SAPIENZA
UNIVERSITÀ DI ROMA

Ph.D. COURSE IN CELL AND DEVELOPMENTAL BIOLOGY
XXXV Cycle (A.Y. 2022/2023)

Symbiotic, opportunistic, and probiotic microbes: new advances in understanding their interaction with immune system

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Allegro Viandante

Ho messo nella mia bisaccia
un fascio di buona volontà
e due di ottimismo,
una manciata di disciplina
e quattro volte tanto
di pazienza,
due grani di buonumore,
un pizzico di amor proprio,
una punta d'ironia.

E continuo la mia strada.

Ho messo
nella mia bisaccia
tutto questo
e cammino cantando

Marcello

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1. GENERAL INTRODUCTION AND AIMS OF THE THESIS

1.1 Summary

In this thesis we analysed the impact of *Saccharomyces cerevisiae*, *Acinetobacter baumannii*, and *Lactobacillus reuteri* on human immune system.

S. cerevisiae is a yeast harboring the human gastrointestinal tract, and the interaction with human blood dendritic cells (DCs) was never been investigated. We found that conventional DC (cDCs) and plasmacytoid DCs (pDCs) sense *S. cerevisiae* and that cDCs induce IL-6 and IL-17 production, while pDCs IFN-α and IL-10 with pro- and anti-inflammatory properties, respectively. These results could have relevant implication in health and diseases associated to microbiota dysbiosis.

A. baumannii is a nosocomial bacteria promoting pathology in immunocompromised hosts. We analysed for the first time the response of human innate immune cells to *A. baumannii* ATCC19606T (low virulence) and ACICU (high virulence). We found that macrophages promote killing of both bacterial strains, monocytes kill preferentially ATCC19606T, DCs and monocytes produce cytokines. Importantly, ACICU induces lower expression of two cytokines that have been associated to protection against *A. baumannii*: IL-10, IFN-α. Thus, we identified mechanisms potentially related to the high virulence of the multidrug-resistant strain.

L. reuteri is a probiotic with beneficial properties. We studied the effect of *L. reuteri* in children with autism spectrum disorder (ASD), characterized by social problems and gastrointestinal dysbiosis. Since we previously demonstrated a pro-social effect of *L. reuteri* supplementation, we analysed the effects of probiotic on immune system and microbiota. We observed an increase of fecal *F.prausnitzii* and *E. rectale*, with anti-inflammatory properties, and a decrease of plasmatic soluble CD40L, with pro-inflammatory properties, in probiotic-treated ASD children. These results indicate an anti-inflammatory role of probiotic *L. reuteri* in ASD children.

Overall, we uncovered new mechanisms involved in the regulation of host/microbe interaction, with potential implication in the response to pathogenic infections, prevention of excessive inflammation, and exploitation of beneficial effect of microbiota and probiotics.

1.2 Synopsis

Host-microbes interactions contribute to shaping the evolutionary process, including diversity of both organisms. In humans, the immune system represents the host component that directly interacts with microorganisms. Thus, the relationship between immune cells and microrganisms is crucial for the human evolutionary process and the human health.

The main objective of this thesis is to better characterize three specific interactions between human primary immune cells and microorganisms. In particular, we analyzed the interaction between immune cells and 1) *Saccharomyces cerevisiae*, a commensal yeast component of the human microbiota; 2) *Acinetobacter baumannii*, an opportunistic multidrug-resistant bacteria responsible of most part of nosocomial human infections; 3) *Lactobacillus reuteri*, a probiotic known to induce health benefits.

The first aim of this thesis arises from the importance of *S. cerevisiae* as one of the most common fungi harbouring the human gastrointestinal tract, and the lack of adequate information related to the human response to *S. cerevisiae*. In fact, previous studies reported that wall components of *S. cerevisiae* activate human monocyte-derived DCs through mannose receptor, DC SIGN, dectin-1, and chitin receptor, thus leading to the production of IL-6 and other inflammatory cytokines. However, the ability of blood circulating human DCs to respond to *S. cerevisiae* has never been investigated. Here, we challenged human blood DC subtypes with *S. cerevisiae.* First, we found a differential microbial recognition by cDCs and pDCs that occur through microbial cell wall and microbial nucleic acid, respectively. Moreover, we found a differential response of cDCs and pDCs, characterized by IL-6 and IFN-α production, with a consequent IL-17 and IL-10 induction in *in vitro* differentiated Th cells, respectively. Thus, we concluded that the exposition of *S. cerevisiae* as well as the prevalence of specific DC targets *in situ* may influence the development and the progression of diseases associated to microbiota-dependent immune dysregulation or fungal opportunistic infection.

The second aim was focused on the study of innate immune response to the opportunistic pathogen *Acinetobacter baumannii*. Although this microbe is a notorious pathogen causing serious infections that are associated with high morbidity and mortality rates, little is known about the human immune response. Previous studies mainly analysed the response of macrophages using cell lines or murine cells. Here, we analysed systematically the response of human monocytes, macrophages, conventional and plasmacytoid dendritic cells to *A. baumannii*.

We used two different *A. baumannii* strains: 1) ATCC19606^{T}, which is the species type strain, reference organism in many laboratories due to its low virulence, amenability to genetic manipulation and extensive antibiotic susceptibility; 2) ACICU, a multidrug-

resistant bacteria, which was isolated from the cerebrospinal fluid of a patient with meningitis at the Hospital S. Giovanni-Addolorata in Rome, Italy.

We found that all innate immune cells sense both strains of *A. baumannii*. However, macrophage exert a strong killing of bacteria at the early phase of infection and do not produce cytokines at late time points of infection; monocytes are able to kill *A. baumannii*, especially ATCC19606^T strain, produce the pro-inflammatory cytokine IL-6 and express co-stimulatory molecules in response to infection; cDC and pDC do not kill bacteria, however they are activated and produce cytokines upon infection. Importantly, the multi-drug resistant *A. baumannii* induces lower expression of regulatory cytokines, such as IL-10 by monocytes and cDC, and IFN-α by pDC. Therefore, higher virulence of ACICU than ATCC19606^T, could be related to a stronger inflammation generated by innate immune cells interacting with the multi-drug resistant strain.

The third aim was focused on the study of the oral administration of probiotic *L. reuteri* in children with autism spectrum disorder (ASD). Autism is a developmental disease characterized by impaired communication and social interaction skills. In addition to the core symptoms, children with ASD suffer from gastrointestinal (GI) problems. In the last years it is emerging that GI problems and behavioural symptoms in ASD are associated to microbiota dysbiosis of the GI tract. Preclinical studies have demonstrated the benefits of probiotic supplementation in correcting microbiota dysbiosis and in reducing behavioural symptoms of autism. In a recent double-blind placebo clinical trial, our collaborators demonstrated a prosocial effect of *L. reuteri* in autistic children. In this thesis we analysed the fecal microbiota and peripheral immune response in ASD children, randomized to receive either *L. reuteri* or placebo, as potential underlying mechanisms of the beneficial effect of *L. reuteri*. Indeed, we analysed immune system from blood, and microbiota from stool of 43 ASD children at baseline, and after 3-6 months of treatment. We observed a significant increase of *L. reuteri* in stool of children treated with probiotic compared to placebo. Although we did not observe a modulation of the global microbiota composition, we found that consumption of probiotic significantly increases the relative abundance of *Faecalibacterium prausnitzii and Eubacterium rectale*, producers of short-chain fatty acids (SCFAs), which have anti-inflammatory properties. Moreover, we found that probiotic induces a significant reduction of soluble CD40L, which may reflect a reduced platelet and lymphocyte activation. However, global peripheral immune response is not modulated by *L. reuteri* supplementation in ASD children and all biological variables analysis does not allow to discriminate placebo- and probiotic-treated ASD children.

In conclusion, the main results of this thesis are the identification of: 1) a differential role of human blood DC subsets in recognition and response to *S. cerevisiae* yeast: pro-

and anti-inflammatory response promoted by cDCs and pDCs, respectively; 2) a differential response of human innate immune cells in response to bacteria *A. baumannii*: killing mainly mediated by macrophages, and late response characterized by production of cytokines and expression of co-stimulatory molecules by monocytes and dendritic cells ; 3) a potential anti-inflammatory role of *L. reuteri* in autistic children characterized by increase of bacteria producing butyrate in the fecal microbiota and reduction of soluble CD40 ligand in the plasma.

1.3 Host-microorganism interaction

The world of microbes includes 10^{11}-10^{12} estimated species (Locey, and Lennon, 2016) of microscopic organisms that require an artificial magnification to be seen. Most of them are unicellular and differ in some characteristics such as structure, organization, metabolism, physiology, reproduction and growth. The main distinction of microorganisms is based on the cellular type: prokaryotic (bacteria, archaea), eukaryotic (fungi, algae, protozoa) and viruses (Pelczar, and Pelczar, 2022).

About 3 billion years ago, the biosphere was dominated by bacteria, archaea, and eukaryotic microbes (Pace, Sapp, Goldenfeld, 2012). Relationship between microbes and other organisms, including human beings, is important for the process of evolution, generation and maintenance of biodiversity.

One of the relationships between host and microorganisms is the host-pathogen interaction (Figure 1), which may lead to different outcomes: clearance mediated by the host immune system; tolerance to the microbes, that leads to a latency of microbe in the host; disease characterized by host damage and rapid replication of the microbe. However, among host-microbes interactions we also include: a) symbiosis; b) opportunistic infection; c) probiotic-host communication (Figure 1).

Symbiosis between host and microbes is defined as a condition where two dissimilar organisms can stably coexist in strong association (De Bary, 1878). This relationship could be beneficial for both the species (mutualism), beneficial for one species while the other is not affected (commensalism), or beneficial for one species and detrimental for the other one (parasitism) (Figure 1) (Olano, et al., 2011).

Opportunistic infections are induced by some microbes, either commensal or not, which promote pathology in immunocompromised hosts (Figure 1) (Bryant, and Baddley, 2017).

Probiotic-host communication is induced by ingestion of microbes that alter the gastrointestinal flora and lead to health benefits (Figure 1) (Williams, 2010).

In this study we will examine these last three host-microbes interactions in human beings.

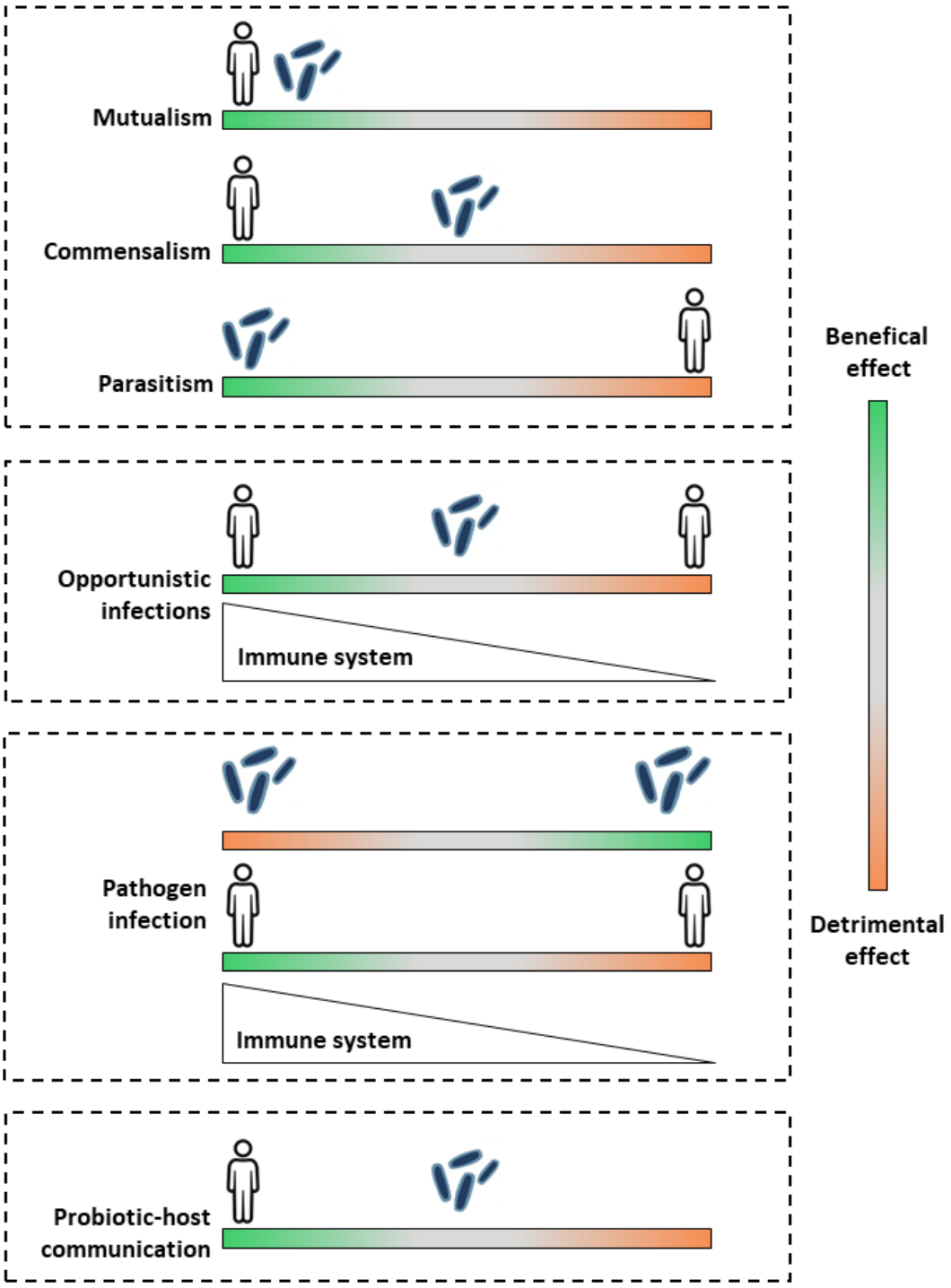

Figure 1. Host-microbe interaction.
Schematic representation of the interactions between human host and different class of microorganisms. Beneficial (green) and detrimental (orange) effect of each interaction is shown for human and microbe.

1.3.1 Symbiosis host-microbes

Microbes co-evolved with human host establishing mutual and/or commensal relations and are considered crucial components of the physiology and metabolism of human beings (Rook, et al., 2017).

The whole complex of symbiotic microbes living in the host is defined microbiota, and includes mainly bacteria, but also fungi, protozoa and viruses. Human body represents an important organism for microbial colonization, able to host a large number of microorganisms in different niches (e.g. skin, oral cavity, nostrils, eyes, urogenital tract). Each tissue in contact with the environment has a protective barrier, skin or mucosa, through which host-microbes communication occurs.

Microbes largely colonized the gastrointestinal tract (gut), where they form the "gut microbiota", a complex of microbial species with the highest richness and diversity of the human microbiota. Most microbes are limited to the surface of the mucous layer in the intestinal tract due to the high viscosity and to the abundance of antimicrobial compounds contained in the mucus. Gut microbiota is crucial for several host functions, such as metabolic activity, development of and communication with the nervous system, and protective role against pathogen microorganisms.

In particular, gut microbes have enzymes required for the utilization of carbohydrates, dietary fibers and undigested proteins, crucial for the conversion of dietary compounds into metabolically bioactive food components. For instance, species such as *Roseburia spp*., *Eubacterium rectale*, and *Faecalibacterium prausnitzii* are the main producers of short-chain fatty acids (SCFAs), derived from the fermentation of dietary fibers in the large intestine (Adak, and Khan, 2019). Microbiota is also important for the vitamin synthesis, such as biotin, thiamine, cobalamin, riboflavin, nicotine, pantothenic acids, vitamin B and K (LeBlanc, Milani, de Giori, Sesma, van Sinderen, and Ventura, 2013). Importantly, microbiota produce neurotransmitters and neurotrophic factors, such as the gamma amino butyric acid (GABA). Moreover, vitamins and SCFAs may affect the central nervous and the enteric nervous systems (Forsythe, Sudo, Dinan, Taylor, and Bienenstock, 2010), and are considered mediators of the gut-brain axis. In particular, SCFAs induce release of serotonin in the gut, able to stimulate the sympathetic nervous system and to influence neurologic processes, such as memory and learning (Silva, Bernardi, and Frozza, 2020; Sarkar, Lehto, Harty, Dinan, Cryan, and Burnet, 2016). In addition, SCFAs protect the integrity of the blood brain barrier (BBB) through increasing production of the tight junction proteins, thus limiting the entry of foreign substances (Mohajeri, La Fata, Steinert, and Weber, 2018).

Finally, microbiota exerts a protective role against microbial pathogens using different mechanisms. The microbial competition for nutrients in the gut is observed in mice, where commensal *Escherichia coli* strains reduce cecal colonization of the enteric

pathogen enterohemorrhagic *E. coli* (EHEC) (Momose, Hirayama, and Itoh, 2008). Moreover, the secretion of toxins and antimicrobial peptides by commensal microbes is another mechanism of defense. For instance, some commensal *Enterobacteriaceae* secrete small antimicrobial peptides called bacteriocins or microcins, which specifically target and kill related competitors, including pathogenic organisms (Rebuffat, 2012).

The composition of human gut microbiota is influenced by different factors, such as age, diet, smoke, exercise, host genetic factors, presence of disease, pharmacologic therapies and use of antibiotics (Gomaa, 2020).

The role of the diet in the modulation of microbial community in the host is particularly important. Specifically, type of macronutrients (proteins, carbohydrates, vitamins), source (animal, vegetable), quantity, and quality (raw, refined) of the food, have a central role in the adjustment of number and/or species of different microbes in the human gut (Zmora, Suez, and Elinav, 2019). Moreover, some microbes are component of the food and their introduction by diet may influence the global microbiota composition.

1.3.2 *Saccharomyces cerevisiae*

Saccharomyces, yeast belonging to fungi kingdom, is one of the most common commensal microbes contained in the food and harboring the gastrointestinal tract. Many members of this genus are considered very important in food production, such as *S. cerevisiae* the bakers' and brewers' yeast (Hallen-Adams, and Suhr, 2017). *S. cerevisiae* is present as harmless and transient digestive commensal microbe in the mucosal surfaces of normal individuals.

Although *S. cerevisiae* is one of the most common dietary fungi, and it is abundant in the human gut microbiota, very little is known about the impact of *S. cerevisiae* on human health. It has been observed that this yeast is able to reduce symptoms of colitis in human (Sokol, Leducq, Aschard, Pham, Jegou, et al., 2017), and mouse (Sivignon, De Valle´e, Barnich, Denizot, Darcha, et al. 2015), and also that has the potential to colonize the mucin-enriched gut environment (Mercurio, Singh, Walden, and Baetz, 2021), representing an important player in the inter-kingdom dynamics of the gut microbes.

