaction with gut microbiota in cases of metabolic disease can still provide a general insight about the topic. Starting with DC activation, the results appear to be in discordance with the literature since it has been proven that microbial activation of DCs (upon binding to TLR4) leads to the expression of co-stimulatory molecules CD40, CD86, and CD80 (Møller *et al*., 2022; Stagg, 2018). Considering that obese individuals may have a dysbiotic gut microbiota, with elevated abundances of Gram-negative bacteria (increased concentration of LPS), it would be expected that activation levels of these co-stimulatory molecules would be higher in infants from obese mothers than in infants from healthy mothers. Furthermore, studies in animal models have proven the impact of a high-fat diet on gut microbiota composition and its link to obesity development. For instance, Cani *et al* (2007) (Cani *et al*., 2007) evaluated intestinal microbiota alterations in mice fed with different food regimens, either a high-fat diet or a control diet. It was observed that mice fed with a high-fat diet presented reduced levels of *Bifidobacterium*, known to reduce the concentration of the endotoxin LPS and improve intestinal barrier integrity. Furthermore, it was found that mice fed with a high-fat diet presented increased endotoxemia – a mechanism in which elevated levels of systemic LPS trigger inflammation (Cani *et al*., 2007). Moreover, changes in diet can deeply impact the composition of intestinal microbiota. A Western-like diet (rich in fat and low in fiber), can deprive commensal bacteria of their source of energy, depleting the gut microbiota of beneficial bacteria and reducing the levels of microbial metabolites (such as propionate and butyrate, which have a beneficial effect in the maintenance of barrier integrity). Thus, a weakened gut barrier will promote translocation and trigger inflammation (Liébana-García *et al*., 2021). But there are few studies that underline the impact

of an obesogenic gut microbiota on DC activation (namely, in the expression of activation markers analyzed in this study), and such, only general comparisons can be made between the results and the literature.

In terms of T cell activation, it has been described that increased levels of intestinal cytotoxic $CD8^+$ T cells are a hallmark of obesity (due to tissue infiltration), often linked to tissue inflammation, and are positively correlated with the host's BMI (Monteiro-Sepulveda *et al*., 2015). However, despite that elevated levels of $CD3^+CD8^+$ T cells would be expected in the "Obese" group, it is important to highlight, once more, that we are assessing the indirect impact of the gut microbiota from obese and lean mothers on their children's immunity, and not the direct influence of gut microbiota from obese or lean children. Thus, we cannot make a proper comparison between our results and those described in the literature. Furthermore, the type of analysis performed in this study is quite distinct from what is typically in similar studies, assessing the role of gut microbiota in obesity: while some authors quantify the number of cytotoxic $CD8^+$ T cells recurred in the intestinal epithelium, we *in vitro* assess the number of cells that express this receptor upon stimulation with microbiota. Regarding T cell activation, the literature has described the importance of commensal microbiota to elevated expression of CD69 in intestinal lymphocytes, further highlighting induction of this activation by the gut microbiota could shape the immune response and prevent invasion of luminal space by external microorganisms (Radulovic & Niess, 2015). As such, considering the state of low-grade inflammation observed in obese individuals, it would be expected to observe higher expression of CD69 in cells treated with gut microbiota from infants of obese mothers. It was also noted that the positive control for T cell activation displayed reduced values compared to the negative control (unstimulated T cells). PMA and ionomycin are typically used for T cell activation studies, since they can bypass the T cell receptor complex on the cell's membrane, and trigger the activation of several intracellular signaling pathways, that will result in T cell activation and release of cytokines (Ai *et al*., 2013). Thus, it would be expected that the positive control for T cell activation would exhibit higher activation levels, and such was not observed in the case of $CD4^+CD69^+$. A hypothesis that might explain these inconsistencies could be that the reagents added were not sufficient to trigger activation in this subset of cells.

