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Final dissertation

Exploring the crosstalk between gut microbiota and macrophages

Investigating the macrophages functional response

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A chi non ha avuto scelta.

Ai miei pilastri. A voi, mamma e papà. E alle mie nonne.

A chi si è sentito sopraffatto ed abbandonato da un sistema che aveva promesso crescita e futuro.

Table of contents

1. Abstract

Studies have identified an evident correlation between the gut microbiota and inflammatory processes. Macrophages play a key role in immune responses and are characterized by phagocytic ability and functional plasticity. In the intestinal environment, they contribute to maintaining the homeostasis. Furthermore, they are responsive to specific changes in the tissue microenvironment. Alterations in the gut microbiota have shown to contribute to inflammation. For this reason since macrophages are highly exposed and sensitive to the microbiota, their interplay is worth investigating. In addition, it was demonstrated that the KO mice microbiota is more enriched in probiotics and protective compared to the WT.

On this ground, this thesis project aims at defining the functional profile, in terms of gene expression and phagocytic capacity, of wild type (WT) macrophages when exposed to fecal microbiota from eight weeks old mice and CD300e knock out mice.

2. Introduction

2.1 Intestinal macrophages

Tissue-resident macrophages are a subset of highly specialized phagocytic cells involved in maintaining tissue homeostasis. Their function is mediated through the capacity to detect and respond to a wide array of challenges, including metabolic fluctuations, tissue injury, and microbial invasion, while fulfilling tissue-specific functions that support the surrounding microenvironment. The functional profile of these cells is profoundly influenced by the tissue in which they reside, necessitating distinct roles across organ systems.[1]

While phagocytosis and other "housekeeping" functions are conserved among tissue-resident macrophages, their phenotypic and functional characteristics are shaped by the unique signals present in their local niche. The development of single-cell RNA sequencing technologies has significantly improved our understanding of the heterogeneity and plasticity of macrophage populations, revealing tissue- and niche-specific differentiation patterns previously unrecognized. Although macrophages were traditionally classified into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes, recent advances have demonstrated that this model is overly simplistic and insufficient to capture the full spectrum of macrophage functionality.[1]

In the gastrointestinal (GI) tract, macrophages are indispensable for mucosal immune surveillance and the regulation of physiological functions such as gut motility and secretion. Their ability to maintain immunological tolerance despite continuous exposure to luminal antigens has positioned them as central players in intestinal immune homeostasis. However, substantial heterogeneity among intestinal macrophage subsets has impeded efforts to define their precise roles. Emerging evidence indicates that distinct macrophage populations contribute differentially to a range of gastrointestinal pathologies, including postoperative ileus, inflammatory bowel disease (IBD), necrotizing enterocolitis, and disorders

associated with HIV infection and Parkinson's disease. These roles are mediated through intricate interactions with cytokine networks and the enteric nervous system (ENS), which vary depending on the pathological context.[2]

Within the intestine, macrophages are distributed across anatomically and functionally distinct niches. A specialized subset resides near the epithelial layer and crypt base, where they interact closely with epithelial stem and Paneth cells. Others are dispersed throughout the villi, while unique populations localize to the submucosal and myenteric plexuses, forming an integrated network that supports ENS-mediated regulation of motility, secretion, and vascular tone. Deeper in the tissue, muscularis macrophages form dense, neuron-associated networks that contribute to neuroimmune communication.[1]

Macrophages were considered terminally differentiated cells derived from circulating bone marrow-origin monocytes, continuously replenishing the tissue resident pool. However, lineage tracing and fate-mapping studies have demonstrated that many tissue-resident macrophages originate during embryogenesis from yolk sac and fetal liver progenitors. In some tissues, these embryonically derived macrophages persist into adulthood as self-renewing populations, while in others, such as the intestinal lamina propria, they are rapidly replaced by bone marrow-derived monocytes. [1]

Monocyte differentiation into tissue-resident intestinal macrophages is primarily driven by colony-stimulating factor 1 (CSF1). Mice deficient in CSF1 or its receptor (CSF1R) exhibit severe depletion of macrophage populations, and pharmacological CSF1R inhibition in adult mice leads to near-total ablation of gut-resident macrophages. This differentiation process, which unfolds over approximately 5-6 days, involves substantial transcriptional reprogramming and culminates in the acquisition of key functional features, including enhanced phagocytic capacity and constitutive interleukin-10 (IL-10) production.[1]