Moreover, *S. cerevisiae* is emerging as etiologic agent of opportunistic fungal infection. In fact, *S. cerevisiae* has been related to a wide variety of infections, which include vaginitis, cutaneous, systemic bloodstream and infections of essential organs in immunocompromised and critically ill patients (Enache-Angoulvant, and Hennequin, 2005; de Llanos, Llopis, Molero, Querol, Gi, and Fernandez-Espinar, 2011).

1.3.3 Opportunistic infections

Opportunistic pathogens are microbes, commensal or environmental, that do not infect healthy hosts but produce infections in hospitals, to immunodepressed individuals or to those affected by other diseases.
Opportunistic pathogens are a large class of pathogens that have the ability to persist and grow in the outside host environment and invade the host under favorable conditions. In the last decades, an increased prevalence of clinical opportunistic pathogens has been reported (Brown, Cornforth, and Mideo, 2012).
Moreover, in the last years the large use of antibiotics, which kill commensals besides pathogens, increased the incidence of opportunistic microorganisms resistant to antibiotics (Bergogne-Bérézin, Decreé, and Joly-Guillou, 1993; D.C. Shanson, 1989).
For instance, opportunistic pathogens, such as *Pseudomonas aeruginosa* or *Burkholderia cepacian* that live in the water or soil, where usually they interact with other organisms than human, develop a virulence activity against humans based on the antibiotic resistance. In fact, the most frequent microbial clones involved in hospital outbreaks are also present in natural ecosystem, indicating that the use of antibiotics for the treatment of infectious diseases selects the environmental microbes with a natural drug resistance (Sanz-García, Gil-Gil, Laborda, Ochoa-Sánchez, Martínez, and Hernando-Amado, 2021).
The opportunistic infections mainly occur in immunodeficient individuals and patients with other diseases, and they represent a critical issue for the health-care system around the world

1.3.4 *Acinetobacter baumannii*

Acinetobacter is a non-fermentative Gram-negative bacterial genus, firstly reported as a nosocomial pathogen in the late 1970s. Organisms belonging to the genus *Acinetobacter* are ubiquitous in nature, recovered from almost all soil and surface water. The more frequent clinical species of *Acinetobacter* is *A. baumannii*, one of the most prevalent antibiotic-resistant microbes in hospitals, associated with infections of the respiratory tract, bloodstream, wound, skin, soft tissues, urinary tract and central nervous system (Kanafani, Zahreddine, Tayyar, Sfeir, Araj, Matar, and Kanj, 2018). It has been observed that genome of *A. baumannii* contains a variety of mobile genetic elements, most notably integrons, transposons and plasmids (Figueiredo, Bonnin, Poirel, Duranteau, and Nordmann, 2012), which may support the antibiotic resistance.
However, *A. baumannii* is rarely part of the normal microflora (Seifert, Dijkshoorn, Gerner-Smidt, Pelzer, Tjernberg, and Vaneechoutte, 1997), and its primary

environmental niche is still not well established (Howard, O'Donoghue, Feeney, Sleator, 2012).

1.3.5 Probiotic-host communication

Since the past century, the beneficial effects of food containing living bacteria have been observed. Probiotics are defined live microorganisms conferring health benefit to the host when administered in adequate amounts (Food and Agriculture Organization of the United Nations and World Health Organization, 2002). In order to be effective, probiotics must: survive in the acidic gastric environment; colonize and reproduce in the gut; adhere to the intestinal epithelium and stabilize the balance of the gut microbiota; not exert pathogenic functions (Food and Agriculture Organization of the United Nations and World Health Organization, 2001; Goldin, 1998). Probiotics are currently used to improve the homeostasis of internal microbiota, to maintain the human intestinal health, and to support the general wellness (Sanders, Heimbach, Pot, Tancredi, Lenoir-Wijnkoop, Lähteenmäki-Uutela, et al., 2011). Although the exact mechanisms associated to the beneficial role of probiotics are still under investigation, it is known that probiotics produce lactic acids, acetic acid, and propionic acid, that are able to lower the intestinal pH and suppress the growth of several pathogenic bacteria, and produce substances, such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants, that are toxic for pathogenic microorganisms (Doron, and Gorbach, 2006).
In the last decades several probiotics have been commercialized: *Lactobacillus spp*., *Bifidobacterium spp*., *Saccharomyces boulardii*, *Propionibacterium spp*., *Streptococcus spp*., *Bacillus spp*., *Enterococcus spp*., and some specific strains of *Escherichia coli* (Kechagia, Basoulis, Konstantopoulou, Dimitriadi, Gyftopoulou, Skarmoutsou, et al., 2013).

1.3.6 Use of probiotic in health and diseases

Although the human response to probiotics is heterogenous because it is influenced by diet, age, genetic background, and gut microbiota composition, common benefits on restoring gut microbiota balance and immune homeostasis have been reported (Reid, Younes, Van der Mei, Gloor, Knight, and Busscher, 2011).
Probiotic supplementation in healthy adults can improve immune functions during infections like rhinoviruses, (Berggren, Lazou Ahrén, Larsson, and Önning. 2011) and bacterial vaginosis (Recine, Palma, Domenici, Giorgini, Imperiale, Sassu, et al., 2016).
The probiotics showed beneficial effects in pathological conditions, such as diarrhea, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), *Helicobacter*

pylori infection, respiratory infections, atopic dermatitis and cardiovascular diseases (Sniffen, McFarland, Evans, and Goldstein, 2018; Huang, Wang, and Hu, 2016). Recently, the use of probiotics resulted beneficial in metabolic disorders and obesity (Green, Arora, and Prakash, 2020).

Moreover, probiotics, mainly *Lactobacillus* and *Bifidobacterium* species, have been observed in modulation of mood, anxiety and cognitive function (Dinan, Stanton, and Cryan, 2013). In this case probiotics are named "psychobiotics", and are considered potential treatment of psychiatric disorders, such as depression, schizophrenia, and anxiety (Mörkl, Butler, Holl, Cryan, and Dinan, 2020). Psychobiotics could be important also in the prevention and/or treatment of neurodegenerative disease (Alzheimer's disease and Parkinson's disease) and neurodevelopmental disorders (Autism spectrum disorder, Attention deficit hyperactivity disorder and Tourette syndrome) (Cheng, Liu, Wu, Wang, and Tsai, 2019).

1.3.7 *Lactobacillus reuteri*

The genus *Lactobacillus*, a heterogeneous group of Gram-positive, nonsporulating, facultative anaerobic bacteria, belongs to the large group of lactic acid bacteria (LAB). *Lactobacillus* genus, largely used as probiotic, includes several species of bacteria living in food products, playing an important role in food fermentation, and present in the gut of humans and animals (Giraffa, et al., 2010). *L. reuteri* is one of the species with multiple beneficial effects for the host health. This microbe grows in oxygen-limited atmospheres, in different pH environments, and it colonizes the gastro-intestinal tract of humans and animals. *L. reuteri* has several beneficial properties, including the reduction of infection by pathogenic microorganisms, the improvement of feed tolerance, the enhancement of nutrients, minerals, and vitamins absorption. The reduction of infections occurs through the secretion of antimicrobial intermediaries and the promotion of gut mucosal integrity, thus reducing bacterial translocation (Sinkiewicz, 2010).

Moreover *L. reuteri* is also associated to a neuromodulatory activity due to the production of gamma-aminobutyric acid (GABA), one of the most important neurotransmitters in the central nervous system (Su, Schlicht, and Ganzle, 2011). Also, *L. reuteri* has an important role in modulating host immune response (Valeur, Engel, Carbajal, Connolly, and Ladefoged, 2004).

1.4 Immune response

The immune system is crucial in the host-microrganism interaction. In fact, the immune system, composed by different cell-types and soluble molecules, interacts with microrganisms and activates a coordinated response to defend the host against microbial infection.

We can distinguish two types of immune response: the innate and the adaptive, which are distinct for timing of activation, specificity against the infectious microorganism, and long-lasting memory.

The innate immunity provides an initial defense, within hours from the antigen recognition, while the adaptive immunity exerts its function after approximately one week from the infection. However, the adaptive immunity is specific for each pathogen and ensures protection against subsequent reinfection with the same pathogen (Abbas, 2012d, 2012e), while innate immunity is mainly not specific and short-lived phenomenon. Moreover, in the last years, it is emerging the concept that also innate immunity shows a long-term adaptation, known as trained immunity, due to a previous stimulation (priming), which results in an enhanced reaction to subsequent challenges (Netea et al., 2020).

Innate immune response consists in physical, chemical and biological barriers, such as epithelium, antimicrobial peptides, and microbiota, which prevent potential infections (Beatriz Aristizábal and Ángel González, 2013). Moreover, in case of infection, other components of innate immunity, including blood factors and innate immune cells, play a crucial role (Figure 2).

Blood factors are cytokines, such as interferon (IFN)-γ, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, produced by innate immune cells and alerting other cells to mount a proper inflammatory response in infected anatomical sites. Blood factors also include proteins of the complement system, which contribute to fight the infection by recruiting immune cells, opsonizing and neutralizing infectious agents (Beatriz Aristizábal and Ángel González, 2013).

Adaptive immune response consists in the activation of defensive mechanisms against microbes localized in several anatomical district in the body (e.g. gastrointestinal tract, bloodstream, and the host cells). The adaptive response is promoted by a large number of lymphocytes specifically generated after the antigen recognition and having the appropriate characteristics to induce the pathogen clearance (Figure 2) (Abbas, 2012d).

Innate immune cells are mast cells, eosinophils, basophils, and phagocytic cells (macrophages, neutrophils, and dendritic cells) (Beatriz Aristizábal and Ángel González, 2013). Other cells, such as natural killer (NK) cells, NK T cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and innate lymphoid cells (ILCs) belong

to innate immunity (Figure 2). However, they also display typical features of the adaptive immune system, such as the specificity against a pathogen and the long-lasting memory against the same pathogen (Vivier et al., 2011; Lanier, 2013; Trottein and Paget, 2018).

Adaptive immune cells are B lymphocytes, which activate the humoral immune response, and T lymphocytes, which activate the cell-mediated immune response (Figure 2).

Humoral immune response is mediated by antibodies that neutralize microbes and toxins, induce opsonisation that favours phagocytosis by activated phagocytes, and activate the complement system (Abbas, 2012b). This response is very important for extracellular microbes, such as parasitic worms.

Cell-mediated immune response is mediated by cytotoxic T lymphocytes (CTL), represented by CD8 T lymphocytes and a small part of CD4 T lymphocytes, called CD4 CTL, which release cytotoxic granules able to damage infected cells and eliminate the pathogens (Abbas, 2012a; Takeuchi and Saito, 2017). This response is critical for the clearance of intracellular viruses and bacteria.

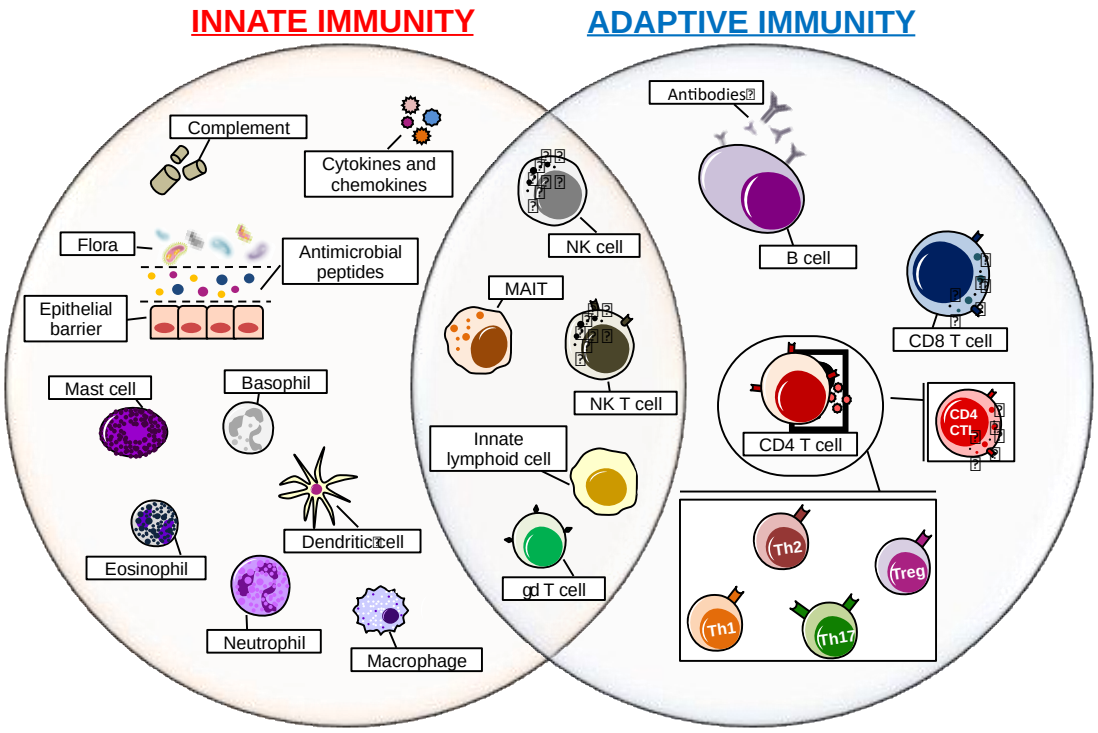

Figure 2. Components of the innate and adaptive immunity.
The innate immunity includes epithelial barrier, antimicrobial peptides, microbial flora, cytokines, chemokines, proteins of complement system and several innate immune cells. The adaptive immunity includes B lymphocytes producing antibodies, CD8 T lymphocytes and CD4 T lymphocytes (T helper subsets and cytotoxic CD4). NK cells, NK T cells, γδ cells, MAIT cells, and innate lymphoid cells share functions with both innate and adaptive immunity.

1.4.1 Interaction between innate immune cells and microrganisms

Macrophages, neutrophils, and dendritic cells are the main immune cells interacting with microorganisms. The recognition of microorganisms occurs through a set of pathogen pattern recognition receptor (PRR), including TLRs, which bind microbial pathogen-associated molecular patterns (PAMPs), such as component of the microbial cell wall (e.g. lipopolysaccharide, peptidoglycan), flagellin found in bacterial flagella, and nucleic acids (Figure 3). Phagocytic cells have cell-surface receptors for the Fc portion of antibodies produced by the adaptive immune system, as well as for the C3b component of complement, whose binding by specific ligands induces actin polymerization at the site of pathogen attachment, which cause the engulfment of pathogen in a large membrane-enclosed phagosome. Once the pathogen has been phagocytosed, the phagosome is acidified by fusion with lysosomes, which contain lysozyme and acid hydrolases that can degrade bacterial cell walls and proteins. The lysosomes also contain defensins, with antimicrobial activity. In addition, the phagocytes assemble an NADPH oxidase complex on the phagosomal membrane that catalyzes the production of a series of highly toxic oxygen-derived compounds,

including superoxide (O2 -), hypochlorite, hydrogen peroxide, hydroxyl radicals, and nitric oxide (NO) (Alberts, Johnson, Lewis, et al., 2003).

Macrophages reside in tissues throughout the body and are especially abundant in areas where infections are likely to arise, including the lungs and the gut. They are also present in large numbers in connective tissues, the liver, and the spleen. These long-lived cells patrol the tissues of the body where encounter invading microbes. Monocytes are the precursor of macrophages; they circulate in the blood and infiltrate tissues in response to microbial stimuli. The main function of monocytes and macrophages is the ingestion and removal of microorganisms, foreign material, and dead or damaged cells.

Similar function is exerted by neutrophils, which are abundant in blood but not present in healthy tissues, and they are rapidly recruited to sites of infection both by activated macrophages and by molecules released by the microbes themselves. However, monocytes and macrophages may also orchestrate the adaptive immune response by releasing cytokines and expressing co-stimulatory molecules.

The main function of dendritic cells (DCs) is the initiation of the adaptive immune responses by activating naïve T lymphocytes, and not only memory T lymphocytes, through the release of cytokines and the expression of co-stimulatory molecules (Banchereau J, Steinman RM., 1998). DCs can be divided into two broad types: those that reside in the peripheral tissues (resident DCs) and those that reside in the blood and then migrate into different sites (blood DCs). Blood DCs can be divided into conventional DCs (cDCs) also called myeloid (mDCs), and plasmacytoid DCs (pDCs) (Shortman K, Liu YJ., 2002). PDCs predominantly recognize viruses and are IFN-α producers (Colonna M., Trinchieri G., and Liu YJ., 2004; Gilliet M., Cao W., and Liu, YJ., 2008; Swiecki M. and Colonna M., 2015), while cDCs are mainly involved in recognition of bacteria and produce inflammatory cytokines, such as IL-6. Notably, blood cDCs can be divided in two cell subsets called cDC1 ($BDCA3^+$ or $CD141^+$) and cDC2 ($BDCA1^+$ or $CD1c^+$). However, cDCs mostly include cDC2 population (Steinman RM. and Inaba K., 1999; MacDonald KPA., Munster DJ., Clark GJ., Dzionek A., Schmitz J. and Hart DNJ., 2002; Collin M., Bigley V., 2017; Balan S., Saxena M., Bhardwaj N., 2019).