It is important to highlight two factors/hypothesis that might explain the inconsistencies observed in the results. The first being that we don't know if our evaluated infants have dysbiotic microbiome. We only know that their obese mother have a dysbiotic microbiome that is vertical transmitted to the child. So, we are not evaluating the direct impact of infant dysbiotic gut microbiota on the activation of immune cells, but the potential influence of maternal obesity

on the infant's immune modulation, exploring the possibility of vertical transfer of dysbiotic microbiota from mother to child, and its effect on the activation on immune cells from healthy donors. Despite the mother representing an important factor in the establishment of early-life gut microbiota, and that maternal bacterial strains tend to remain in the infant's gut until 1 year postpartum (Mulligan & Friedman, 2017), other factors that influence the baby's intestinal microbiota must be considered, such as early-life feeding strategies and probiotic intervention (Milani *et al*., 2017). These external factors can potentially dampen the impact of a dysbiotic maternal gut microbiota on the offspring's gut microbiota, and consequently, on the maturation of the immune system. However, a larger number of participants will be needed to draw conclusion on these topics.

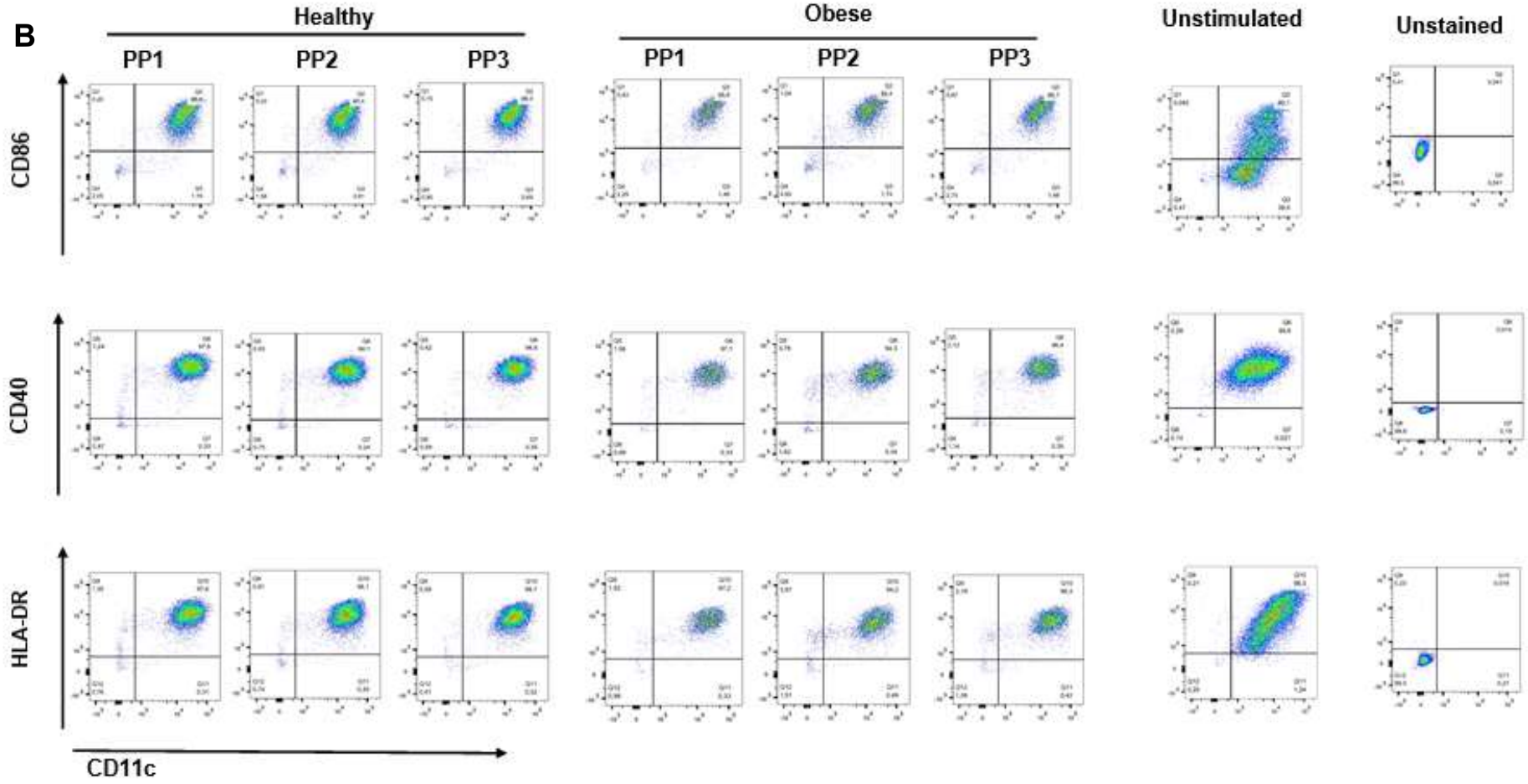
Another hypothesis that was suggested by the research group was immune cell exhaustion by long-term stimulus. Studies have shown that, while short-term stimulation of DCs with LPS leads to increased secretion of cytokines and upregulation of co-stimulatory molecules (such as CD86 and HLA-DR), long term stimulation (24h or more) can make DCs resistant to further stimulation (Choi *et al.*, 2022). Moreover, it has been reported that prolonged contact with a stimulus can trigger apoptosis in DCs, leading to a reduction of live cells in cell culture (Carstensen *et al*., 2019). T cells can also become "exhausted" and display reduced cytokine secretion, and losing its effector abilities upon prolonged contact with an antigen, (however it should be noted that most studies that come to this conclusion derived from the long-term contact of T cells with viruses, and not bacteria) (Wherry, 2011; Wherry & Kurachi, 2015). However, this hypothesis was soon discarded since the time frame in which the cells are placed in contact with the stimulus, is not sufficient to lead to cell exhaustion. Thus, it is more likely that these inconsistencies might be related to the reduced number of samples (n), which did not allow to observe any significant differences.