Finally, tissue-resident macrophages express a diverse array of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors, C-type lectins, and scavenger receptors. These enable them to recognize both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), facilitating rapid immune responses and maintenance of tissue integrity.[3]

2.2 Gut microbiota and crosstalk with macrophages

Microorganisms are distributed throughout the human body, colonizing both external surfaces such as the conjunctiva, oral mucosa, and skin, and internal environments including the gastrointestinal tract and saliva. Growing evidence suggests that these microbial communities exert significant influence over various physiological processes, including metabolic regulation, inflammatory responses, and immune system function.[4]

The gut microbiota is defined by several key features: stability, resilience, and diversity. In healthy adults, the composition of the gut microbial community remains relatively stable over time, a reflection of robust homeostatic mechanisms that include complex feedback loops. Nevertheless, the thresholds at which microbial communities transition into alternative, potentially dysbiotic, stable states remain poorly understood. Interactions among microbes, ranging from mutualism and commensalism to competition and amensalism, as well as symbiotic relationships with the host, are critical to shaping the structure, stability, and adaptability of the gut ecosystem.[4]

Recent advancements in multi-omics technologies, including metagenomics, metabolomics, and transcriptomics, have facilitated deeper insights into the functional landscape of the gut microbiota. These tools have enabled researchers to explore how microbial communities respond to perturbations such as dietary changes, disease states, and host genetic variation. One major challenge in interpreting these data is the concept of functional redundancy,

wherein different microbial taxa perform overlapping roles, complicating the linkage between compositional changes and functional consequences.[4]

The gut microbiota is essential for preserving intestinal barrier integrity and overall gastrointestinal homeostasis. This system is under constant surveillance by the intestinal immune network, which monitors microbial composition and responds to perturbations. Dysbiosis, a detrimental imbalance in the microbial community, can lead to mucosal inflammation and has been implicated in the pathogenesis of various systemic diseases. In healthy states, the host provides nutrients and a hospitable environment, while the microbiota reciprocates by contributing to host metabolism and by producing metabolites that drive immune system development and function.[4]

The gut microbiome is now recognized as a central regulator of host immune responses. It participates in immunosurveillance and shapes immune tolerance, allowing the host to eliminate pathogenic threats while maintaining tolerance toward commensal organisms. Germ-free (GF) animal models have provided key insights into the essential role of microbes in immune development. These models exhibit immature gut-associated lymphoid tissues (GALT), thinner mucus layers, and reduced immune cell diversity, highlighting the importance of microbial colonization in shaping immune architecture.[4] In this sense, murine models are frequently employed to investigate host-microbiota interactions and the influence of dietary, microbial, and genetic factors on intestinal physiology. While many features of the intestinal tract are conserved between mice and humans, key anatomical and physiological differences must be considered when designing experiments and interpreting data.[5]

Macrophages, central to the innate immune response, not only defend against pathogens but also interact with commensal microbes. For instance, Bacteroides fragilis has been shown to promote M1 polarization of macrophages, enhancing their phagocytic capacity. Furthermore, gut microbiota facilitate crosstalk between interleukin-1β-producing macrophages and colony-

stimulating factor 2 (Csf2)-secreting RORγt^{+} group 3 innate lymphoid cells (ILC3s). Microbial metabolites such as n-butyrate influence macrophage function by suppressing pro-inflammatory cytokines, thereby promoting tolerance toward commensals. Butyrate has also been linked to enhanced antimicrobial activity via modulation of macrophage metabolism and increased LC3-associated antimicrobial clearance.[4]

Efforts to therapeutically modulate the gut microbiota have led to the exploration of several strategies, including fecal microbiota transplantation (FMT), dietary modifications, probiotics, prebiotics, and engineered microbial consortia. Among these, FMT remains the most clinically established approach, currently approved by the U.S. Food and Drug Administration for specific indications such as recurrent *Clostridioides difficile* infection. FMT involves transferring fecal matter from a healthy donor to a recipient in order to restore microbial balance. Despite its clinical promise, many microbiota-based interventions remain in early stages of development, with most studies focused on correlative observations rather than mechanistic causation. Future research must prioritize the development of targeted, reproducible, and mechanistically informed strategies to harness the microbiome for therapeutic benefit.[4]