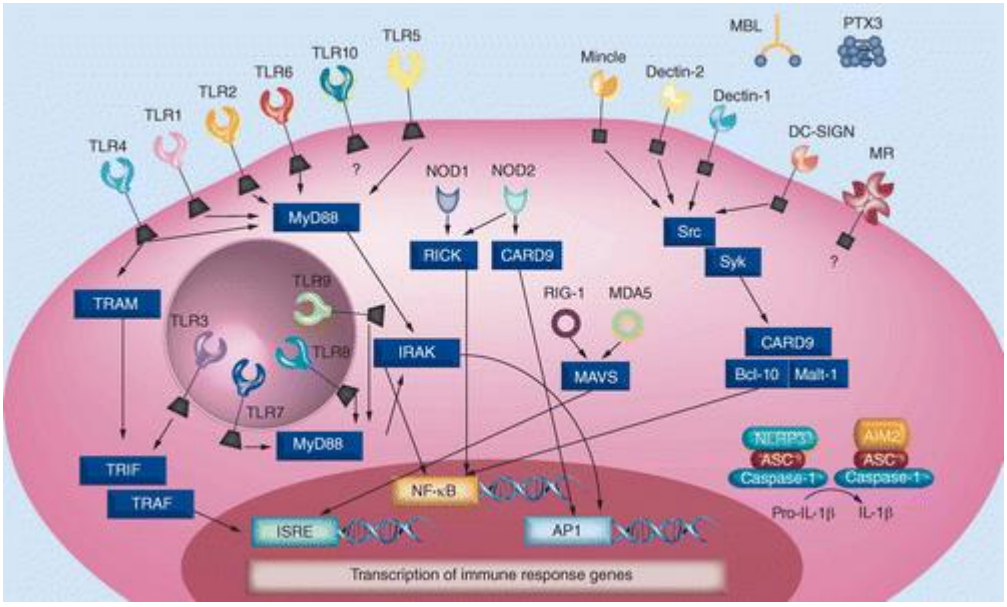

Figure 3. Schematic representation of the main pattern recognition receptor for the recognition of microorganisms by human cells

The PRR families include membrane and endosomal TLRs, C-Type Lectin Receptors, and NOD-Like Receptors. (Adapted from Jaeger, Stappers, Joosten, Gyssens, Netea, 2015).

1.4.2 Activation of adaptive immune cells

Antigen presentation is the crucial process that induces activation of adaptive immune response.

It consists in the interaction between the major histocompatibility complex (MHC), which is present on the surface of innate immune cells and the receptors present on the surface of adaptive immune cells. Importantly, during this process MHC molecules contain the components of the microbial pathogens, which are digested by antigen presenting cells and are called antigens. In fact, the cells involved in this process are defined antigen-presenting cells (APC), and include monocytes, macrophages, dendritic cells and B lymphocytes (Abbas, 2012c, 2012d). Moreover, cytokines produced by APC during the antigen presentation process, are critical for the establishment and the amplification of the proper adaptive immune response.

CD4 T helper lymphocytes are the first cells activated by the antigen presentation process, then they activate either the humoral than the cell-mediated adaptive immune response, by releasing soluble factors, known as cytokines and chemokines, directed towards other immune cells (Abbas, 2012a). In particular, cytokines activate or inhibit immune cells, while chemokines recruit immune cells.

Importantly, we can distinguish CD4 T lymphocytes with inhibitory functions, called T regulatory (Treg) cells, and those with activating functions, called T helper (Th) cells. Treg are crucial for the suppression of the adaptive immune response after the clearance of viral, bacterial and parasitic infections. They produce IL-10 and TGF-β, and are

involved in suppressing humoral and cell-mediated immune responses once the pathogen has been cleared. On the other side, Th cells are critical for the establishment of the proper adaptive immune response (humoral or cell-mediated) against viruses, bacteria, and parasites.

The initial step is the activation of naïve CD4 T cells by dendritic cells. In fact DCs encounter the infectious agent in periphery, migrate into secondary lymphoid tissues, and present antigen to naïve CD4 T cells, which express membrane receptors, such as CD45RA, CD27, and C-C chemokine receptor type (CCR)7, which ensure their main presence into secondary lymphoid tissues (lymph nodes, spleen and mucosal-associated lymphoid tissue, such as Peyer's patches in the small intestine) (McLachlan and Jenkins, 2007; Ferrando-Martinez, Ruiz-Mateos and Leal, 2010; Caccamo et al., 2018). Once activated naïve CD4 T cells acquire a memory phenotype and specific effector functions. Then, memory CD4 T cells migrate into the site of infection and activate the proper adaptive immune response aimed to eradicate the pathogen.

The activation of naïve CD4 T cells, also called differentiation or polarization, requires three signals. The first signal is the interaction between antigen presented on MHC molecule exposed on dendritic cell surface, and T-cell receptor (TCR) on naïve CD4 T cell surface (Thomas J Kindt; Richard A Goldsby; Barbara Anne Osborne; Janis Kuby, 2007). The second signal is the binding between co-stimulatory molecules on T cell surface and corresponding ligands on dendritic cell surface. CD28 is the main co-stimulatory molecule constitutively expressed by T cells, whose interaction with CD80 and CD86 on mature dendritic cells leads to the proliferation and expansion of CD4 T cells (Jenkins et al., 2001; Magee, Boenisch and Najafian, 2012; Porciello, and Tuosto, 2016). The third signal involves interaction of naïve CD4 T cells with cytokines released by dendritic cells through specific cytokine receptors. Interestingly, different sets of cytokines may activate distinct differentiation programs leading to Th1, Th2, Th17 or Treg profiles. Th1 and Th2 differentiation are driven by IL-12 and IL-4, respectively. Th1 produce IFN-γ (Mosmann et al., 1986; Mosmann and Coffman, 1989), while Th2 produce IL-4, IL-5, and IL-13 (Mosmann et al., 1986). Th1 cells induce cell-mediated inflammatory responses (macrophages and CD8 T cells) important for the clearance of intracellular bacteria, while Th2 cells activate B cells and promote a protective humoral response important against helminth infection (Mosmann and Coffman, 1989; Paul and Seder, 1994). Th17 cells differentiate in presence of the cytokines IL-1β, IL-6, IL-23 and TGF-β (Veldhoen, Hocking, Atkins, et al., 2006; Volpe et al., 2008), leading to release of IL-17A and IL-17F. In general, Th17 immune response induces the recruitment of neutrophils and the production of antimicrobial-peptides by epithelial cells, that improves epithelial-barrier integrity and is critical for mucosal host defence against extracellular bacteria and fungi (Weaver et al., 2006; Bettelli et al., 2008). Treg

cells differentiate in presence of transforming growth factor-β (TGF-β) and IL-2 (Rodríguez-Perea et al., 2016).

1.4.3 Interaction between microbiota and immune cells

In the mucosal tissues of the host there is a strong communication between immune cells and microbiota.

In the gastrointestinal tract, commensal microbes express signals able to enhance the host defense mechanisms. For instance, commensal microbes primes barrier immunity by driving expression of a) mucin by Goblet cells, found in the columnar epithelium; b) immunoglobulin A (IgA) by B lymphocytes; c) antimicrobial molecule α-defensins by Paneth cells, specialized secretory epithelial intestinal cells with a key role in the control of enteric pathogens growth (Salzman, Hung, Haribhai, Chu, et al., 2010).

Moreover, commensal microbes may modulate the immune response. In fact, microbial-derived product SCFAs, interact with monocytes, inducing the diminished expression of monocyte chemotactic protein-1 (MCP-1) and production of TNF, IFN-γ and IL-10 in response to treatment with LPS (Cox, et al., 2009), indicating that SCFAs modulates monocyte-activity in response to pathogen infection simulated by LPS. In dendritic cells, treatment with butyrate, produced by bacteria, leads to a decreased expression of pro-inflammatory cytokines IL-12 and IFN-γ, increased expression of IL-10 and IL-23 (Berndt, et al., 2012), and downregulation of antigen-presentation machinery, including CD40, CD80, CD86 and major histocompatibility complex class II molecules (Liu, et al., 2012). The Segmented Filamentous Bacteria (SFB) induce Th17 cells (Ivanov, et al., 2009), while *Clostridium spp*. generates an environment rich in TGF-β and colonic Treg cells (Atarashi, et al., 2011). Bacterial polysaccharide (PSA) from the gut microorganism *Bacteroides fragilis* is sensed by intestinal DCs and lead to regulation of Th1/Th2 lineage differentiation, which may contribute to protection from disease by creating an appropriate immune response (Mazmanian, Liu, Tzianabos, and Kasper, 2005).

However, not only bacteria, but also commensal fungi modulate the immune system. In fact, fungal β-glucans and *C. albicans* phospholipomannans (PLMs) is recognized by TLR2 (Bourgeois, and Kuchler, 2012), *C. neoformans* glucuronoxylomannan (GXM) and *C. albicans* mannans (O-linked) by TLR4, *Malassezia spp.* and *C. albicans* interact with C-type lectin on immune cells. These interactions induce the release of chemokines and cytokines against fungi (Miyake, Oh-hora, and Yamasaki, 2015). Importantly, interactions between β-glucans and α-mannans on fungal cell wall and Dectin-1/2, expressed by macrophages and DCs, result in the differentiation of Th17 cells (Saijo, Ikeda, Yamabe, et al., 2010), which are crucial in the anti-fungal response (Weaver et al., 2006; Bettelli et al., 2008).

Moreover, it is known that the wall components of *S. cerevisiae* activate human monocyte-derived DCs through mannose receptor, DC SIGN, dectin-1, and chitin receptor, thus leading to the production of IL-6 and other inflammatory cytokines (Rizzetto, Kuka, De Filippo, et al., 2010; Rizzetto, et al., 2016; Di Paola, Rizzetto, Stefanini, et al., 2020).

1.4.4 Interaction between opportunistic microbes and immune cells

The opportunistic microbes, such as *A. baumannii* and P. aeruginosa, are recognized by innate immune cells (Wong, Nielsen, Bonomo, Pantapalangkoor, Luna, and Spellberg, 2017).

The recognition of *A. baumannii* by innate immune cells occurs through the binding between bacterial LPS and TLR4/ TLR2 on immune cells. Neutrophils are the first to arrive and are the most effective phagocytic cell for controlling and eliminating *A. baumannii* through the release of ROS, myeloperoxidase, and β-defensins (Wong, Nielsen, Bonomo, Pantapalangkoor, Luna, and Spellberg, 2017). Macrophages also phagocytose and kill *A. baumannii* at the early stage of infection, releasing critical proinflammatory cytokines, such as tumor necrosis factor (TNF) and IL-8 (Erridge, Moncayo-Nieto, Morgan, Young, Poxton, 2007), and chemokines for the subsequent further recruitment of neutrophils and other innate immune cells (Bruhn, Pantapalangkoor, Nielsen, Tan, Junus, Hujer, et al., 2015).

However, outer membrane protein A (OmpA) of *A. baumannii* activates DCs, inducing their maturation and activation of MAP kinase and NF-κB signaling pathway, with consequent induction of Th1 cell response (Lees-Miller, Iwashkiw, Scott, Seper, Vinogradov, Schild, et al., 2013).

P. aeruginosa is recognized by the host through TLR4 and TLR5, which bind LPS and flagellin, respectively (Feuillet, Medjane, Mondor, Demaria, Pagni, Galan, Flavell, and Alexopoulou, 2006). This interaction leads to bacterial internalization, secretion of TNF-α, IL-6, IL-1β, and killing of bacteria (Raoust, Balloy, Garcia-Verdugo, Touqui, Ramphal, and Chignard, 2009; Mijares, Wangdi, Sokol, Homer, Medzhitov, and Kazmierczak, 2011).

1.4.5 Interaction between probiotic microbes and immune cells

Lactobacillus species, contained in the most probiotics mixtures, induce a stimulation of regulatory DCs, expressing high levels of IL-10, TGF-β, COX-2, and indoleamine 2,3-dioxygenase, able to promote the generation of Treg cells, and concomitant downregulation of Th1, Th2 and Th17 subsets (Kwon, Lee, So, et al., 2010). It has been observed that *L. plantarum, L. paracasei and L. rhamnosus* (LGG) are able to induce

DCs activation and production of IL-12p70, TNF-α and IL-10 (Mileti, Matteoli, Iliev, and Rescigno, 2009). Moreover, *L. rhamnosus* modulates dendritic cell functions by upregulating the expression of CD86, CD83, HLA-DR, TLR4, and downregulating DC-SIGN, MR, and CD14 (Evrard, Coudeyras, Dosgilbert, et al., 2011).

Other probiotic mixtures, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Bifidobacterium bifidium, and Streptococcus thermophilus*, induce $Foxp3^+$ Treg cells (Kwon, Lee, So, et al., 2010).

In addition, *L. reuteri* is able to activate ILCs and induce their secretion of IL-22, which reduce colonization of *Candida albicans*, thus contributing to protection of the host against the opportunistic pathogens (Zelante, Iannitti, Cunha, et al., 2013), while the probiotic strain of *L. casei* induces IL-10 production by Th2 lymphocytes and macrophages (Lemme-Dumit, Polti, Perdigón, et al., 2018).

1.5 Aims of the thesis

Global objective of this study was to investigate the cross-talk between immune system and different type of microorganisms.

In particular, we distinguish three specific aims:

1) Study of the immune response induced by *Saccharomyces cerevisiae* and human dendritic cells;
2) Characterization of the interaction between *Acinetobacter baumannii* and human innate immune cells;
3) Analysis of the immunomodulatory effect of probiotic *Lactobacillus reuteri* in autistic children.

2. HUMAN CONVENTIONAL AND PLASMACYTOID DENDRITIC CELLS DIFFER IN THEIR ABILITY TO RESPOND TO *SACCHAROMYCES CEREVISIAE*

Results of Section 2 address the Aim 1, and have been included in:

Human conventional and plasmacytoid dendritic cells differ in their ability to respond to *Saccharomyces cerevisiae*

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Front Immunol. 2022;13:850404 doi:10.3389/fimmu.2022.850404

2.1 Introduction

In the last years it is emerging a crucial role of microbiota in the development of pathologies involving the immune system, such as allergies, inflammatory disorders, and autoimmune diseases.

Microbiota is the set of microbes, including viruses, bacteria, and fungi living in the host organism. Microbiota is essential for the protection against pathogens, the synthesis of molecules, and the metabolism of substances derived from diet (Prakash, Rodes, Coussa-Charley, Tomaro-Duchesneau, 2011). In healthy condition, all of these microbes are balanced with each other and are able to induce immune tolerance. However, an alteration in number and/or localization of different microbes (dysbiosis) may promote diseases associated to immune dysregulation. Microbiota influences the immune system through the interaction with innate immune cells (Belkaid, and Hand, 2014). In particular, dendritic cells (DCs) interact with microorganisms through a set of pattern recognition receptors (PRRs), such as toll like receptor (TLR), and initiate the adaptive immune response by activating naïve T lymphocytes through the release of cytokines and the expression of co-stimulatory molecules (Banchereau, and Steinman, 1998). Blood DCs can be divided into conventional (cDCs) and plasmacytoid DCs (pDCs) (Shortman, and Liu, 2002; See, Dutertre, Chen, Gunther, McGovern, Irac, et al., 2017). pDCs predominantly recognize viruses and are IFN-α producers (Colonna, Trinchieri, and Liu, 2004; Alculumbre, Raieli, Hoffmann, Chelbi, Danlos, and Soumelis, 2019), while cDCs are involved in the recognition of several microorganisms, including bacteria and viruses, and produce inflammatory cytokines, such as interleukin (IL) -6 (Segura, Touzot, Bohineust, Cappuccio, Chiocchia, and Hosmalin, et al., 2013).

However, bacteria and viruses are not the unique microbes composing microbiota. Fungi kingdom is an important component of the microbiota, called mycobiota. Interestingly, the composition of the mycobiota is particularly unstable compared to the rest of microbiota (Hallen-Adams, and Suhr, 2017). Thus, fungi derived from dietary or environmental sources may contribute to mycobiota diversity and may strongly

influence innate immunity. The most common genus of fungi originating from food and environment, and harbouring the gastrointestinal tract, is *Saccharomyces.* Many members of this genus are considered very important in food production, such as *S. cerevisiae,* the bakers' and brewers' yeast (Hallen-Adams, and Suhr, 2017).

However, *S. cerevisiae* is also an etiologic agent of opportunistic fungal infection in immunocompromised patients (Zelante, Montagnoli, Bozza, Gaziano, Bellocchio, Bonifazi P, et al., 2007), and the ability to colonize and give rise to disease depends on the host immune response. Although immune response to *A. fumigatus, C. albicans, Cryptococcus neoformans, Malassezia* have been largely characterized (Romani, 2011), little is known about the interactions between *S. cerevisiae* and host defense cells. Previous studies reported that wall components of *S. cerevisiae* activate human monocyte-derived DCs through mannose receptor, DC SIGN, dectin-1, and chitin receptor, thus leading to the production of IL-6 and other inflammatory cytokines (Rizzetto, Kuka, De Filippo, Cambi, Netea, Beltrame, et al., 2010; Di Paola, Rizzetto, Stefanini, Vitali, Massi-Benedetti, Tocci, et al., 2020). However, the ability of blood circulating human DCs to respond to *S. cerevisiae* has never been investigated. Here, we challenged human blood DC subtypes with *S. cerevisiae,* and we found a differential response of cDCs and pDCs, characterized by IL-6 and IFN-α production, with a consequent IL-17 and IL-10 induction in *in vitro* differentiated Th cells, respectively. Thus, the exposition of *S. cerevisiae* as well as the prevalence of specific DC targets *in situ* may influence the development and the progression of diseases associated to microbiota-dependent immune dysregulation or fungal opportunistic infection.

2.2 Results

The laboratory strain of *Saccharomyces cerevisiae* SK-1 promotes activation of human blood DCs

In order to test blood DCs response to *S. cerevisiae*, human pDCs and cDCs were purified from peripheral blood of healthy donors (Supplementary Figure S1, S2), and stimulated with a laboratory strain of *S. cerevisiae*, called SK-1. Specifically, we cultured DCs in presence of SK-1 at multiplicity of infection (MOI) of 5 (CFU SK-1/DC). We used as positive control Resiquimod (R848), which is an imidazoquinoline compound known to be a potent immune activator of both pDCs and cDCs because it is an agonist of TLR7 and TLR8, expressed by pDCs and cDCs, respectively. Unstimulated DCs were used as negative control. We evaluated the levels of CD80 and CD86, two molecules binding CD28 on T cell surface and inducing T cell activation and proliferation, and the expression of programmed death-ligand 1 (PD-L1), which is a co-inhibitory molecule known to reduce T cell proliferation through the binding with PD-1 on T cell surface. Our results showed that SK-1 induces a significant increase of CD80 and CD86 expression by pDCs and cDCs (Figure 1A). Surprisingly, we also found a significant increase of PD-L1 expression induced by SK-1 in both DC subsets, especially in pDCs (Figure 1A). Consistently, the increase of CD80+, CD86+, and PD-L1+ cells in DCs is associated to an overall high median fluorescence intensity (MFI) (Figure 1B). In addition, we measured IFN-α and IL-6 production by SK-1 stimulated-pDCs and cDCs, respectively. We found that SK-1 induces IFN-α production by pDCs (Figure 1C), and IL-6 production by cDCs (Figure 1D), compared to unstimulated DCs.