Despite the apparent lack of differences in the expression of the activation markers in stimulated DCs and T cells, there are other variables that were not included in this study and whose results could provide further insight of the impact of dysbiotic gut microbiota on immune system priming. For instance, obtaining a cytokine secretion profile and/or understanding which subtype of T helper cells are being expressed upon interaction with the gut microbiota (Th1, Th2, or Th17) would be helpful to understand the overall scenario of the interaction of obesogenic microbiota and immunity.

Finally, it seems relevant to explain the apparent lack of differences in the expression of activation markers (both in DC and T cells analysis) between the timepoints of infant development: 1 month, 6 months and 1 year. As mentioned in previous chapters, at birth, the

infant's immunity is highly suppressed, in part to prevent an abnormal immune response by the mother that could lead to termination of the pregnancy. After birth, the infant comes in contact with a wide array of microorganisms, that represent the trigger necessary to stimulate the baby's immune system (Milani C. *et al*, 2017). Thus, it would be expected that infant gut microbiota at 1 month postpartum, would not induce a potent immune response. However, as the infant grows, and it's exposed to other various sources of microorganisms, it would be expected that at later timepoints (6 months and 1 year postpartum) there would be differences in the expression of activation markers, and particularly between infants from obese and lean mothers. As mentioned above, the inconsistencies observed in the results might be due to the reduced number of samples included in the study, that did not enable to observe differences between the tested timepoints.

Considering the differences in the gut microbiota composition of obese and healthy people, and the influence of gut microbiota composition on the host's metabolic status, it would be expected to observe differences in the activation levels in both groups. Such was not observed, and it might be due to the low number of participants included in this work (n=3). Future experiments should include a higher number of samples for analysis.