2.3 CD300e

CD300e, previously referred to as Immune Receptor Expressed by Myeloid cells-2 (IREM-2), is a glycosylated surface receptor characterized by a single extracellular immunoglobulin-like domain. It is a member of the CD300 receptor family, which includes both activating and inhibitory receptors expressed across myeloid and lymphoid lineages. Although the endogenous ligand of CD300e remains unidentified, its function has been studied using an agonistic monoclonal antibody. Experimental data indicate that CD300e engagement in human monocytes and myeloid dendritic cells promotes cell survival, induces

activation marker expression, and stimulates the release of pro-inflammatory cytokines, suggesting its role as an activating immune receptor.[6]

The presence of a lysine residue within the transmembrane domain has led to the hypothesis that CD300e may associate with adaptor proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs). Supporting this, CD300e has been shown to associate with DNAX-activating protein 12 (DAP12) in transfected COS-7 cells. However, this interaction has not been conclusively demonstrated in primary human monocytes, leaving the downstream signaling pathways of CD300e activation incompletely understood.[6]

The CD300 family comprises both activating and inhibitory receptors that fine-tune immune responses. In humans, CD300e is primarily expressed in monocytes and myeloid dendritic cells, where it transmits activating signals via DAP12. The murine ortholog (mCD300e), however, remains less well-characterized. Notably, mCD300e is predominantly expressed in non-classical ($CD14^{low}$ $CD16^{+}$) and intermediate ($CD14^{+}$ $CD16^{+}$) monocyte subsets, whereas its human counterpart is expressed across all monocyte subsets, including classical ($CD14^{+}$ $CD16^{-}$) monocytes, albeit with higher levels in the intermediate and non-classical subsets. [7]

During in vitro monocyte-to-macrophage differentiation, CD300e expression is typically downregulated. However, recent findings demonstrate that this downregulation can be reversed by inhibiting miR-4270, which restores CD300e expression in monocyte-derived macrophages. [8]

In vitro studies support the classification of CD300e as an activating receptor involved in inflammatory and immune functions regulation. Under physiological conditions, CD300e expression is relatively low in tissues such as muscle, lung, kidney, and adipose tissue. [8] Notably, bone marrow exhibits the highest expression of CD300e, consistent with the high expression observed in

monocytes and myeloid dendritic cells. As monocytes differentiate into macrophages, CD300e expression is markedly downregulated.[8]

Emerging evidence points to dysregulated CD300e expression in various pathological conditions, where it appears to exert immune-activating and pro-inflammatory effects. [7]

Functionally, CD300e activation induces the secretion of pro-inflammatory cytokines, such as IL-1β and IL-6, in macrophages. This response, sustained for up to 24 hours post-infection, is accompanied by suppression of MHC class II–mediated antigen presentation pathways.[8]

3. Methods

3.1 Macrophage isolation and differentiation

Bone marrow-derived macrophages (BMDM) were differentiated from bone marrow (BM) cells isolated from the bone marrow of 8-week old WT mice. To induce macrophages differentiation BM cells were seeded either in 24-well plates (200 000 cells/well) or in petri dishes (500000 cells/petri) and cultured in RMPI 10% FBS + penicillin/streptomycin (P/S) supplemented with 10% L929 conditioned medium as a source of m-CSF.

The differentiation period lasted 7 days and, on the fourth, the culture medium was replaced with fresh medium. At the end of the 7-day period, non-polarized macrophages (M0) were used for the experiments.

3.2 Microbiota isolation from WT and KO feces

Microbiota was isolated from feces WT and KO from 8-weeks old mice. One fecal pellet was resuspended in 2ml RPMI 10% FBS and desegregated by vortexing and pipetting. Then, the fecal material was filtered first using a 70um cell strainer and then a 30µm filter to remove debris. Finally, bacteria were quantified reading the optical density at 600 nm (OD600) and considering OD=0,5 corresponding to $1x10^8$ CFU/mL.

3.3 Experimental condition setting or definition

The WT BMDM were stimulated separately with WT microbiota and KO microbiota. The stimulation was done with an MOI for 6 hours 24 hours.