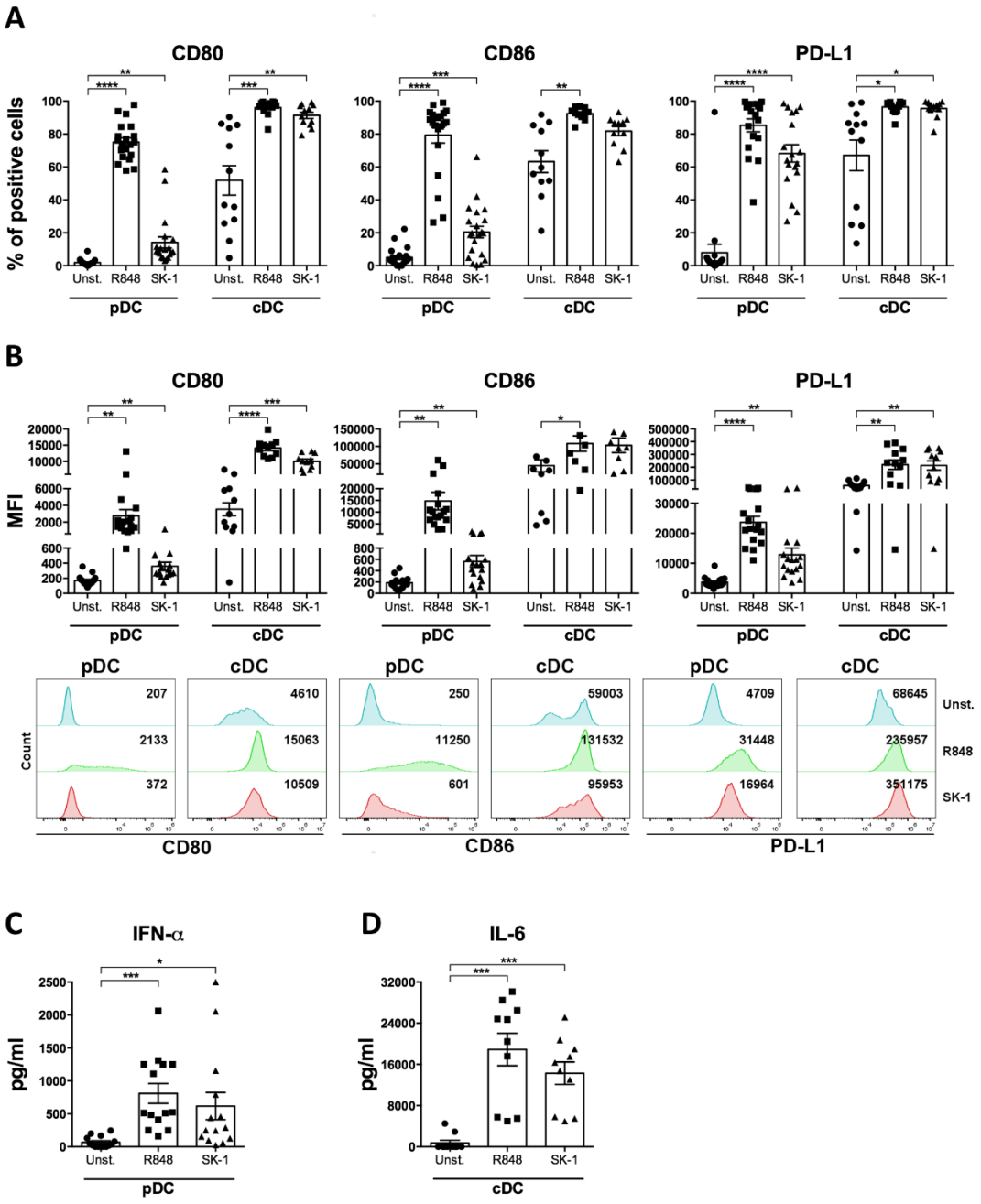

Figure 1. The laboratory strain of Saccharomyces cerevisiae SK-1 promotes the activation of human blood DCs.

Human pDCs and cDCs purified from peripheral blood of healthy donors were cultured for 48h without stimulation (Unst.), with R848 (1µg/ml) as positive control, or with the laboratory strain of Saccharomyces cerevisiae SK-1 at MOI 5 (CFU SK-1/pDC). Expression of molecules CD80, CD86, and PD-L1 was analysed by flow cytometry and percentage of positive cells (A) and median fluorescence intensity (MFI) (B) were reported. Levels of IFN-α (C) and IL-6 (D) were measured in the culture supernatants by ELISA assays. Data are the mean of 11 independent experiments, each from different donors. One-way ANOVA was used to compare different experimental conditions (p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001).*

SK-1 induces differentiation of P1-subpopulation of human pDCs

To further insight into pDC activation by SK-1, we cultured pDCs in presence of SK-1 at different MOI. After 48 hours (h) of culture we firstly evaluated formation of cell clusters, which reflects pDC activation and pDC viability by optical microscopy. Our results showed that stimulation with SK-1 leads to cell cluster formation (Figure 2A). As expected, pDCs stimulated with the positive control (R848) form cell clusters, while unstimulated pDCs do not form any cell cluster (Figure 2A). Moreover, we found that different doses of SK-1 lead to a progressive increase of CD80, CD86, and PD-L1 within viable pDCs in a dose-dependent manner (Figure 2B). We found that SK-1 at MOI 5, and not at MOI 10, induces the highest levels of IFN-α (Figure 2C), likely due to the lethal effect of high dose of SK-1 (data not shown). These results confirm that SK-1 (MOI 5) promotes pDC activation and viability with the concomitant IFN-α production. Recently it has been observed that the differential expression of the surface molecules PD-L1 and CD80, define three specific pDC subpopulations with distinct functions: P1 (PD-L1$^+$ CD80$^-$), P2 (PD-L1$^+$ CD80$^+$), P3 (PD-L1$^-$ CD80$^+$) (Alculumbre, Saint-Andre, Di Domizio, Vargas, Sirven, Bost, et al., 2018). Given the high expression of PD-L1 induced by SK-1 in pDCs, we hypothesized that P1-pDC subpopulation is preferentially induced upon stimulation with the laboratory strain of *S. cerevisiae*. Thus, we analyzed pDC-subpopulations in our experimental conditions by flow cytometry. Our results showed that SK-1 is able to induce P1-pDC subpopulation (Figure 2D,E). In particular we observed a dose-dependent induction of the percentage of P1-pDCs at increasing doses of SK-1. The positive control R848 induces all three subpopulations (Figure 2D,E), as previously demonstrated (Alculumbre, Saint-Andre, Di Domizio, Vargas, Sirven, Bost, et al., 2018). The fluorescence-minus-one experiment demonstrates the specificity of PD-L1 staining (Supplementary Figure S3).

Moreover, it has been reported that IFN-α-producing pDCs are mostly P1-pDCs (Alculumbre, Raieli, Hoffmann, Chelbi, Danlos, and Soumelis, 2019). Therefore, we investigated whether induction of P1-pDCs obtained upon stimulation with SK-1 was associated to IFN-α production in the same experimental conditions. To address this relationship, we correlated the levels of IFN-α and the percentage of P1-pDCs in several pDCs-SK-1 cultures. Interestingly, we found a positive correlation between P1-pDCs and IFN-α (Figure 2F), suggesting that SK-1 induces PD-L1$^+$ CD80$^-$ pDCs, which in turn produce IFN-α.

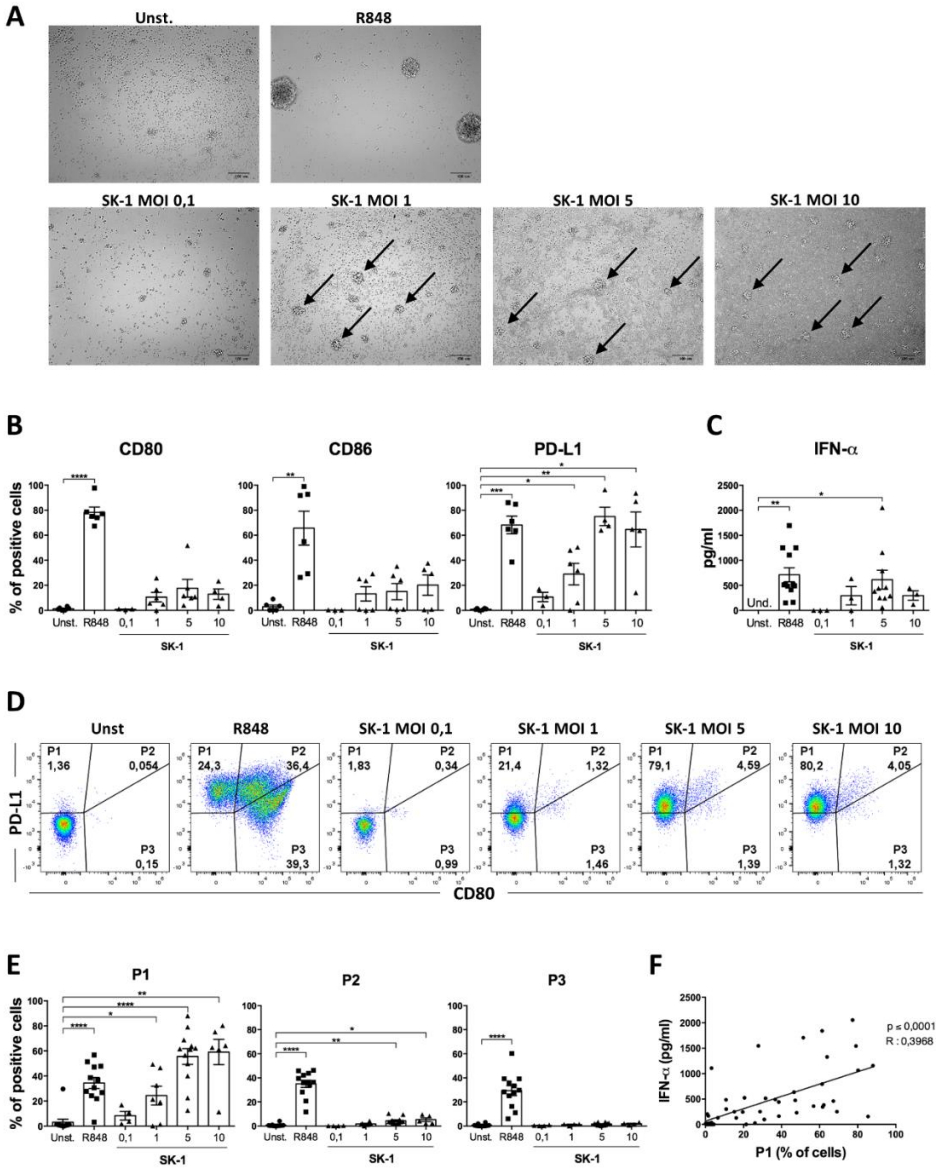

Figure 2. SK-1 induces differentiation of P1-subpopulation of human pDCs

*Human pDCs purified from peripheral blood of healthy donors were cultured for 48h without stimulation (Unst.), with R848 (1μg/ml) as positive control, or with laboratory strain of Saccharomyces cerevisiae SK-1 at different MOI 0,1-1-5-10 (CFU SK-1/pDC). Photos by optical microscope show cell clusters, indicated by arrows, formed by pDCs upon activation. Pictures are representative of three independent experiments, each from different donor (A). Expression of molecules CD80, CD86, and PD-L1 was analysed by flow cytometry and reported as percentage of positive cells (B). Levels of IFN-α were measured in the culture supernatants by ELISA assay (C). Expression of PD-L1 and CD80 was analysed by flow cytometry. Representative plots show the percentage of three pDC subpopulations (D) and graphs show the corresponding quantification of more experiments (E). Data in B, C, and E are the mean of six or more independent experiments, each from different donors. Error bars represent SEM. One-way ANOVA was used to compare different experimental conditions (** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001). Percentages of P1 subpopulation, obtained from all experimental conditions of 4 independent experiments, were correlated to their IFN-α levels using Pearson correlation (F). R value indicates the correlation coefficient.*

Fungal nucleic acids activate human pDCs

In order to investigate the mechanism inducing blood DC activation by *S. cerevisiae*, we firstly analyzed the involvement of TLR7 and TLR8, able to recognize microbial RNA, in SK-1 mediated DC activation. We performed a set of experiments in presence of a synthetic antagonist inhibitor of TLR7 in pDCs and TLR8 in cDCs. First, human purified DCs were pre-treated for 30 minutes with the inhibitor, and then stimulated for 48h with SK-1 at MOI 5. We analyzed the IFN-α production in pDCs, and IL-6 production in cDCs, and we compared results with those obtained in SK-1 stimulated-DCs without inhibitor. Interestingly, IFN-α production in pDCs, and not IL-6 in cDCs, is significantly reduced in presence of TLR7-8 inhibitor (Figure 3A). Importantly, we tested different doses of TLR7-8 inhibitor and results confirmed that TLR8 is not involved in IL-6 production by cDCs stimulated with SK-1 (Supplementary Figure S4A), while it is involved in those stimulated with R848, which is the ligand of TLR7-8 (Supplementary Figure S4B). Since pDCs express also TLR9, which recognizes microbial DNA, we cultured SK-1 stimulated pDCs in presence of TLR7-9 inhibitor. We observed a reduction of IFN-α levels in pDCs stimulated with SK-1 and pre-treated with TLR7-9 inhibitor (Figure 3B). However, we could not appreciate an additive effect due to the inhibition of both TLR7 and TLR9 signaling, compared to the inhibition of TLR7 alone (Figure 3B). In contrast, the expression of CD80, CD86, and PD-L1 seems to be not mediated by fungal nucleic acids. In fact, PD-L1 expression in SK-1-stimulated pDCs and cDCs was not affected by the presence of TLR7-8-9 inhibitors (Figure 3C-F), and CD80 and CD86 expression were weakly increased in pDCs in presence of TLR7-8 inhibitor (Figure 3C, D). These results suggest that IFN-α production in pDCs could be mediated by the interaction between yeast nucleic acids and TLR, while upregulation of surface markers on pDC surface depend on other pathways, which are further activated in response to TLR7 inhibition. On the other hand, recognition of whole SK-1 by cDCs occur through other pathways independent on TLR8.

In order to directly assess whether *S. cerevisiae* nuclei acids induce IFN-α production by pDCs, we stimulated blood pDCs with RNA and DNA extracted from SK-1. We found that both nucleic acids induce cell cluster formation typical of pDC activation (Figure 4A) and IFN-α production (Figure 4B). To further characterize this response, we performed experiments in presence of nucleases specifically degrading single strand (ss) or double strands (ds) yeast nucleic acids. We found that degradation of ssRNA, ssDNA, and dsDNA of SK-1 nucleic acids inhibits IFN-α production by human pDCs (Figure 4C).

Interestingly, nucleic acids from SK-1 do not induce production of the pro-inflammatory cytokine IL-6 by cDCs (Supplementary Figure S5). Moreover, RNA and DNA from SK-1 induce P1-pDC differentiation (Figure 4D), upregulation of the activation markers

(CD80 and CD86) and the inhibitory marker PD-L1 at a similar extent to whole SK-1 (Figure 4E,F). Collectively these data indicate that yeast nucleic acids specifically activate human pDCs, leading to IFN-α, CD80, CD86, and PD-L1 overexpression. In order to investigate the role of TLRs in recognizing SK-1 nucleic acid in pDCs, we used TLR inhibitors in yeast DNA- and yeast RNA-treated cells, and we found that TLR7 and TLR7/9 inhibitors partially reduce IFN-α production by SK-1 RNA and DNA, respectively (Figure 5A). The percentage of P1-pDC subpopulation (Figure 5B,C) and the expression of CD80, CD86, and PD-L1 are not affected by the presence of TLR7 and TLR9 inhibitors (Figure 5D,E).

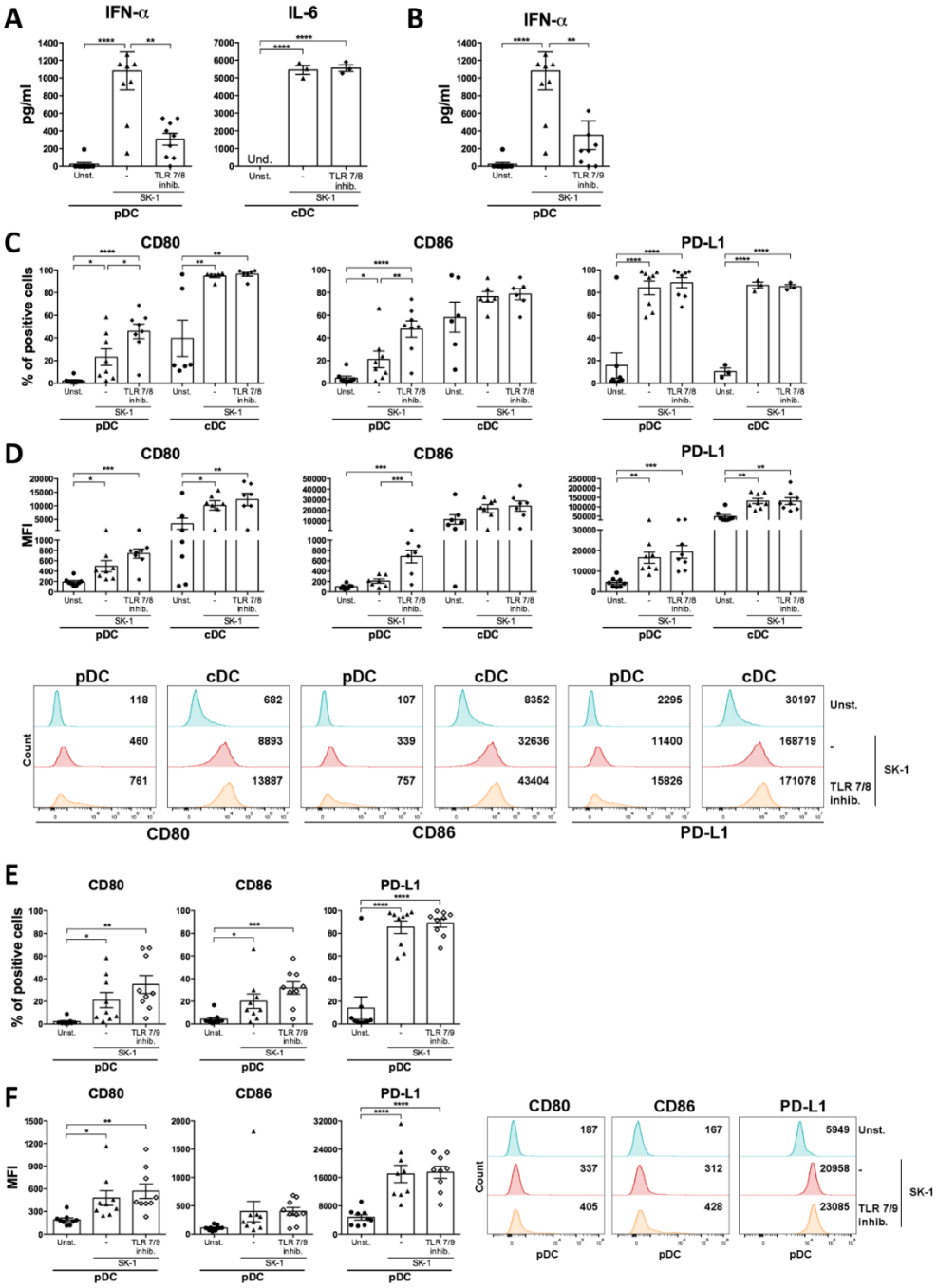

Figure 3. IFN-a production by SK-1-stimulated pDCs is mediated by sensors of nucleic acids. *Human pDCs and cDCs purified from peripheral blood of healthy donors were pre-treated for 30 minutes with TLR 7/8 (1μM), or with TLR 7/9 (1μM), and cultured for 48h without stimulation (Unst.), or with the laboratory strain of Saccharomyces cerevisiae SK-1 at MOI 5 (SK-1/pDC). Levels of IFN-α or IL-6 were measured in the culture supernatants by ELISA assays (A,B). Expression of molecules CD80, CD86, and PD-L1 was analysed by flow cytometry and reported as percentage of positive cells and median fluorescence intensity (MFI) (C-F). Graphs show mean ± SEM of three or more independent experiments, each from different donors. Two-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).*