Labels

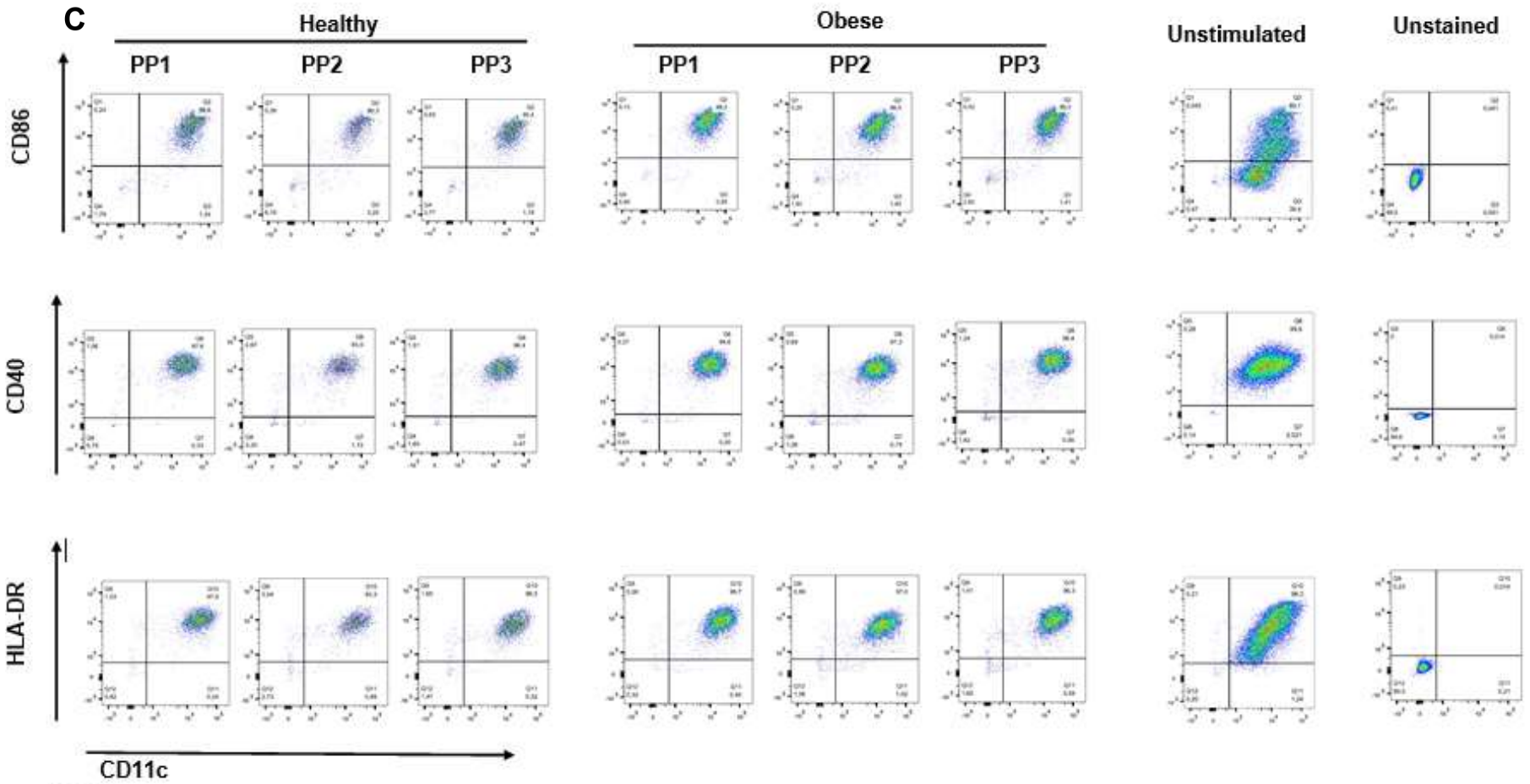
PP1 – 1 month postpartum

PP2 – 6 months postpartum

PP3 – 1 year postpartum

Unstimulated – Dendritic cells in co-culture with T cells, non-stimulated