After 7-day differentiation period, the differentiation medium was removed and two washings with PBS were performed to remove any remaining volume of antibiotic.

Then, 500µl of fresh RPMI 10% FBS additioned with bacteria from the microbiota was added to each well of the plate.
After 6 hours of infection, the medium was removed, the wells were washed with PBS and 300µl/well of Trizol were added. After 10 minutes incubation at RT, Trizol was transferred in Eppendorf tubes 1 RNAase and DNAase free, and the samples were stored at -80 C˚ for the subsequent RNA extraction and gene expression evaluation.

On the other hand, after 24 hours of infection, BMDM were detached for flow cytometry analysis. To do so NaEDTA 5mM was added. The samples were then centrifuged at 300g for 5 minutes and pelleted.
For the flow cytometry 1µl of fluorophore APC-Cy-7 conjugated live/dead was added to each tube, which binds to damaged cell membranes of dead cells.
After 15 minutes one wash was performed and they were pelleted at 300g for 5 minutes. Subsequently, they were fixed with PFA 4% to stop degradative processes and preserve the cells that will be later analyzed. After 15 minutes two more washings were performed.

3.4 Gene expression analysis

3.4.1 RNA extraction

To extract the total RNA for the PCR 100µl of chloroform were added to each tube and incubated at RT for 2-3 minutes.

After vortixing for 15 seconds, the samples were centrifuged at 12.000 at 4C° for 15 minutes, in order to obtain phase separatIon. Consequentially three phases were obtained: one at the bottom that is the phenol-chloroform, then an interphase, and above a colorless aqueous phase. The latter, which contains RNA, was collected for each sample and transferred in a new tube.

Then, 250µl of pre-cooled isopropanol were added to the tubed, in order to precipitate RNA. After having mixed, the samples were incubated at RT for 10 minutes and centrifuged at 12000g at 4C° for 10 minutes.

Subsequently, supernatant was removed and the RNA pellet was resuspended by adding 500 µl of pre-chilled 75% ethanol. Afterwards, the samples were vortexed for about 3 second and centrifuged at 7500g at 4C° for 5 minutes.

The supernatant was discarded and the pellet left to dry for 5-10 minutes. The dried pellet was then resuspended in diethylpyrocarbonate (DEPC)-treated water.

Then, the tubed were incubated in a heat block Thermomixer comfort set at 60C° for 15 minutes, in order to increase RNA solubility.

Finally, NanoDrop Lite (Thermo Scientific) was used to quantify the RNA, measuring the absorbance at 260nm and 280nm. Furthermore, the A260/A280 ratio was used to evaluate the purity, with a ratio between 1.8 and 2.0 as an indicator of purity.

Retrotranscription

Retrotranscription indicates the process by which a complementary DNA is synthesized starting from an RNA template. A Reverse Transcriptase enzyme, an RNA dependent DNA polymerase catalyzes the aforementioned process.

Using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) 1µg of RNA was retrotrotranscribed from each sample.

The retrotranscription mixture is indicated in the following table.

Reagent	Volume	Final concentration
RNA template	1 µg	1 µg/20 µL
RT Buffer 10X	2 µL	1X
dNTPs 100 mM	0,8 µL	0,4 mM
Random primers 10X	2 µL	1X
MultiScribe Reverse Transcriptase (50 U/µL)	1 µL	50 U/20 µL
DEPC H_2O	4,2 µL	-
Total volume	20 µL	

Table 1. Showing the retrotranscription mixture x1.

For the process the thermocycler GeneAmp PCR System 9700 (Applied Byosystems) was used and it include four steps as follows (Table 2).

Step	Temperature	Duration
1	25°C	10 min
2	37°C	2 hrs
3	85°C	5 min
4	4°C	Hold

Table 2. Showing reverse transcription program.

Quantitative PCR

Quantitative PCR represent a powerful molecular biology technique that allow the monitoring of the amplification of a target DNA in real time (for this reason it is also known as real time PCR).

The qPCR was carried out by using the SensiFAST SYBR Lo-ROX Kit, which includes SYBR green and all the reagents needed but the primers and the cDNA.