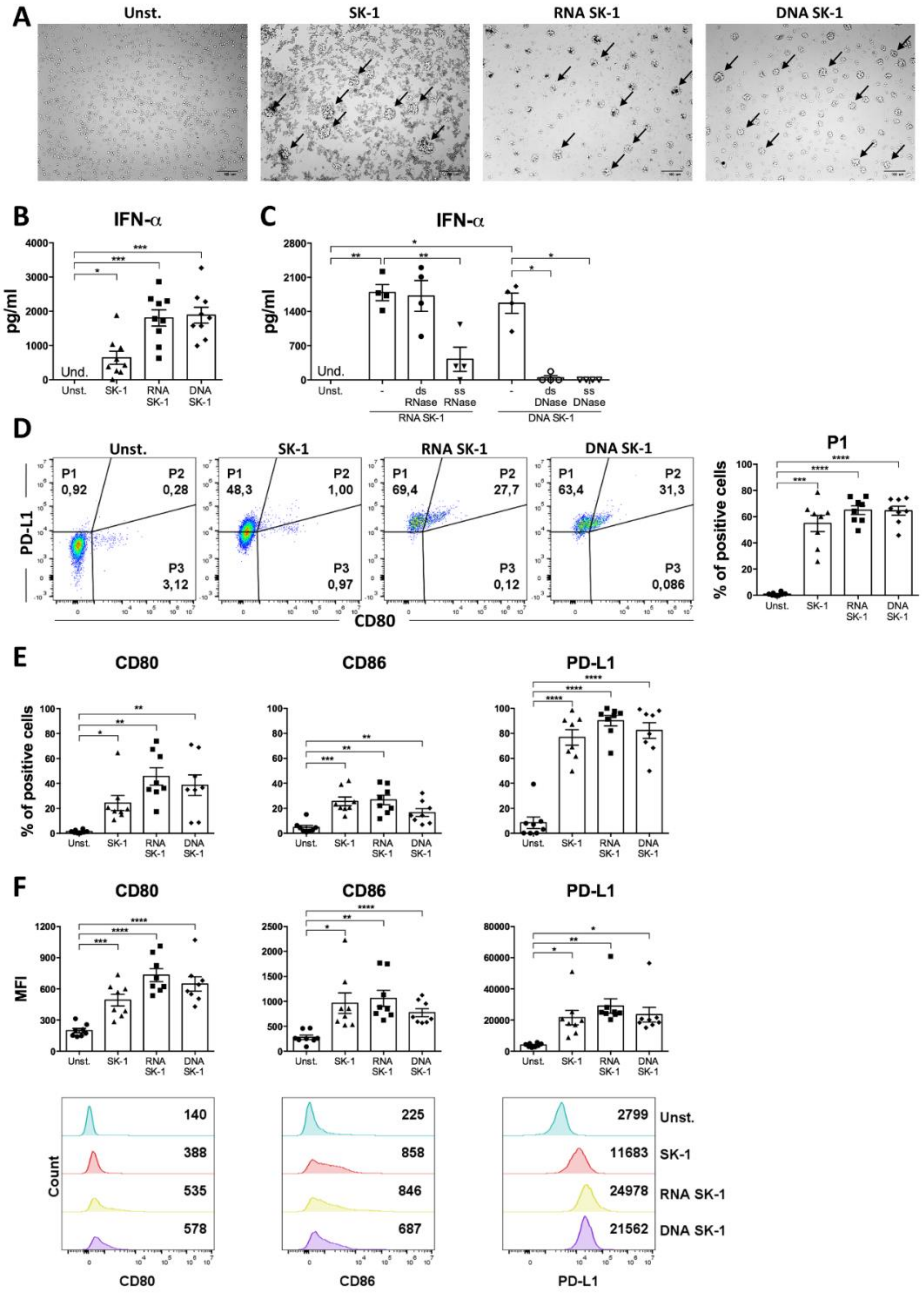

Figure 4. RNA and DNA of SK-1 induce activation of human pDCs.

Human pDCs purified from peripheral blood of healthy donors were cultured for 48h with RNA or DNA (0,2µg) extracted from Saccharomyces cerevisiae SK-1 and pre-treated with Dotap (10µl/µg of nucleic acids) for 30 minutes at 37°C, without stimulation (Unst.), with the laboratory strain of Saccharomyces cerevisiae SK-1 at MOI 5 (SK-1/pDC). Photos by optical microscope show cell clusters formed by pDCs upon activation highlighted by arrows (A). Pictures are representative of four experiments, each from different donor. DNA and RNA were pre-treated with nucleases, where indicated. Levels of IFN-α were measured in the culture supernatants by ELISA assay (B, C).

*Percentage of P1-pDC subpopulation was evaluated by flow cytometry (D). Percentages and median fluorescence intensity (MFI) of costimulatory molecules CD80, CD86, and PD-L1 were analysed by flow cytometry (E,F). Graphs show mean ± SEM of eight independent experiments, each from different donors. One-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).*

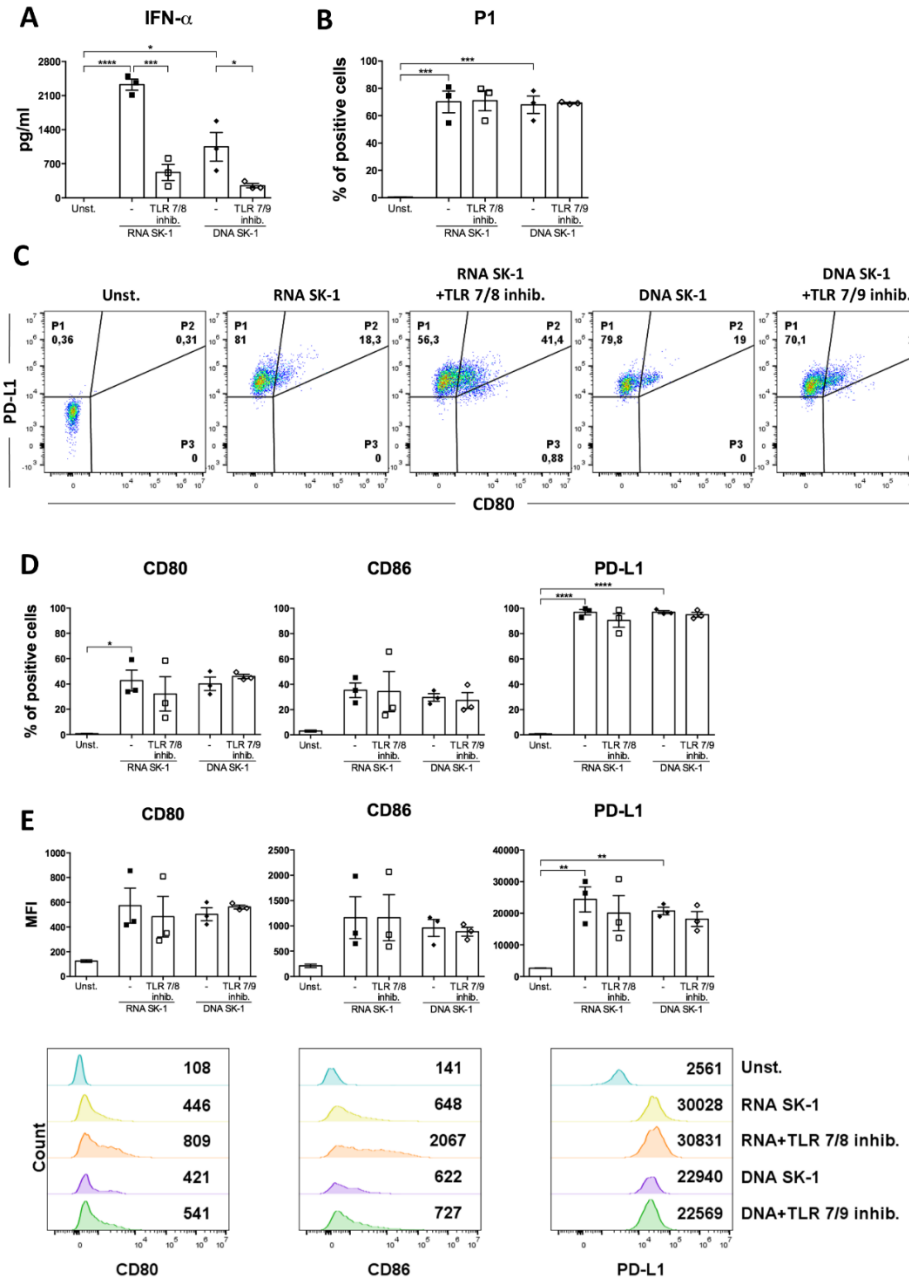

Figure 5. IFN-a production by human pDCs stimulated with SK-1 nucleic acids is partially mediated by TLR7 and TLR9.

*Human pDCs purified from peripheral blood of healthy donors were pre-treated for 30 minutes with TLR 7/8 (1µM), or with TLR 7/9 (1µM), cultured for 48h with RNA or DNA (0,2µg) after incubation with Dotap (10µl/µg of nucleic acids) for 30 minutes at 37°C, without stimulation (Unst.). Levels of IFN-α were measured in the culture supernatants by ELISA assay (A). Percentage of P1-pDC subpopulation was evaluated by flow cytometry (B,C). Percentages and median fluorescence intensity (MFI) of costimulatory molecules CD80, CD86, and PD-L1 were analysed by flow cytometry (D,E). Graphs show mean ± SEM of three independent experiments, each from different donors. Two-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).*

SK-1-primed pDCs and cDCs induce IL-10- and IL-17-producing CD4 T cells

Activated DCs perform important adaptive functions in naïve CD4 T cell priming and polarization. Given the differential response of pDCs and cDCs to *S. cerevisiae* in terms of cytokine production and costimulatory molecules expression, we hypothesized a functional specialization towards distinct T helper (Th) profiles. We stimulated naïve CD4 T cells with anti-CD3, to simulate the antigen recognition, and with SK-1-primed DCs, to study the impact of the up-regulation of their cytokines and co-stimulatory receptors on Th polarization. We analyzed production of typical Th cytokines (IFN-α for Th1; IL-4 for Th2; IL-17 for Th17; IL-10 for T regulatory cells) in T cells co-cultured with SK-1-primed DCs, compared to T cells co-cultured with unstimulated DCs, and T cells stimulated with anti-CD3 and anti-CD28.

We found that IL-4 is not significantly modulated in the different experimental conditions (Figure 6A,B), while SK-1-primed cDCs induce a significant IL-17 production compared to unstimulated cDCs (Figure 6B), and SK-1-primed pDCs induce a significant IL-10 production compared to unstimulated pDCs (Figure 6A). Concomitantly to IL-17 production, we observed a significant reduction of IFN-α by naïve CD4 T cells co-cultured with SK-1-primed cDCs (Figure 6B).

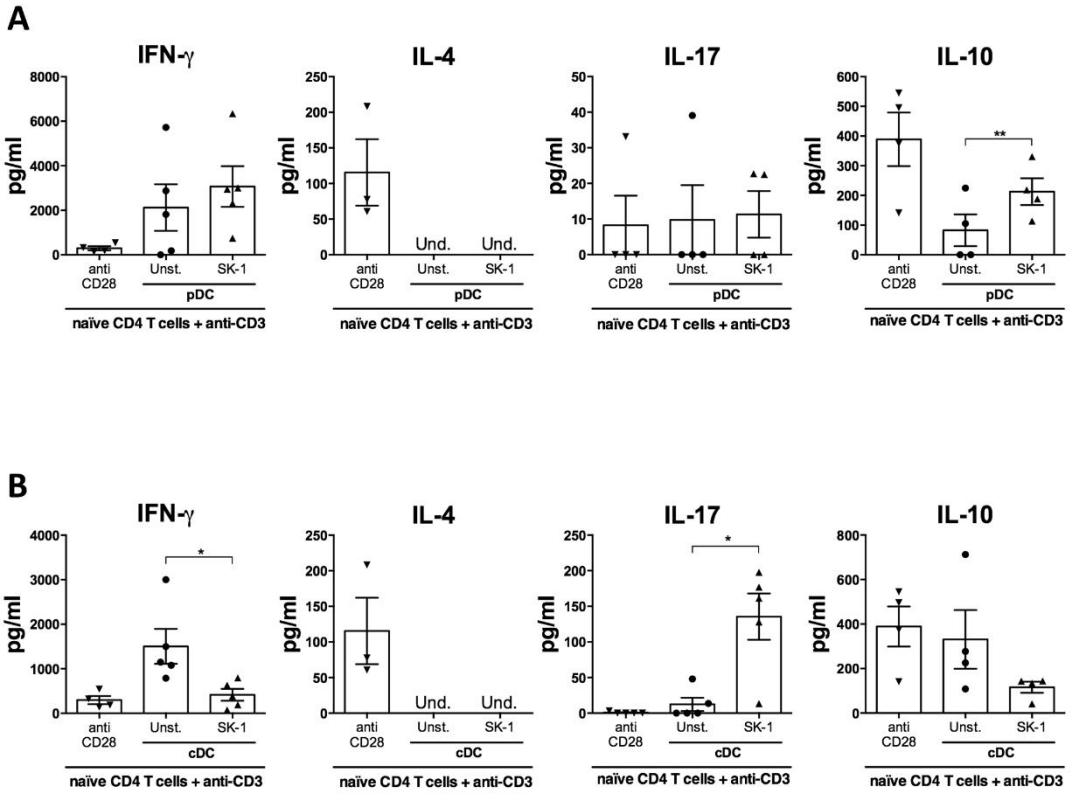

Figure 6. pDCs and cDCs primed with SK-1 induce IL-10- and IL-17-producing CD4 T cells, respectively

*Human naïve CD4 T cells were cultured in presence of anti-CD3/CD28 beads or placed in co-culture for 5 days with unstimulated or SK-1-primed pDCs or cDCs in presence of anti-CD3. After 5 days, the cells were re-stimulated with anti-CD3 (co-culture) or anti-CD3/CD28 beads (CD4 T cells alone). Supernatants were collected after 24h of re-stimulation, and levels of IFN-γ, IL-4, IL-17, and IL-10 in co-cultures with pDCs (A) and cDCs (B) were measured by ELISA assays. Graphs show mean ± SEM of four or five independent experiments, each from different donors. Paired student's t test was used to compare unstimulated and stimulated DCs (*p-value ≤ 0.05).*

2.3 Discussion

SK-1 is a fungus with phylogenetic similarity to yeasts isolated from fermentation in the African area (Stefanini, Dapporto, Legras, Calabretta, Di Paola, De Filippo, et al., 2012), and is most likely one of the constituents of the human microbiota derived from the ingestion of fermentation products. However, *S. cerevisiae* has also been found in the bloodstream of immunocompromised patients (Herbrecht, and Nivoix, 2005; Taylor, Buchanan-Chell, Kirkland, McKenzie, and Wiens, 1994) considering this fungus an emerging opportunistic pathogen (de Llanos, Fernandez-Espinar, and Querol, 2006). Thus, our study is useful for understanding the interaction of mucosal DCs with intestinal microbiota, and for analysis of the anti-fungal immunity of blood DCs against pathogenic *S. cerevisiae*. Results from this study revealed that SK-1 is able to activate human blood pDCs and cDCs, indicating that both DC subsets have an important role in modulating the immune response following a fungal stimulus. Importantly, we observed a differential activation of pDCs and cDCs by SK-1, suggesting a distinct role of each DC subset in the anti-fungal immunity, and in the interaction with intestinal microbiota. Specifically, SK-1 induces high induction of IFN-α and expansion of a subpopulation of pDCs, called P1, characterized by the expression of PD-L1 molecule on the cell surface. However, SK-1 does not stimulate a strong maturation process in these cells, in fact the co-stimulatory molecules CD80 and CD86 are only weakly induced, unlike what happens as a consequence of their activation mediated by viruses (Cella, Facchetti, Lanzavecchia, and Colonna, 2000), which in our system is simulated by R848. A robust IFN-α production associated to a weak induction of costimulatory molecules at the pDC surface is a typical response of CpG-A (Kerkmann, Costa, Richter, Rothenfusser, Battiany, Hornung, et al., 2005). Thus, similarly to CpG-A, fungal nucleic acids could form a large multimeric complex upon internalization that is retained in early endosomes and signals through MyD88 and IRF-7 (Guiducci, Ott, Chan, Damon, Calacsan, Matray, et al., 2006).