*Samples collected from infants of obese or healthy mothers



Labels

PP1 – 1 month postpartum

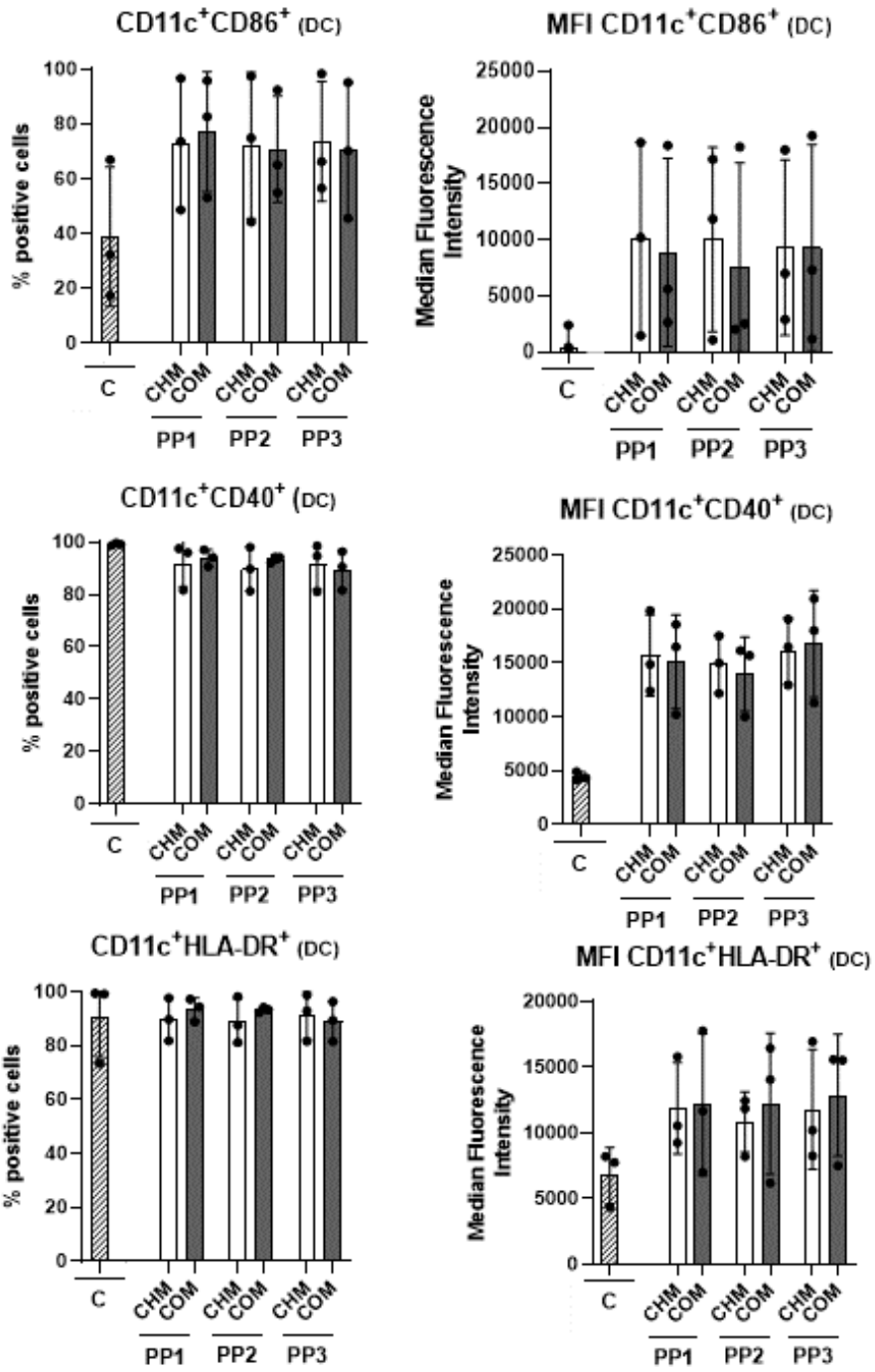
PP2 – 6 months postpartum

PP3 – 1 year postpartum

Unstimulated – Dendritic cells in