Reagent	Volume
SensiFAST SYBR Lo-ROX Mix 2X	4 µL
Primer forward 50 µM	0,05 µL
Primer reverse 50 µM	0,05 µL
DEPC H_2O	0,20 µL
cDNA 3.75 ng/µL	4 µL

Table 3. Showing q-PCR reaction mixture x1.

The reaction mixture was aliquoted in a 384-well plate (Sarstedt), while the PCR was performed using QuantoStudio5 Real-Time PCR System. The cycling parameters indicated in the following table were used.

Step	Temperature	Duration	Cycle
Hold stage	95°C	2 minutes	1
PCR stage	95°C	5 seconds	40
	60°C	20 seconds	
Melt Curve stage	95°C	15 seconds	Continuous
	60°C	15 seconds	
	95°C	15 seconds	

Table 4. Showing cycling parameters of q-PCR.

3.5 Cytofluorimetric analysis

Flow cytometry is a standard laser based technique that allows to analyze specific cell populations. It is used in the detection and measurement of physical and chemical characteristics of either cells or particles in a heterogeneous fluid mixture. The properties measured by this process include size, granularity or internal complexity and fluorescence intensity of a particle/cell. Flow cytometry is based on the measurement of light scattered by particles and the fluorescence observed when said particles are passed in a stream through a laser beam. The extent to which a particle deflects incidents laser light depends on the physical properties of the particle. The forward-scattered light (FSC) is proportional to the cell-surface area or size of the cell. The side-scattered light (SSC) shows the cell granularity. On the other hand, the fluorescent compound absorbs light energy over a range of wavelengths that is characteristic of that compound. Fluorescent markers are used to detect the cellular molecules expression, like proteins or nucleic acids. The fluorescence pattern of each subpopulation, and the FSC and SSC data, are used to identify the cells present in a sample. It also useful to count their relative percentages.

For the scope of this thesis project, flow cytometry was used to evaluate the percentage of living macrophages, after 24 hours of incubation with WT or KO microbiota. The flow cytometer used was the LSRFortessa X-20 Cell Analyzer (BD

Biosciences, Franklin Lakes, New Jersey, USA). The data were later analyzed using FlowJo software.

3.6 Phagocytoses assay

For the phagocytosis assay, BMDM were differentiated in petri dishes and, on the day of the experiment, they were detached with 5 mL/petri dish of NaEDTA 5mM. They were counted and 100.000 BMDM in 500 ul of RPMI 10% FBS were seeded in each well of a 24-well plate with a roundish coverslip deposited to the bottom. After seeding, plates were incubated for 2 hours at 37°C to allow macrophages adhesion to the coverslip.

The WT BMDM were stimulated with WT microbiota or KO microbiota. Before phagocytosis assay, bacteria from the microbiota were stained with a fluorescent compound. To do so, bacteria were isolated from feces as previously described and quantified. Then, $1x10^8$ bacteria were transferred inside a bacterial tube and 5 ul of DiO was added per 1 mL of bacteria resuspension. The tubes were incubated at 37°C with agitation (180 rpm) and, subsequently, the excess of dye was removed by performing two washings with PBS. Afterwards, fluorescently labelled bacteria were resuspended in 1 ml of starvation medium, RPMI 0,2% BSA.
Before phagocytosis, 500 ul/well of starvation were added and the plates were incubated on ice 4°C for 30 minutes. As a control, one well per each experimental condition was additioned with Cytocalasine D.

Completed the starvation period, the solution containing the bacteria was added, with an MOI 1:50, and the plates were incubated at 37C for 1h hour.
After incubation, two washing with PBS were performed and then 500 ul/well of Trypan 0,2% in PBS were added for 3 minutes to quench the fluorescence of extracellular bacteria. Then, three washes with PBS were performed and samples were fixed by adding 500 ul/well of para-formaldehyde 4%. Two final washes were performed in order to remove the excess of paraformaldehyde.

Immunofluorescence

Macrophages on round coverslips were permeabilized with 500 ul/well of 0.5% Triton X-100 in PBS, which acts on the plasma membrane and allows the antibody to bind to intracellular molecules, and incubated for 1 hour at RT.. Then after two washes in PBS, cells were blocked adding 500 ul/well of 3% BSA in PBS for 30 minutes at RT, to avoid unspecific antibody binding.