We observed that SK-1 promotes the expansion of P1-pDC subpopulation (PD-L1^{+} CD80^{-}), specialized in the production of IFN-α and in the induction of the anti-inflammatory cytokine IL-10 by T cell lymphocytes (Alculumbre, Saint-Andre, Di Domizio, Vargas, Sirven, Bost, et al., 2018). Consistently, our data showed that pDCs stimulated with SK-1 induce IL-10-producing T cells. It is known that IL-10 and IFN-α, synergistically, promote the differentiation of a particular population of regulatory T lymphocytes, known as Tr1 (IL-10^{+}, IFN-γ+, IL-2$^{-/low}$, IL4^{-}), capable of suppressing the proliferation of T lymphocytes (Levings, Sangregorio, Galbiati, Squadrone, de Waal Malefyt, and Roncarolo, 2001). All together, these results suggest that SK-1, by inducing pDCs to produce IFN-α and the consequent generation of IL-10-producing T cells, may

contribute to the generation of immunoregulatory T cells. The activation of a regulatory response by pDCs has been described in previous papers (Gilliet, and Liu, 2002; Ito, Yang, Wang, Lande, Gregorio, Perng, et al., 2007; Ruocco, Rossi, Motta, Macchiarulo, Barbieri, De Bardi, et al., 2015) suggesting that this is a typical property of pDCs, regardless of the nature of the activating stimulus.

Moreover, the immunosuppressive activity of T regulatory cells has been described in fungal infections (Montagnoli, Bacci, Bozza, Gaziano, Fiorucci, Spreca, et al., 2001; Hori, Carvalho, and Demengeot, 2002). Specifically, the anti-inflammatory role of DCs in response to fungi is mediated by the enzyme indoleamine 2,3-dioxygenase (IDO) (Grohmann, Fallarino, and Puccetti, 2003), which is associated with induction of IL-10-producing T regulatory cells (Montagnoli, Bacci, Bozza, Gaziano, Mosci, Sharpe, et al., 2002). Thus, the metabolic pathway involving tryptophan catabolism could be involved in pDC response to SK-1 and local tolerogenic responses. Given the importance of the anti-inflammatory responses in chronic autoimmune diseases, such as multiple sclerosis and Crohn's disease, pDC response to SK-1 could play a protective role in these diseases.

In contrast to pDCs, cDCs express high levels of CD80 and CD86 in response to SK-1, produce high levels of the inflammatory cytokine IL-6, and promote a Th17 response, suggesting that interaction between *S. cerevisiae* and cDCs in the gut contributes to mucosal inflammation and to mucosal host defense against fungal infection (Huang, Na, Fidel, and Schwarzenberger, 2004). Importantly, cDCs promote a simultaneous reduction of IFN-γ production by CD4 T cells that suggests a prominent role of *S. cerevisiae* and cDCs in regulating the balance between IL-17-producing (Th17) and IL-17/IFNγ-producing (Th1/17) cells.

It is already known that yeast cell wall, characterized by galactose and glucosamine (chitin moieties), glucose (beta-glucan) and mannose (mannans), induces the inflammatory response in monocyte-derived DCs (Rizzetto, Kuka, De Filippo, Cambi, Netea, Beltrame, et al., 2010). Our data suggest that components of yeast cell wall are also involved in the activation of blood cDCs, cells sharing numerous similarities with monocyte-derived DCs.

However, here, for the first time, we reported that nucleic acids of *S. cerevisiae* specifically induce cell activation and IFN-α production in pDCs. In particular ssRNA, ssDNA, and dsDNA derived from SK-1 are involved in pDC activation. Importantly, the induction of IFN-α is partially mediated by TLR7 and TLR9, while the up-regulation of co-stimulatory molecules in pDCs is mediated by other pathways. Among the potential receptors of yeast PAMPs, we suppose that galectin-3 and Fc-γ receptor, recognizing yeast mannans, and DNA-PK, cGAS (CGAS), MRE11, DHX36, DHX9,

DDX41, and DDX60, sensing yeast nuclei acids, may have a role in SK-1-mediated activation because they are expressed by human pDCs (Supplementary Figure S8A,B).
We do not observe an additive effect in IFN-α blocking due to the inhibition of both TLR7 and TLR9 signaling, compared to the inhibition of TLR7 alone that could be due to a different effectiveness exerted by TLR7/8 and by TLR7/9 inhibitors on TLR7, or to the involvement of other DNA sensors in pDCs. In this context, it has been reported that pDCs express DHX36, DHX9 (Kim, Pazhoor, Bao, Zhang, Hanabuchi, Facchinetti, et al., 2010), cGAS (Bode, Fox, Tewary, Steinhagen, Ellerkmann, Klinman, et al., 2016) (Supplementary Figure S8B), which are TLR9 independent DNA sensors.
Previous studies reported that nucleic acids induce maturation of DCs and generate anti-fungal immune response (Bozza, Perruccio, Montagnoli, Gaziano, Bellocchio, Burchielli, et al., 2003; Bacci, Montagnoli, Perruccio, Bozza, Gaziano, Pitzurra, et al., 2002). Specifically, DNA is recognized by TLR9 in *C. albicans* (van de Veerdonk, Netea, Jansen, Jacobs, Verschueren, van der Meer, et al., 2008; Miyazato, Nakamura, Yamamoto, Mora-Montes, Tanaka, Abe, et al., 2009), *A. fumigatus* (Ramirez-Ortiz, Specht, Wang, Lee, Bartholomeu, Gazzinelli, et al., 2008), *C. neoformans* (Nakamura, Miyazato, Xiao, Hatta, Inden, Aoyagi, et al., 2008) infection, while *S. cerevisiae* and *C. albicans* RNA are recognized by TLR7 (Bacci, Montagnoli, Perruccio, Bozza, Gaziano, Pitzurra, et al., 2002; Biondo, Malara, Costa, Signorino, Cardile, Midiri, et al., 2012), and *C. albicans* and *A. fumigatus* RNA by MDA5 (Bozza, Perruccio, Montagnoli, Gaziano, Bellocchio, Burchielli, et al., 2003; Bacci, Montagnoli, Perruccio, Bozza, Gaziano, Pitzurra, et al., 2002; Jaeger, van der Lee, Cheng, Johnson, Kumar, Ng, et al. 2015; Wang, Caffrey-Carr, Liu, Espinosa, Croteau, Dhingra, et al., 2020).
Interestingly, our data indicate that TLR8 inhibitor does not reduce neither IL-6 production nor up-regulation of co-stimulatory molecules in cDCs, indicating that SK-1 cell wall components are the main stimuli for cDCs, and that the response to SK-1 nucleic acids is a typical feature of pDCs.
All together, these results indicate that exposure to *S. cerevisiae* triggers pro- or anti-inflammatory responses depending on the interaction with cDCs or pDCs, respectively. Importantly, nucleic acids of *S. cerevisiae* are a specific anti-inflammatory trigger for pDCs. This information indicates that the opposite role of pDCs and cDCs in response to *S. cerevisiae* could mediate the equilibrium between the pro-inflammatory and the anti-inflammatory immune response in the gut, which is important either for the gut homeostasis than during a *S. cerevisiae* opportunistic infection. The immune dysregulation, characterized by an imbalanced prevalence of cDCs or pDCs, could alter this equilibrium and could lead to development of autoimmune diseases, or exacerbate fungal infections.

Future studies aimed to investigate the molecular mechanisms leading to IFN-α production by pDCs, such as the identification of the activating RNA or DNA sequence of *S. cerevisiae*, could open new perspectives towards therapeutic approaches for dysbiosis-related diseases, such as probiotic intervention. On the other side, the identification of the molecular mechanisms leading to the pro-inflammatory activity of cDCs by *S. cerevisiae* could be useful for the therapeutic targeting of chronic inflammatory diseases, and for a better understanding of the mechanisms underlying immune response during fungal infections in immunocompromised patients.

2.4 Material and methods

Purification of DC subpopulations (cDCs and pDCs) from adult blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (GE Healthcare) from whole blood of healthy donors (Santa Lucia Foundation, Rome, Italy).

Approval by the ethics committee of the Santa Lucia Foundation, Rome (Italy), and written informed consent in accordance with the Declaration of Helsinki from all participants, were obtained before the study was initiated. PBMCs ($400x10^6$) were enriched using the Human Pan-DC Pre-Enrichment Kit (Stemcell Technologies), specific for the purification of all DC type, by negative selection. After the isolation, cells were stained with the following antibodies: anti-human CD11c PE-Dazzle594 (BD Biosciences), anti-human CD4 PC7 (Beckman Coulter), anti-human CD3 PE (Beckman Coulter), anti-human CD14 PE (Immunological Science), anti-human CD16 PE (Miltenyi Biotec), anti-human CD56 PE (Beckman Coulter), anti-human CD19 PE (Miltenyi Biotec), anti-human CD235α (eBioscience).

Highly purified pDC and cDC cells were selected and sorted by a 6-way sorter MoFlo Astrios (Beckman Coulter), by using the following analysis, previously described (O'Doherty, Peng, Gezelter, Swiggard, Betjes, Bhardwaj, et al., 1994): Lineage (CD3, CD14, CD16, CD56, CD19, CD235α)$^-$, $CD4^+$, $CD11c^-$ for pDCs and Lineage (CD3, CD14, CD16, CD56, CD19, CD235α)$^-$, $CD4^+$, $CD11c^+$ for cDCs. Sorted cells had a purity of over 95% and 94% respectively for pDCs and cDCs, as confirmed by flow cytometry analysis (Supplementary Figure S1A, B). This gating strategy includes only pDCs $CD123^+$, and cDCs HLA-DR^+ cells (Supplementary Figure S2).

***In vitro* stimulation of pDCs and cDCs**

pDCs and cDCs were cultured separately in 96-well flat-bottom half-area plates (Corning) at a density of $5x10^4$ per well in RPMI 1640 with 5% of human serum for 48 hours at 37°C with 5% of CO_2, in absence of stimuli or in presence of R848 (Invivogen) (100 ng/mL), SK-1 (*Saccharomyces cerevisiae*) (MOI 0,1-1-5-10 SK-1 cells/cDC or pDC). For the SK-1 nucleic acid stimulation experiments, the RNA and DNA (0,2μg) were extracted from SK-1 as previously described (Di Paola, Rizzetto, Stefanini, Vitali, Massi-Benedetti, Tocci, et al., 2020) and their quality and purity was verified by agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermofisher) (Supplementary Figure S6). SK-1 RNA and SK-1 DNA samples were used for pDC stimulation pre-treated with Dotap (Merck) (10μl/μg of nucleic acids). Pre-treatment of nucleic acids with ezDNase or RNase III (Thermofisher) was performed to degrade dsDNA and dsRNA, respectively, while S1 nuclease (Thermofisher) was used to

degrade ssDNA and ssRNA. For the TLR inhibition experiments, cDCs and pDCs were pre-treated for 30 minutes with TLR 7/8 (Miltenyi Biotec ODN 2087) or TLR 7/9 (Miltenyi Biotec ODN 2088) inhibitors (1μM), and then stimulated with SK-1 (MOI 5).

Naïve CD4 T cells and DC subset coculture

pDC and cDC subsets from healthy donors were cultured separately in 96-well flat-bottom half-area plates (Corning) at a density of $5x10^4$ per well in RPMI 1640 with 5% of human serum, in absence of stimuli, or in presence of SK-1 (MOI 5). CD4 T lymphocytes were purified from PBMCs of the same healthy donors by immunomagnetic selection, using the anti-Mouse IgG MicroBeads (Miltenyi Biotec). After the isolation, the cells were stained with anti-human CD4 PC7 (Beckman Coulter), anti-human CD45RA BV421 (BD Biosciences), anti-human CD45RO PE (BD Biosciences), anti-human CD27 APC (Beckman Coulter), and CD4 naïve T cells were sorted by Astrios high-speed cell sorter (Beckman Coulter) as $CD4^{high}$, $CD45RA^{high}$, $CD45RO^{-}$, and $CD27^{+}$. Sorted cells had a purity of over 97%, as shown by flow cytometry (Supplementary Figure S7).

After 24h, stimulated DCs were collected and cocultured with naive CD4 T cells at a density of $5x10^4$ DCs and $5x10^4$ lymphocytes (ratio 1:1) per well, in X-VIVO 15 serum free medium (Lonza) in 96-well round-bottom plates (Falcon) coated with anti-CD3 (BD Biosciences) (10μg/ml). Naïve CD4 T cells stimulated with Dynabeads CD3/CD28 T cell expander (1 bead per cell) (Life Technologies) were used as control. After 5 days, cells were harvested, washed, and viability was determined by Trypan Blue exclusion. Then, cells were resuspended in X-VIVO 15 serum free medium (Lonza) at the concentration of 1×10^6 cells/ml and restimulated with anti-CD3 for 24h in 96-well flat-bottom plates (Falcon). The conditions of incubation were stable (temperature 37°C with 5% of CO_2).

Flow cytometry analysis

pDCs and cDCs were harvested after 48h of culture and resuspended in an EDTA-containing medium, then stained for 15 minutes at 4°C with the following antibodies: anti-human CD4 PC7 (Beckman Coulter), anti-human PD-L1 PE (Biolegend), anti-human CD86 APC (Miltenyi Biotec) and anti-human CD80 BV650 (BD Bioscience). Samples were washed in EDTA-containing medium, acquired using CytoFLEX cytometer (Beckman Coulter) and analyzed by FlowJo-10 software (version 10.3.0).

Analysis of cytokine production

Cytokines were measured in supernatants from pDC and cDC cultures, respectively, using IFN-α ELISA (Invitrogen, Human IFN alpha Antibody Pair Kit), IL-6 ELISA

(R&D Systems, Human IL-6), IL-17 ELISA (R&D Systems, Human IL-17), IL-4 ELISA (R&D Systems, Human IL-4), IFN-γ ELISA (R&D Systems, Human IFN-γ), and IL-10 ELISA (R&D Systems, Human IL-10) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using one-way ANOVA, two-way ANOVA, or Student's t test, depending on the number of experimental conditions and independent variables. We used GraphPad Prism software (version 6.01, GraphPad Software). Data were presented as the mean ± standard error (SEM). The p values (p) of 0.05 or less were considered statistically significant.

2.5 Supplementary materials

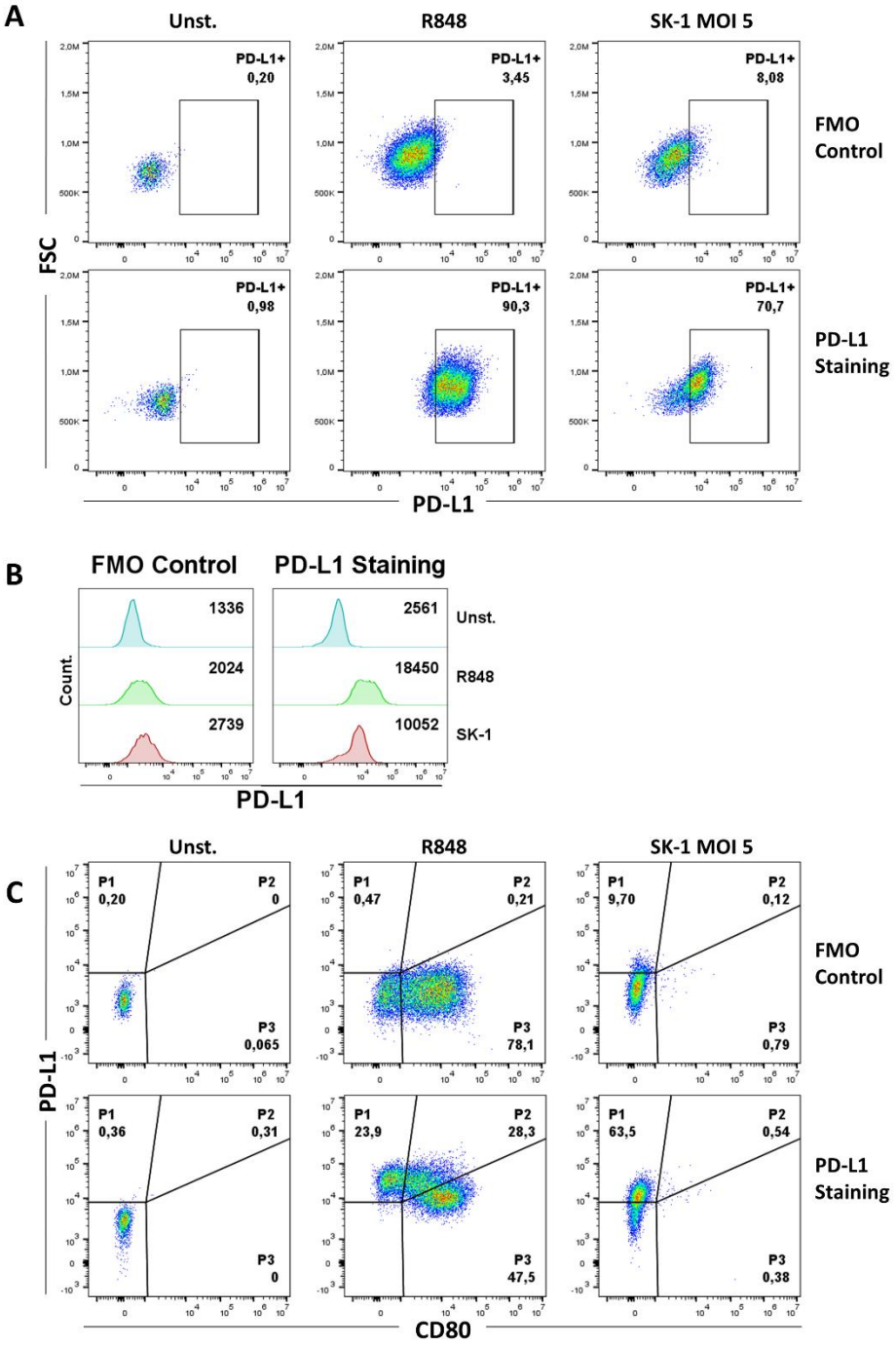

Figure S1. Gating strategy for the purification of blood DCs
Human PBMCs purified from peripheral blood of healthy donors were enriched using the Human Pan-DC Pre-Enrichment Kit, then labelled with specific antibodies conjugated with a fluorochrome. Using a cell sorter, the pDCs were isolated as Lineage (CD3, CD14, CD16, CD19, CD56, CD235α)$^-$ CD4$^+$, CD11c$^-$, while cDCs were isolated as Lineage (CD3, CD14, CD16, CD19, CD56, CD235α)$^-$ CD4$^+$, CD11c$^+$ (A). The purity of the isolated pDCs and cDCs, evaluated by flow cytometry after purification, is more than 94% (B).