co-culture with T cells, non-stimulated

*Samples collected from infants of obese or healthy mothers

D **Dendritic cells (DC)**



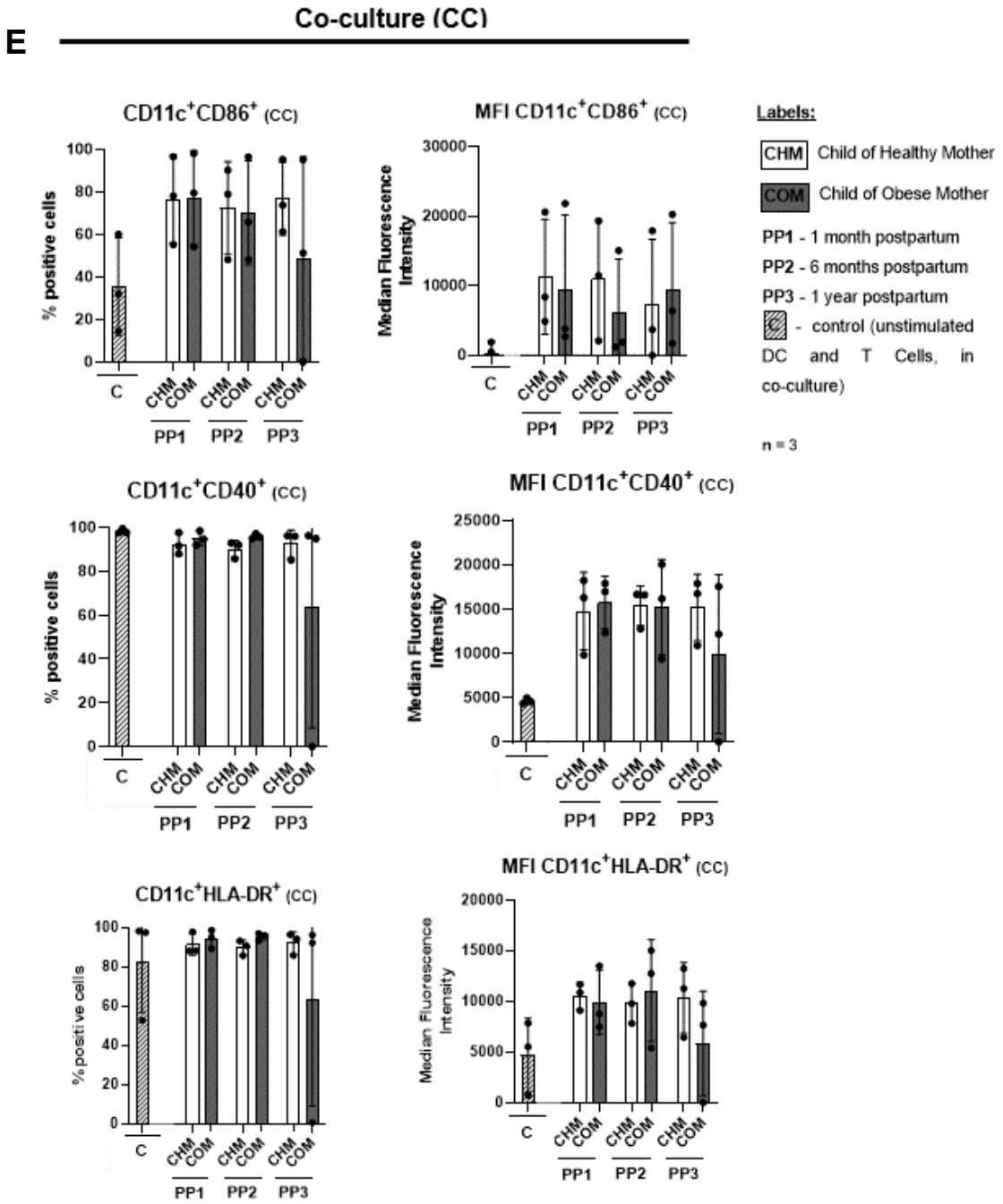
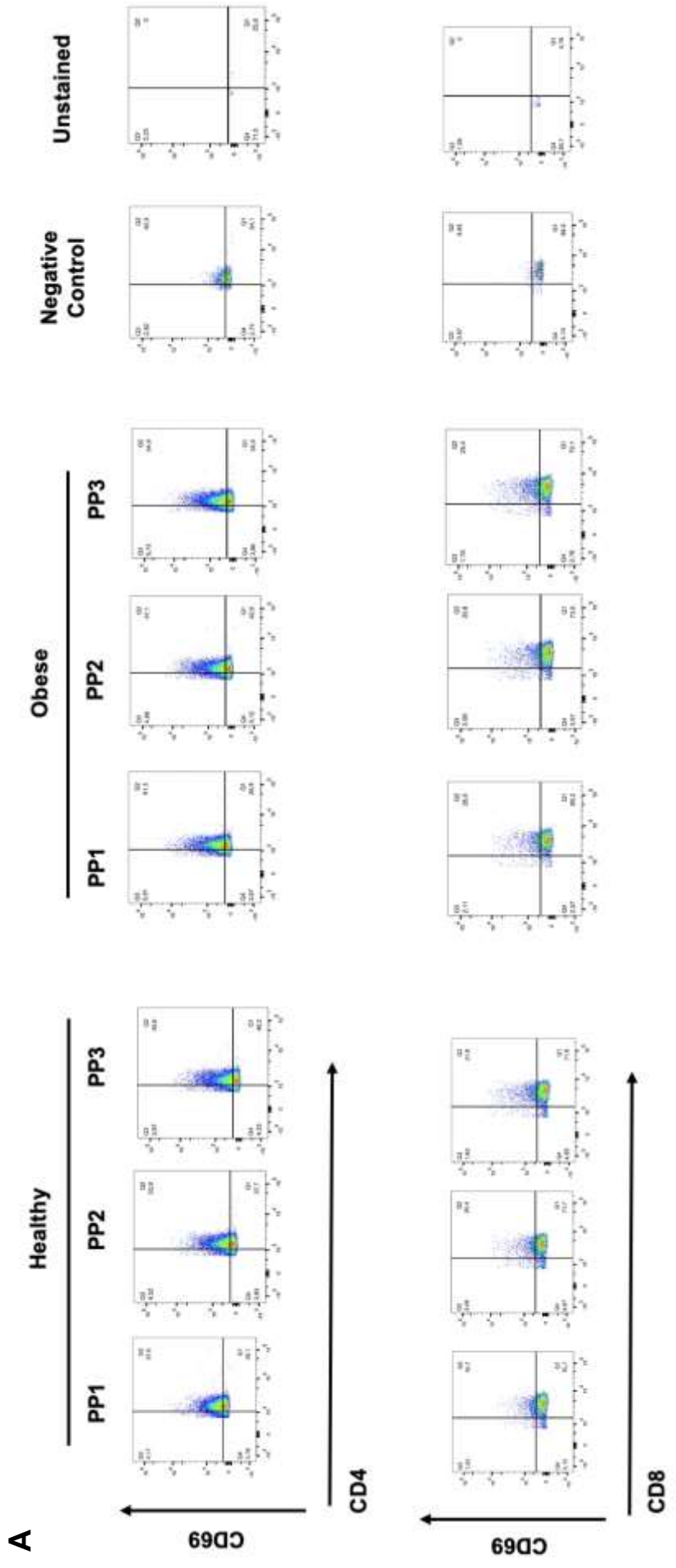