As a primary antibody rat-anti-mouse anti-F4/80 diluted 1:50 in 0,1% Triton X-100, 1% BSA in PBS was added to each coverslips and they were then incubated overnight in a humified chamber. The day after, two washes were performed with PBS in order to remove the possible excess of the primary antibody. Then, the secondary antibody goat anti-rat conjugated to Alexa Fluor 594 diluted 1:200 was added to the coverslip and then incubated in a humified chamber for one hour at room temperature. In addition, DAPI for the counterstaining of the nuclei of the cell, diluted 1:1000, was added in a mix with the Alexa Fluor 594.

Two more washes with PBS were performed and finally, the glass slides were mounted using ProLong DiamondTM An fade Mountant (Invitrogen). The glass were left to dry for 24 hours.
Subsequently, images were acquired using a Zeiss LSM900 confocal microscope. The images were then analyzed using ImageJ software, processed as z-stack. The bacteria inside the macrophages were counted manually.

4. Results

4.1 Evaluation of the WT macrophage's inflammatory profile in response to WT and KO microbiota

4.1.1 Gene expression evaluation

BMDM isolated from WT mice were infected with microbiota KO/WT, and after 6 hours of infection, the macrophages transcriptional response was investigated.

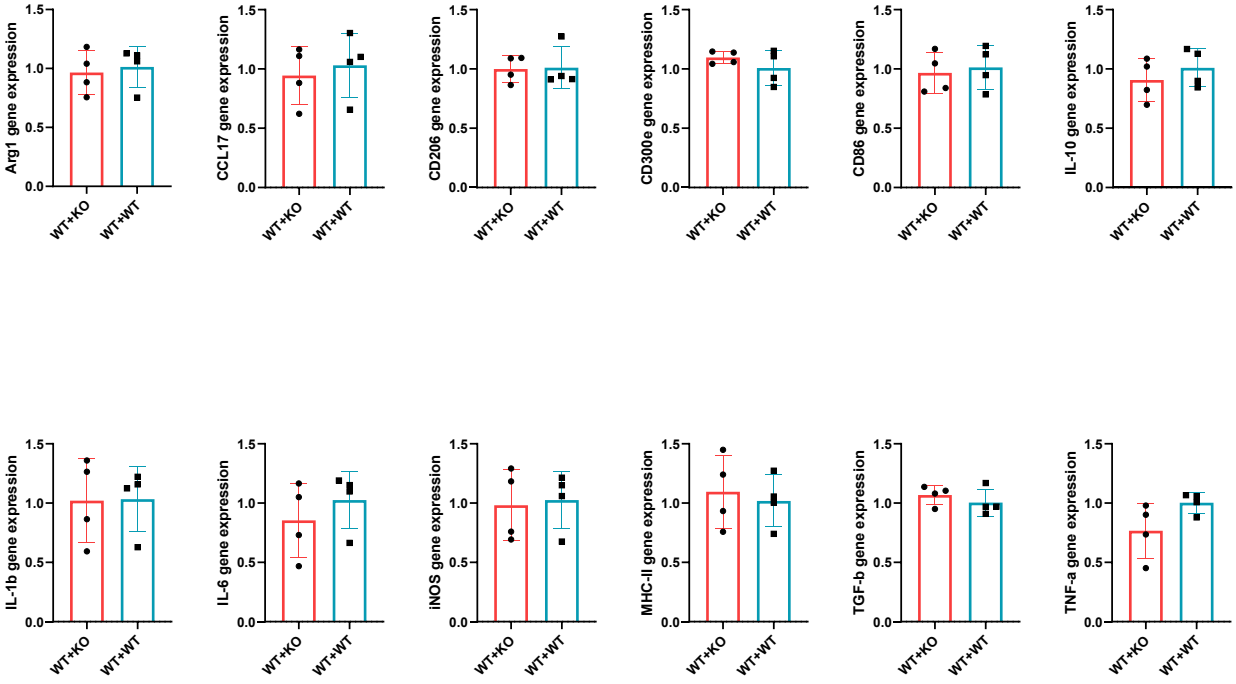

Figure 1. Graphs comparing the pro-inflammatory and anti-inflammatory genes expression. The gene expression was evaluated by performing a RT-qPCR. For the purpose of this thesis project, genes encoding for pro-inflammatory (IL-1β, IL-6, iNOS, MHC-II, and TNF-α)and anti-inflammatory (Arg1, CCL17, CD206, CD300e, CD86, IL-10, and TGF-β) markers were analyzed.