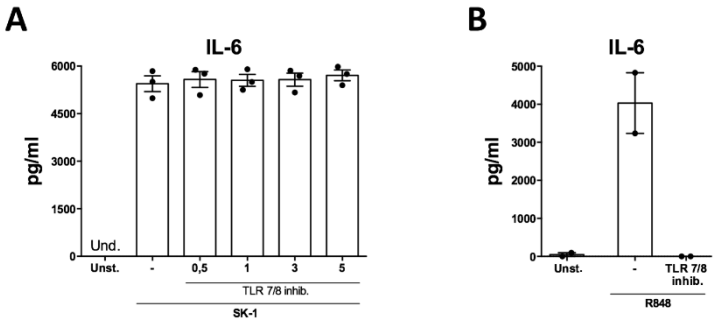

Figure S2. Human blood pDCs and cDCs used in this study are CD123$^+$ and HLA-DR$^+$ cells, respectively

Human PBMCs purified from peripheral blood of healthy donors were labelled with specific antibodies conjugated with a fluorochrome. PDCs were identified as Lineage (CD3, CD14, CD16, CD19, CD56, CD235α)$^-$ CD4$^+$, CD11c$^-$, while cDCs as Lineage (CD3, CD14, CD16, CD19, CD56, CD235α)$^-$ CD4$^+$, CD11c$^+$. Staining of CD123 and HLA-DR before (A) and after sorting (B) reveals that pDCs are CD123$^+$ cells, while cDCs are HLA-DR$^+$ cells. The plots show the data of a representative of more experiments.

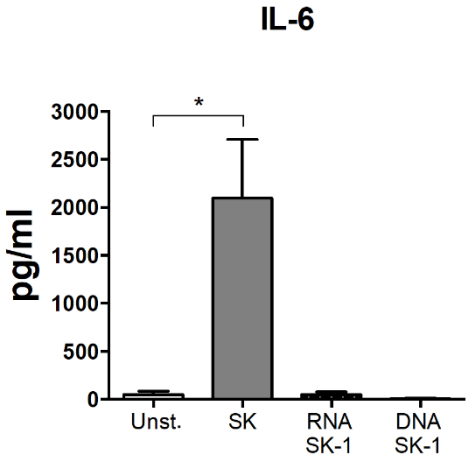

Figure S3. Fluorescence-minus-one (FMO) control for PD-L1 staining

Human pDCs purified from peripheral blood of healthy donors were cultured for 48h without stimulation (Unst.), with R848 (1μg/ml), or with laboratory strain of Saccharomyces cerevisiae SK-1 at MOI 5 (CFU SK-1/pDC). Cells were stained with anti-CD80 BV650 (FMO control) or anti-PD-L1 PE and anti-CD80 BV650 (PDL-1 staining). Percentage of PD-L1$^+$ cells (A), MFI of PD-L1 (B), and percentage of P1, P2, P3 pDC subpopulations (C) were represented. Plots and histograms are from one representative of more experiments

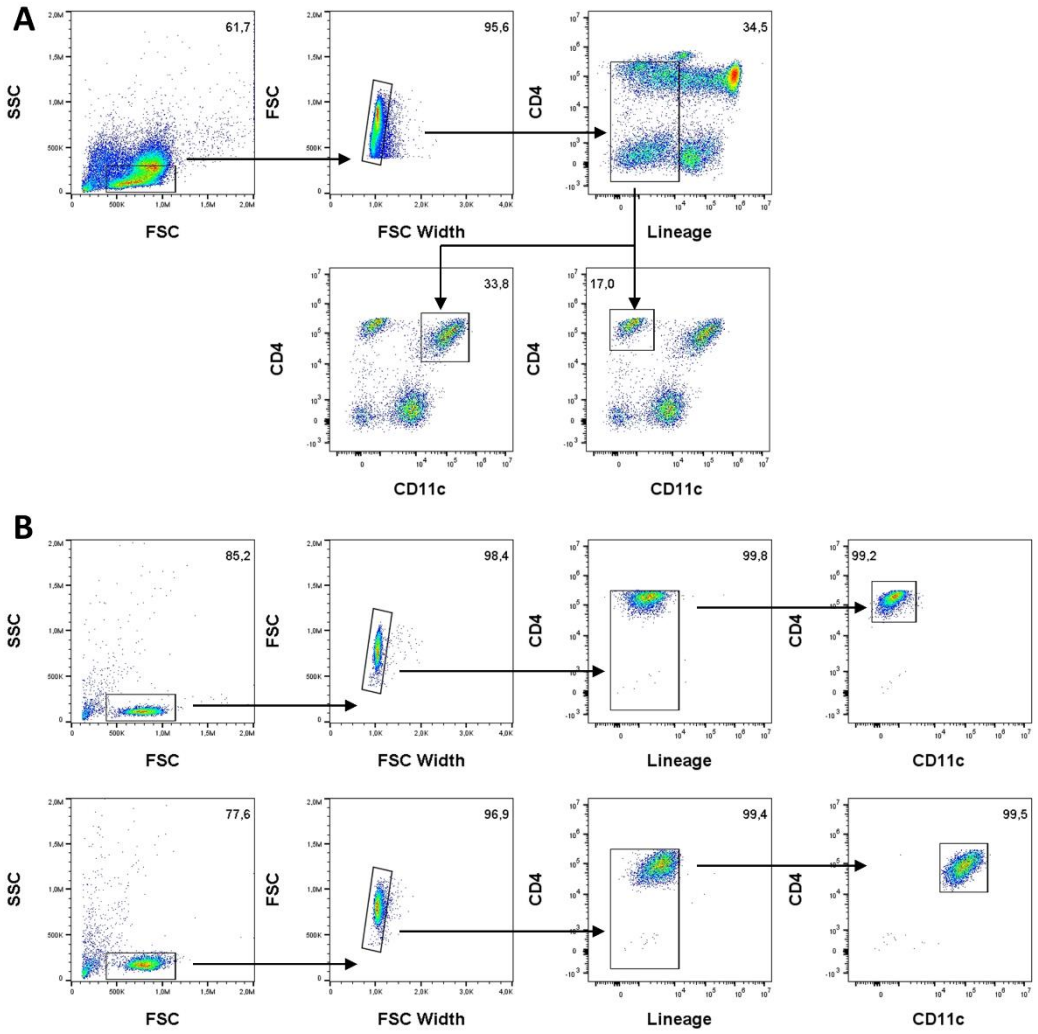

Figure S4. Human cDC activation by SK-1 is not affected by TLR 8

*Human cDCs purified from peripheral blood of healthy donors were cultured for 48h without stimulation (Unst.), pre-treated for 30 minutes with different doses of TLR inhibitor, as indicated, and with the laboratory strain of Saccharomyces cerevisiae SK-1 at MOI (SK-1/pDCs) 5 (A), or with 5□g/ml TLR8 inhibitor and R848 (0,1µg/ml) (B). Levels of IL-6 were measured in the culture supernatants by ELISA assay. Graphs show mean ± SEM of 3 independent experiments, each from different donors. Two-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05).*

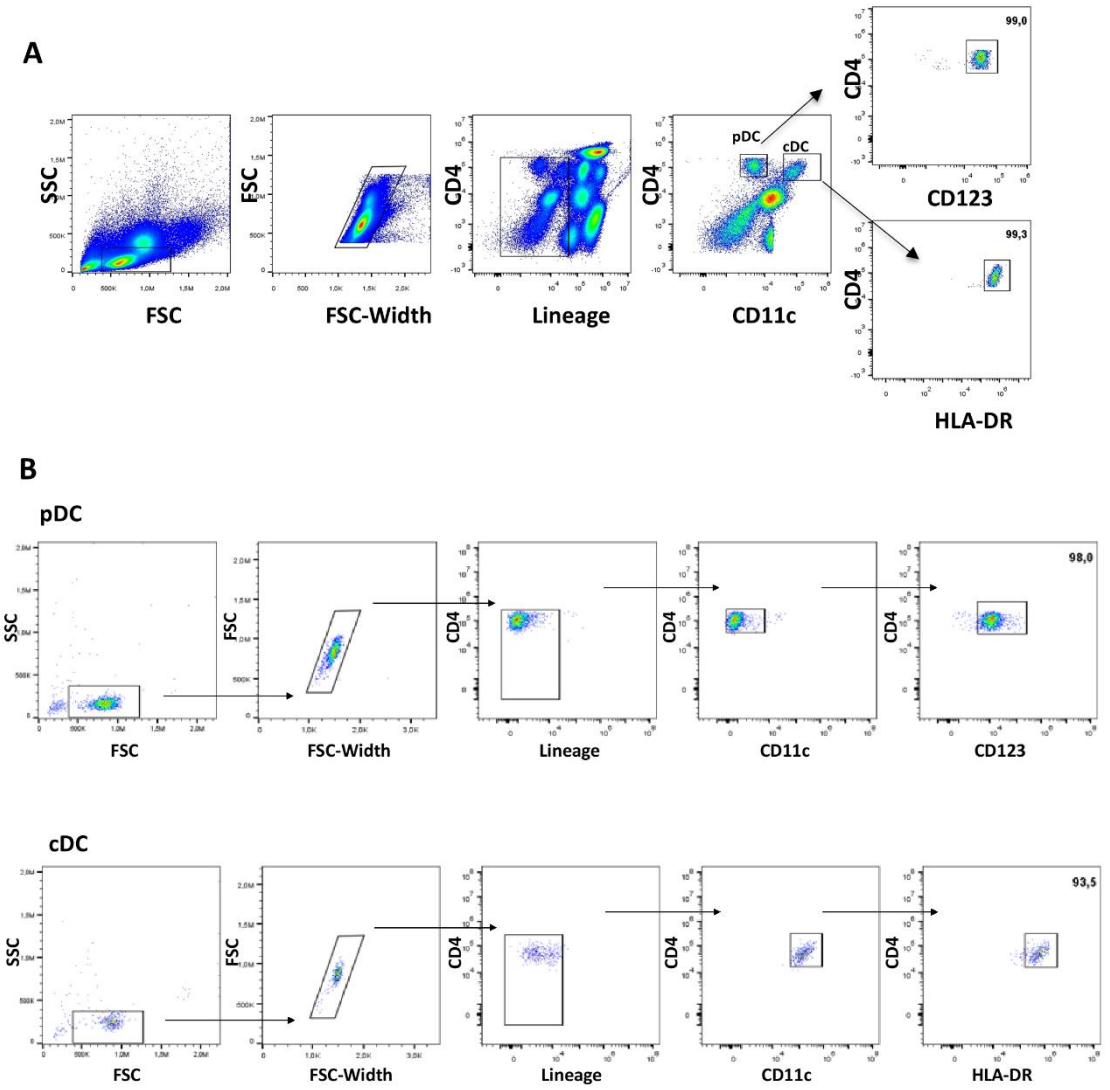

Figure S5. Nucleic acids from S. cerevisiae SK-1 does not induce production of IL-6 by cDCs *Human cDCs purified from peripheral blood of healthy donors were cultured for 48h with RNA or DNA (0,2µg) extracted from Saccharomyces cerevisiae SK-1 and pre-treated with Dotap (10µl/µg of nucleic acids) for 30 minutes at 37°C, without stimulation (Unst.), with the laboratory strain of Saccharomyces cerevisiae SK-1 at MOI 5 (SK-1/pDC). Levels of IL-6 were measured in the culture supernatants by ELISA assay. Graphs show mean ± SEM of three independent experiments, each from different donors. One-way ANOVA was used to compare different experimental conditions (*p-value ≤ 0.05).*

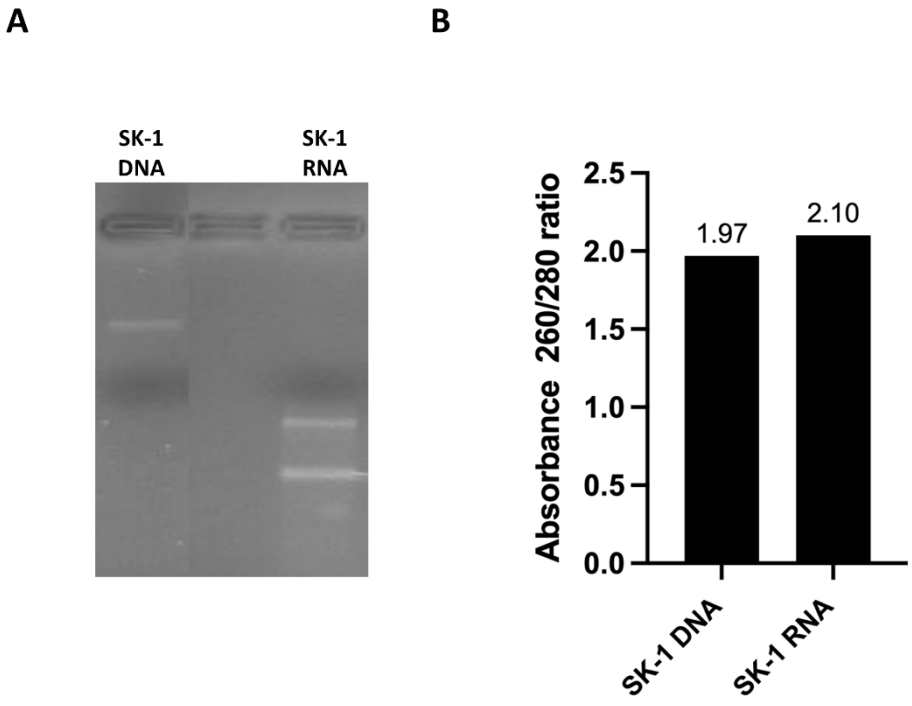

Figure S6. Quality and purity of DNA and RNA extracted from SK-1
The integrity of DNA and RNA samples isolated from SK-1 was analyzed by 1% agarose gel electrophoresis (A). Measurements of absorbance 260/280 ratio of DNA and RNA samples were performed by Nanodrop spectrophotometer (B).

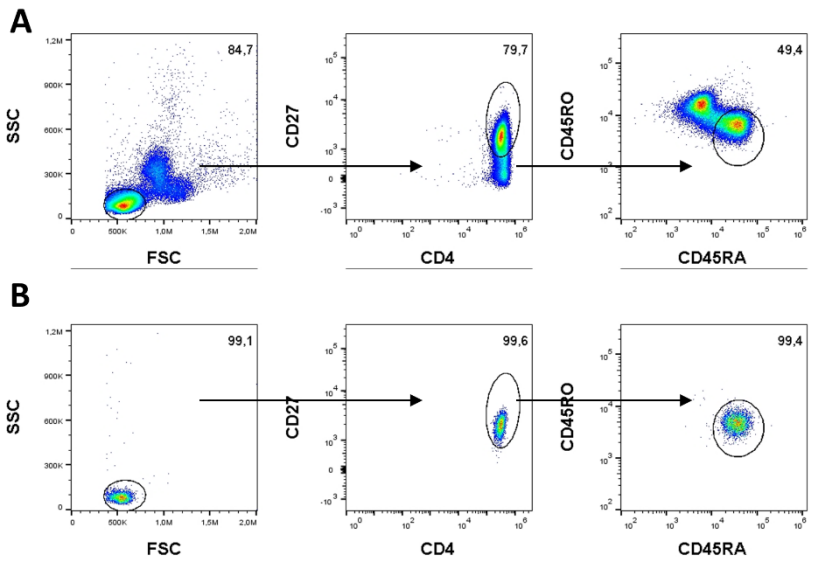

Figure S7. Gating strategy for the purification of naïve CD4 T cells
Human PBMCs purified from peripheral blood of healthy donors were labelled with specific antibodies conjugated with a fluorochrome and, by using a cell sorter, naïve CD4 T cells were isolated as $CD4^{high}$, $CD45RA^{high}$, $CD45RO^{-}$ and $CD27^{+}$ (A). The purity of the isolated naïve CD4 T cells, evaluated by flow cytometry after purification, is more than 97% (B). The plots show the data of a representative of all experiments performed with DCs or naïve CD4 T cells.

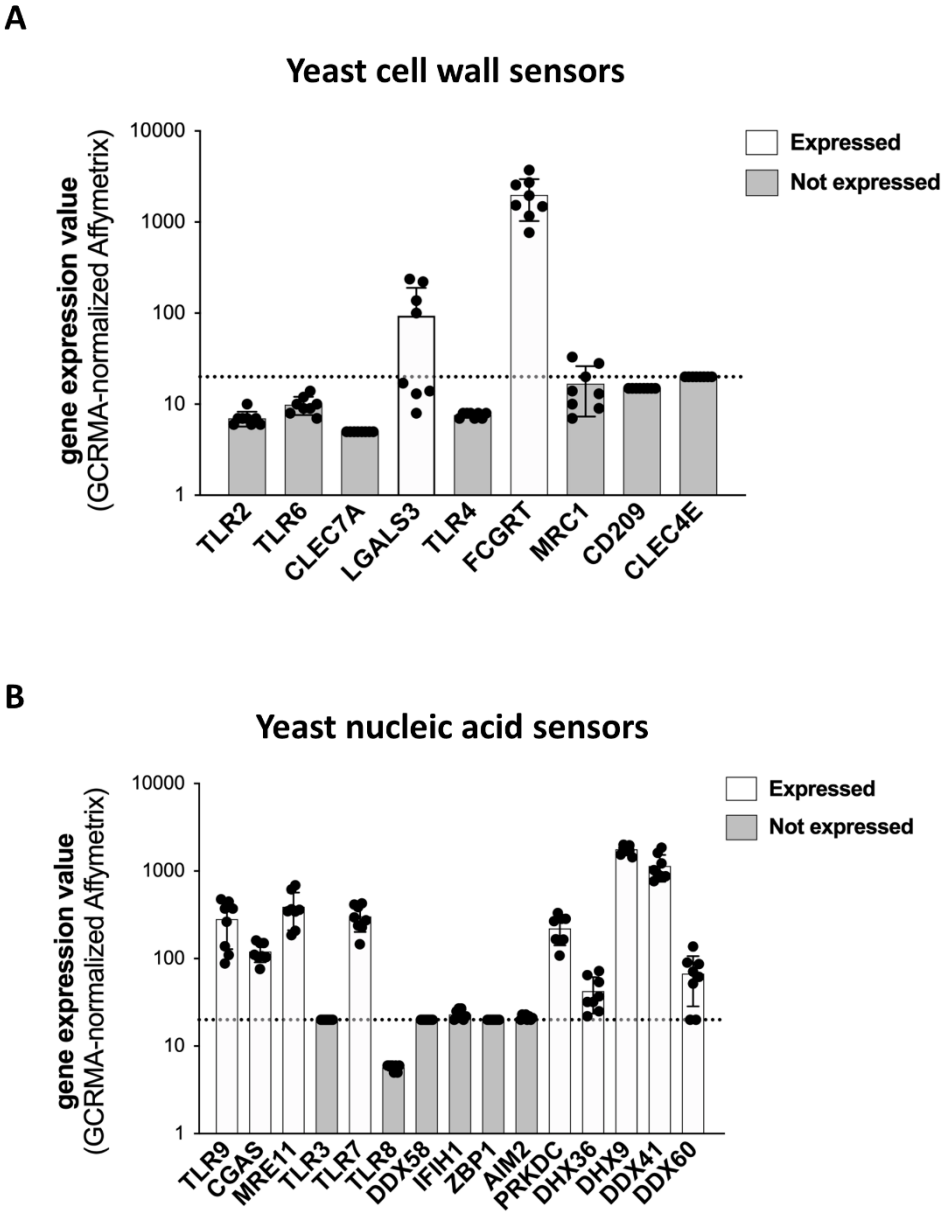

Figure S8. Expression of yeast receptor in human pDCs

Expression values of yeast pattern recognition receptors, cell wall (A) and nucleic acid sensors (B), on pDCs freshly isolated from the blood of healthy donors were extracted from Affymetrix data (Human Genome U133 Plus 2.0 arrays) (48). The dashed line is the threshold of signal detection. White bars represent the mRNA levels of the receptors expressed on pDCs; gray bars, the receptors not detectable. Data are the mean of seven independent experiments each from different donors. Error bars represent standard deviation (SD).