Figure 3.10 – Expression of activation markers in DCs by flow cytometry – CD86, CD40 and HLA-DR expression was determined by flow cytometry in DCs and DCs in co-culture with T cells, stimulated with gut microbiota from infants of obese or healthy mothers, at three timepoints. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. The gating strategy applied was the same as indicated in previous figures. (B -C) Dot plots representing expression of the activation markers in DCs and DCs in co-

culture with T cells, stimulated with gut microbiota from infants of obese or healthy mothers, at three timepoints. (C) Graphs with representation of double positive cells (left panel) and intensity of median fluorescence (right panel) in DCs and DCs in co-culture with T cells, stimulated with gut microbiota from infants of obese or healthy mothers, at three timepoints (N=3).


A
Healthy
PP1
PP2
PP3
Obese
PP1
PP2
PP3
Negative Control
Unstained
CD69
CD4
CD69
CD8

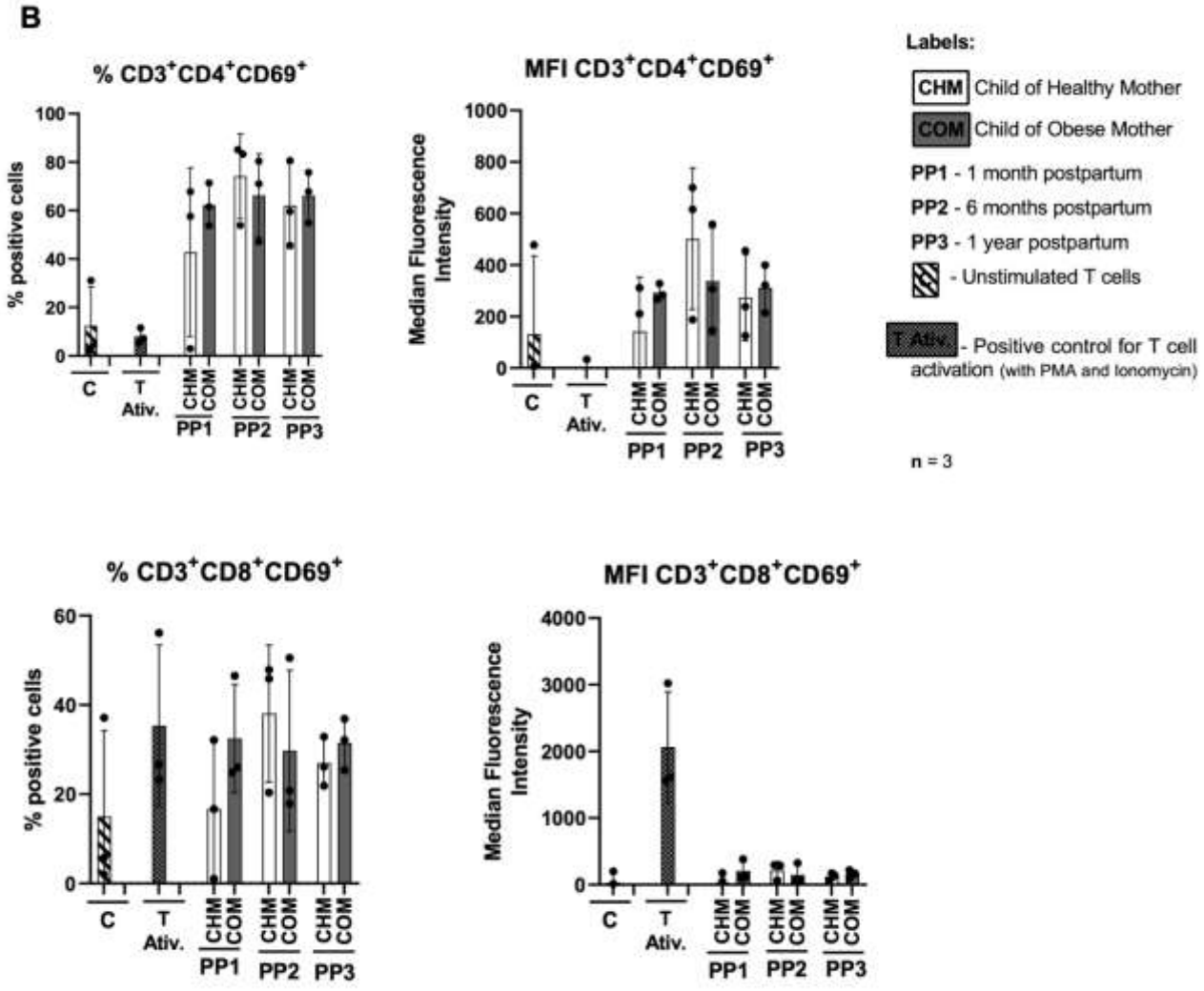


Figure 3.14 – Expression of activation markers in T cells by flow cytometry – CD4/CD8 and CD69 expression was determined by flow cytometry in T cells and DCs in co-culture with T cells, stimulated with gut microbiota from infants of obese or healthy mothers, in three timepoints. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. The gating strategy applied was the same as indicated in previous figures. (B) Pseudo color plots with representation of expression of activation markers in T cells and DCs in co-culture with T cells, stimulated with gut microbiota from infants of obese or healthy mothers, in three timepoints. (C) Graphs representing percentage of double positive cells and intensity of median fluorescence in DCs in co-culture with T cells, stimulated with gut microbiota from infants of healthy or obese mothers, in three timepoints.

3.4. Limitations of the study

A limitation that potentially impacted the results was the low number of samples analyzed in this study, which did not allow us to obtain definitive conclusions about the main hypothesis. The analysis of additional participants' samples is currently underway.