As shown in the graph (Figure 1), no significant difference was observed in the gene expression levels of the WT macrophages when infected with the WT/KO microbiota.

Genes associated with anti-inflammatory phenotypes, including Arg1, CCL17, CD206, CD300e, CD86, IL-10, and TGF-β, displayed comparable expression between the WT infected with KO microbiota and WT infected with WT microbiota. Similarly, pro-inflammatory genes such as IL-1β, IL-6, iNOS, MHC-II, and TNF-α showed no notable variation in their expression between the two experimental groups.

These results suggest that, at a 6 h time point, exposure to microbiota from CD300e-deficient mice does not induce significant changes in the transcriptional activation of inflammatory markers.

4.1.2 Flow cytometry data analysis

BMDM isolated from wild type (WT) mice were infected for 24 h with microbiota derived from either KO or WT mice, and viability was determined by APC-Cy7 live/dead staining on a flow cytometer.

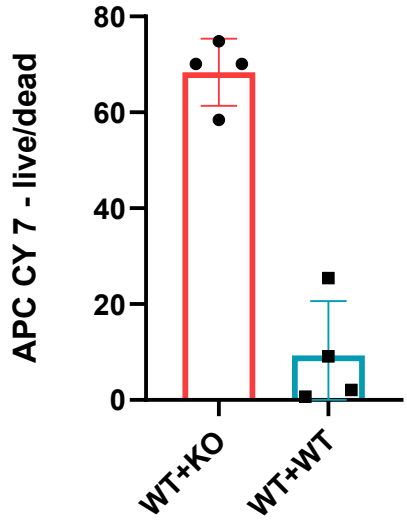

Figure 2. Graph representing the percentage of surviving WT macrophages when infected with either WT or KO microbiota. The percentage was acquired using through flow cytometry analysis. The live/dead stains dead cells, since it can enter through the damaged membranes, binding to intracellular amine.

As shown in Figure 2, infection with microbiota KO resulted in a mean macrophage survival rate of 67.3 %, whereas exposure to WT derived microbiota yielded an evident lower survival rate of 8.7%.

4.2 Evaluation of Macrophages Phagocytic Ability in Relation to the WT and KO Microbiota

The phagocytic activity of wild type (WT) macrophages was evaluated following infection with either WT or KO microbiota.

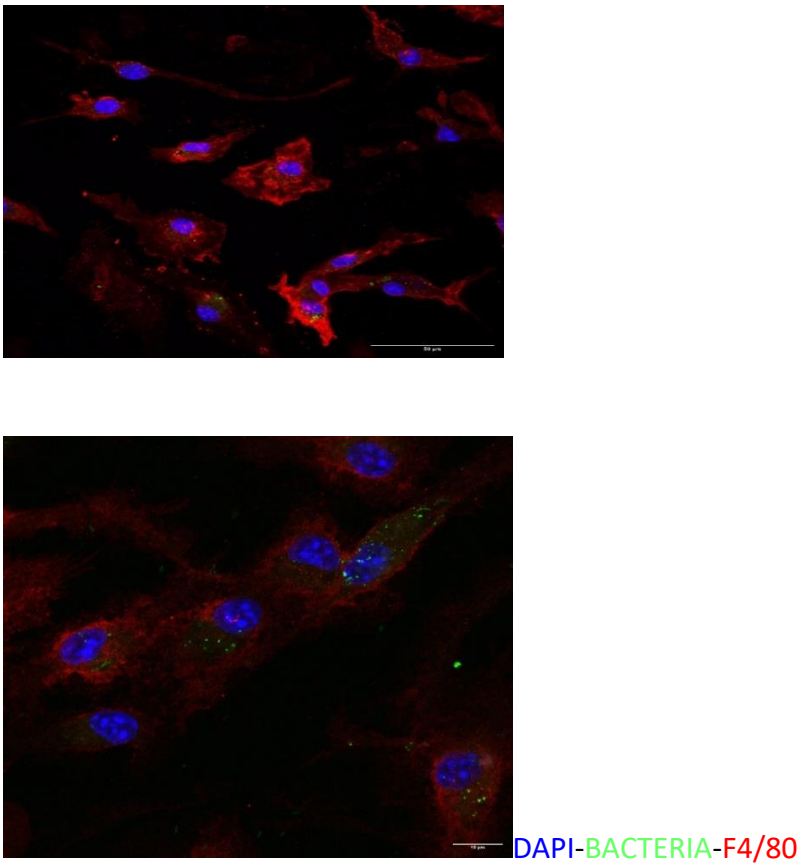

Figure 3. Images acquired with Zeiss LSM900 confocal microscope and analyzed with Imagej, showing WT macrophages after the phagocytosis assay in red, the nuclei stained with DAPI in blue, and bacteria from KO microbiota (abova) and WT microbiota (on the bottom) in green.

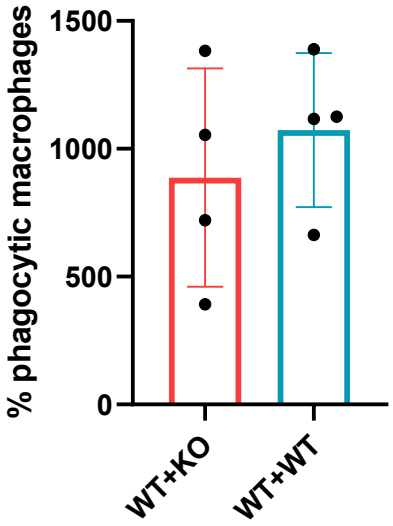

Figure 4. Graph aimed at comparing the phagocytic index of the WT macrophages when stimulated with either WT or KO microbiota. The phagocytic index was calculated by counting manually the phagocytic macrophages and the total number of bacteria.

As shown in Figure 4, WT macrophages infected with WT microbiota exhibited a higher phagocytic percentages compared to those infected with KO microbiota. This may be due to a different proliferation of the bacteria derived from WT/KO microbiota, with WT microbiota bacteria proliferating more. In turn, this might have compromised the phagocytic function of the WT macrophages.

5. Discussion and conclusion

In conclusion, these results demonstrate that microbiota derived from CD300e-deficient (KO) mice differentially modulate distinct aspects of wild type (WT) macrophage function.

The transcriptomic analysis at a 6 h time point revealed no significant differences in the expression of classical anti-inflammatory (*Arg1, IL-10, TGF-β*) or pro-inflammatory (*IL-1β, IL-6, TNF-α*) markers between macrophages infected with KO versus WT microbiota. After 24 h infection with microbiota, the results suggested a contrasting effect on cell viability: macrophages infected with KO microbiota displayed significantly higher survival rate (67.3%) compared to those infected with WT microbiota (8.7%). This may imply that the absence of CD300e in the KO microbiota might have affected the bacteria proliferation, allowing the survival of a higher percentage of macrophages. Nonetheless, it might have also influenced the macrophages response, which was not possible to evaluate in this thesis project (due to the fact that the WT macrophages stimulated with WT microbiota did not survive, rendering not possible to make a marker evaluation and comparison), but will further be evaluated in future experiments.

For what concerns the phagocytic activity of the macrophages, the results showed a slight difference in the phagocytic index, with WT macrophages stimulated with WT microbiota having a higher percentage. These results could indicate that KO microbiota affects the phagocytic function of WT macrophages or that there might have been a different proliferation of the different microbiota derived bacteria.

These findings could suggest that CD300e-associated microbial components may simultaneously affect macrophage phagocytic activity while increasing cytotoxicity, pointing to a complex influence of microbiota on innate immune function. On the other hand, it should not be ruled out that the CD300e did not affect the phagocytic ability of the macrophages, but rather the proliferation of the bacteria derived from the microbiota (and as such, influencing the phagocytic index of the macrophages), Further research will be needed to attest weather the CD300e deletion influences the phagocytic activity of the macrophages and the inflammatory profile. For this reason, future experiments will focus on the evaluation of the phagocytic ability and

inflammatory profile (through gene expression analysis and flow cytometry) of KO macrophages, using the same experimental setting described in this thesis project. In addition, the experiment will be repeated by changing the setting condition, possibly by reducing the time point for the flow cytometry analysis, in order to evaluate the inflammatory markers.

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