Additionally, other variables that were not included in the study, namely microbiome analysis, cytokine secretion or the effect of specific bacterial strains, known to be present in obese individuals, could provide better understanding at the interaction between gut microbiota and immunity.

4. General Conclusions

4.1. Conclusions

In this study, a protocol for the separation and inactivation of infant gut microbiota, and consequent immune cell stimulation, was successfully optimized. Moreover, it was observed that UV-inactivated infant gut microbiota enabled monocyte-derived dendritic cell and T cell activation.

The preliminary results, with a very limited number of samples, showed that, although infants' microbiota led to immune cell activation, there were no significant differences between activation levels of immune cells stimulated (namely in the expression of activation markers in DCs and T cells) with gut microbiota from infants of obese or healthy mothers. Despite the knowledge that obesity has a profound impact in immune modulation, and that mothers have a profound impact on the child development, still there is not sufficient evidence to clarify how maternal obesity may impact the immune system primming in early-life.

4.2. Future work

The next stage of the experimental work includes the increase in the number of participants, to conclude if there are significant differences in immune stimulation of infants from obese and lean mothers.

Additionally, since it is known that the gut microbiota of obese individuals displays a different composition than their lean counterparts, it would be interesting to analyze the gut microbiota composition of the mother/child dyad and confirm if the mother's gut microbiota is transferred to the child.

Conditioned media from the co-cultures, that was collected, in co-culture will be used to perform cytokine analysis and to observe if there are any differences in the cytokine profile of infants from obese and lean mothers.

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