3. DIFFERENTIAL IMMUNE RESPONSE OF HUMAN MONOCYTES, MACROPHAGES, AND DENDRITIC CELLS TO *ACINETOBACTER BAUMANNII*

Results of Section 3 address the Aim 2, and have been included in:

Differential immune response of human monocytes, macrophages, and dendritic cells to *Acinetobacter baumannii*

Andrea Sabatini[*], Massimiliano Lucidi[*], Serena Ciolfi, Claudia Vuotto, Luca Battistini, Paolo Visca, Daniela Visaggio[#], Elisabetta Volpe[#] *([*,#] equal contribution) (manuscript in preparation).*

3.1 Introduction

A. baumannii is Gram-negative bacterium and important opportunistic pathogen, associated with nosocomial infections such as bacteraemia, pneumonia, urinary tract infections, wound infections and meningitis (Antunes, Visca, and Towner, 2014). Moreover, *A. baumannii* rapidly developed multidrug resistance (Kanafani, Zahreddine, Tayyar, Sfeir, Araj, Matar, et al., 2018), thus becoming one of the most important human pathogen in the hospital environment.

The pathogenicity of *A. baumannii* is amplified in immunocompromised individuals (Munier, Biard, Legrand, Rousseau, Lafaurie, Donay, et al., 2019), indicating that host immune response plays a crucial role in the defense against this pathogen.

It is known that *A. baumannii* is recognized by the host immune system at a very early stage of infection (within hours), which largely determine the host fate of the infection (Bruhn, Pantapalangkoor, Nielsen, Tan, Junus, Hujer, et al., 2015). Neutrophils are essential for the control of *A. baumannii* infection, reaching a rapid recruitment at the site of infection (already in the first hours), with a peak after 24 hours post infection. The involvement of these cells is associated with the increase of cytokines and chemokines, such as TNF-α, MIP-1, MIP-2, (van Faassen, KuoLee, Harris, Zhao, Conlan, and Chen, 2007) as well as bactericidal mechanisms including the oxidative burst (Sato, Unno, Miyazaki, Ubagai, and Ono, 2019; Qiu, Kuolee, Harris, and Chen, 2009). However, other innate immune cells are activated in response to *A.baumanii*, such as macrophages. Similarly to neutrophils, macrophages phagocytose bacteria in the early stages of infection, produce nitric oxide (NO) and reactive oxygen species (ROS), and produce MIP-2, IL-6, TNF-α, IL-10 and IL-1β (Qiu, KuoLee, Harris, Van Rooijen, Patel, and Chen, 2012). Dendritic cells are activated by *A. baumannii* and the activation leads to high expression of molecules involved in antigen presentation and production of the inflammatory cytokine IL-12, with consequent polarization of T helper (Th)1 effector cells (Lee, Choi, Kim, and Lee, 2010; Kim, Jeong, Lee, Jeon, Park, Kang, et al., 2013). Importantly, previous studies analysing the innate immune response to *A. baumannii* used mouse model of infection, human and murine macrophage cell lines, or

murine dendritic cells. Here, for the first time we analysed human blood dendritic cells, monocytes and monocyte-derived macrophages in response to *A. baumannii* (Moffatt, Harper, Harrison, Hale, Vinogradov, Seemann, et al., 2010). Moreover, we analysed the human innate immune response to the epidemic, multidrug-resistant *Acinetobacter baumannii* strain ACICU (Iacono, Villa, Fortini, Bordoni, Imperi, Bonnal, et al., 2008).

3.2 Results

Human monocytes and macrophages interact with *Acinetobacter baumannii* leading to a differential killing and activation.

In order to study the interaction between human monocyte/macrophages and *Acinetobacter baumannii* strains, we performed experiments using immune cells isolated from blood of healthy donors and two different strains of *A. baumannii*, called ATCC 19606^T and ACICU. We incubated for 1hour (h) bacteria and immune cells using multiplicity of infection (MOI) of 100 (colony-forming unit microbe/monocyte). Previous confocal microscopy experiments revealed that MOI 100 and 1h of stimulation was neither lethal for bacteria nor for immune cells (data not shown), indicating that this experimental setting was optimal for the visualization and the study of immune cell-microbe interaction. Then, we performed a first qualitative analysis, using the scanning electron microscopy (SEM) showing the interaction between bacteria and immune cells. We observed an interaction between bacteria from the ACICU and ATCC 19606^T strain and monocytes (Figure 1A), and bacteria from ACICU strain and macrophages (Figure 2A), while we did not observe neither contact nor extracellular bacteria in macrophages infected with ATCC 19606^T (Figure 2A), suggesting that in this condition bacteria are mainly internalized by immune cells.

The second qualitative analysis was performed by confocal fluorescence microscopy, using GFP-bacteria, concanavalin A-TRITC, as surface marker of immune cells, and DAPI, staining nuclei of immune cells.

This analysis confirmed that both bacterial strains (green) interact with monocytes (red and cyan), especially the ATCC 19606^T, where we observed the yellow staining as merge of TRITC and GFP stains. Although the antibiotic-resistant strain seems to interact less with monocytes, compared to the ATCC 19606^T strain, we did not find a significant differential interaction (Figure 1B). The analysis of macrophages indicates that *A. baumannii* are mainly internalized (Figure 2B). Importantly, we observed clusters of bacteria in macrophages infected with ACICU (Figure 2B), that could be bacterial biofilm at the surface of macrophages (Schulze, Mitterer, Pombo, and Schild, 2021)

In order to investigate the effect of the interaction between immune cells and bacteria, we evaluated the percentage of viable bacteria associated to cells (phagocytosed or bound to cell wall) and percentage of bacteria remained in the extracellular compartment upon 1h of infection. Moreover, the percentage of killed bacteria was calculated by subtracting all viable bacteria (associated to immune cells and in the extracellular compartment) from bacteria used for the infection. This analysis revealed that

monocytes kill ATCC 19606^T and partially ACICU (Figure 1C), while macrophages kill most part of bacteria, either ATCC 19606^T than ACICU (Figure 2C).

Then, we analysed the effect of the infection on immune cell side and we found that monocytes produce IL-6 and IL-10 in response to infection (Figure 1D), and increase expression of co-activation molecules, such as CD14, CD80 and PD-L1 (Figure 1E, F) after 24h of infection with heat-killed (HK) ATCC 19606T and ACICU strains at MOI 100. In contrast, macrophages upon infection do not produce significant levels of cytokines, and do not increase significantly expression of activation molecules typical of macrophages, such as CX3CR1, MR1, CD11b, CD68, CD163 (Figure 2E,F).

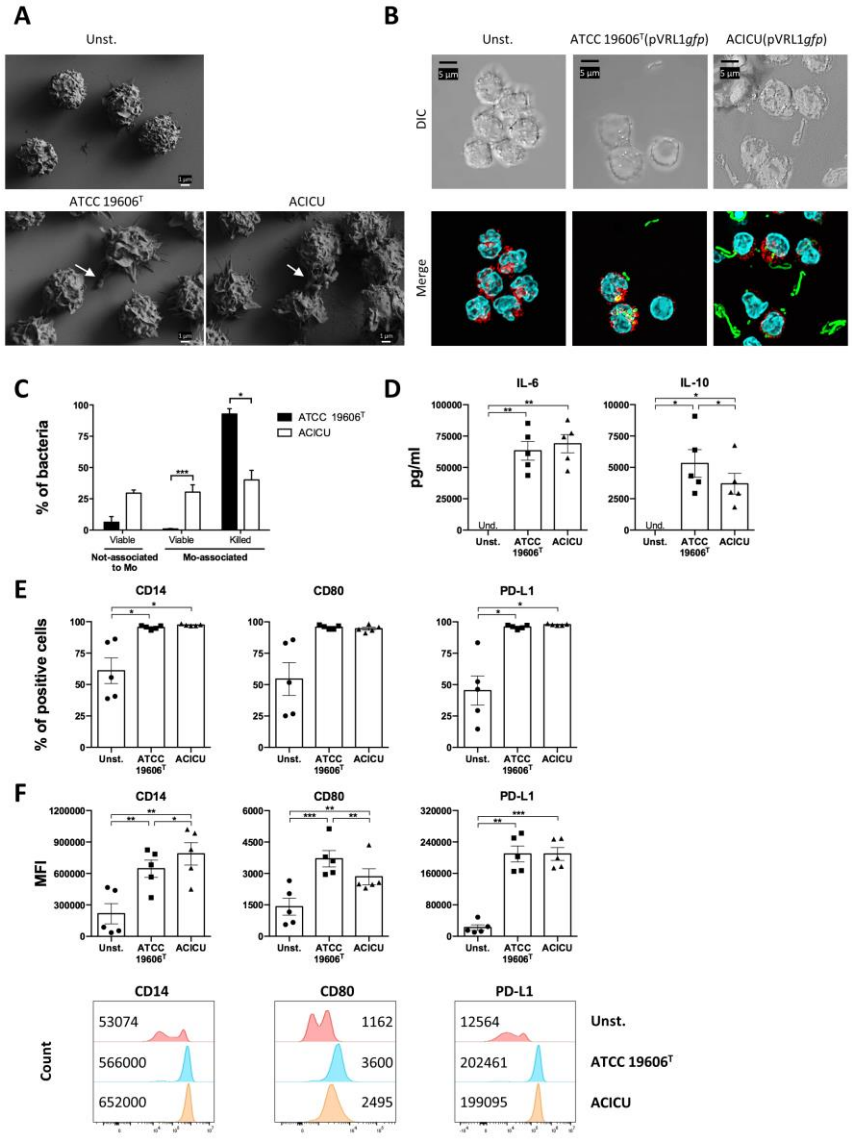

Figure 1. Interaction between human monocytes and Acinetobacter baumannii leads to monocyte activation and bacterial killing

Human monocytes purified from peripheral blood of healthy donors were cultured for 1h without stimulation (Unst.), or with the strain of Acinetobacter baumannii ATCC 19606T or ACICU at MOI 100 (bacterial CFU/monocyte). Pictures of cells after 1h of infection were acquired by scanning electron microscopy, and arrows indicate microbes (A), and by confocal fluorescence microscopy (DAPI: cyan; ConA: red; Bacterial pVRL1gfp: green) (B). Pictures are representative of three independent experiments.

The percentage of viable bacteria obtained by lysed host cells (associated to monocytes) or in the culture supernatants (not-associated) after 1h of infection were determined by CFU assay, and compared to CFU derived from bacteria used for the inoculum to obtain the percentage of killed bacteria (C). Data are the mean of 3 independent experiments, each from different donors. Error bars represent standard error of the mean (SEM).

Human monocytes were infected for 24h with heat-killed bacteria at MOI 100, levels of IL-6 and IL-10 were measured in the culture supernatants by ELISA assays (D); expression of molecules CD14, CD80, PD-L1 analysed by flow cytometry was reported as percentage of positive cells (E) and median fluorescence intensity (MFI) (F). Data are the mean of 5 independent experiments, each from different donors. Error bars represent standard error of the mean (SEM). One-way ANOVA was used to compare different experimental conditions (p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001).*

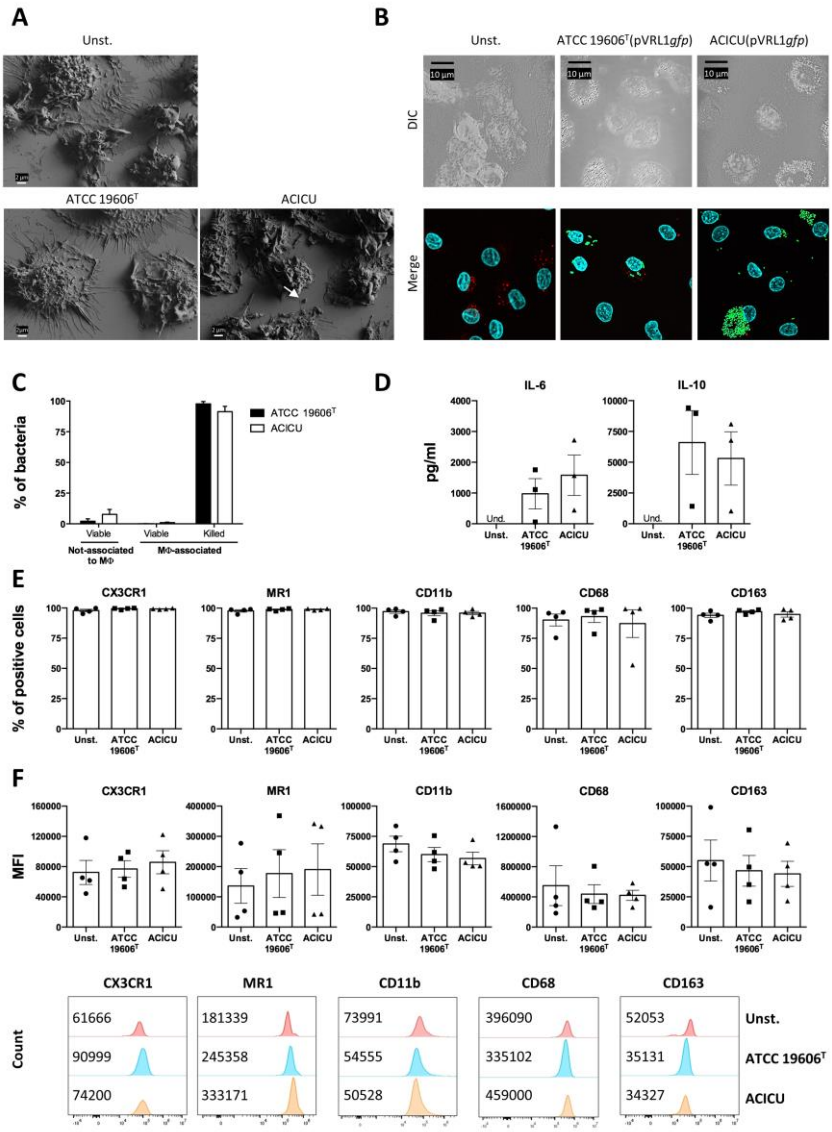

Figure 2. Interaction between human macrophages and Acinetobacter baumannii leads to bacterial killing

Human monocyte-derived macrophages purified from peripheral blood of healthy donors were cultured for 1h without stimulation (Unst.), or with the strain of Acinetobacter baumannii ATCC 19606T or ACICU at MOI 100 (bacterial CFU/macrophage). Pictures of cells after 1h of infection were acquired by scanning electron microscopy, and arrows indicate microbes (A), and by confocal fluorescence microscopy (DAPI: cyan; ConA: red; Bacterial pVRL1gfp: green) (B). Pictures are representative of three independent experiments.

The percentage of viable bacteria obtained by lysed host cells (associated to macrophages) or in the culture supernatants (not-associated) after 1h of infection were determined by CFU assay, and compared to CFU derived from bacteria used for the inoculum to obtain the percentage of killed bacteria (C). Data are the mean of 3 independent experiments, each from different donors. Error bars represent standard error of the mean (SEM).

Human macrophages were infected for 24h with heat-killed bacteria at MOI 100, levels of IL-6 and IL-10 were measured in the culture supernatants by ELISA assays (D); expression of molecules CXCR1, MR1, CD11b, CD68, CD163 analysed by flow cytometry was reported as percentage of positive cells (E) and median fluorescence intensity (MFI) (F). Data are the mean of 4 independent experiments, each from different donors. Error bars represent standard error of the mean (SEM). One-way ANOVA was used to compare different experimental conditions.

Acinetobacter baumannii **induces activation of human dendritic cells**

To further insight in innate immunity activation, we investigated the interaction between *A. baumannii* and two human blood DC subsets, cDCs and pDCs. Human cDCs and pDCs derived from healthy donors were stimulated with ATCC 19606^T and ACICU strains at MOI 100.

Bacterial counts after infection revealed that DCs are not able to kill any bacterial strains (Figure 3A; 4A). Moreover, we found that bacteria either associate with immune cells (almost 50%) or remain in the extracellular compartment (almost 50%) (Figure 3A; 4A). However, both strains of *A. baumannii* activate DCs. In particular, we found increased levels of IL-6 and IL-10 produced by cDCs, IFN-α produced by pDCs and increased level of expression of PD-L1, CD86, CD80, and HLA-DR by both DC subsets, after 24h infection with heat-killed (HK) ATCC 19606^T and ACICU strains at MOI 100 (Figure 3B,C,D; Figure 4B,C,D,E).

In addition, we evaluated the formation of cell clusters, which reflects pDC activation and pDC viability by optical microscopy, and we found that both bacterial strains leads to cell clusters formation (Figure 4B).

Levels of IL-10 induced by ACICU in monocytes (Figure 1D) and cDCs (Figure 3B) compared to those induced by ATCC 19606^T were significantly lower, suggesting an high level of inflammation in presence of the multidrug-resistant strain. Consistently, in pDCs the levels of IFN-α and PD-L1, molecules associated to regulatory functions, were lower in ACICU- compared to ATCC19606^T-infected cells (Figure 4C,D).

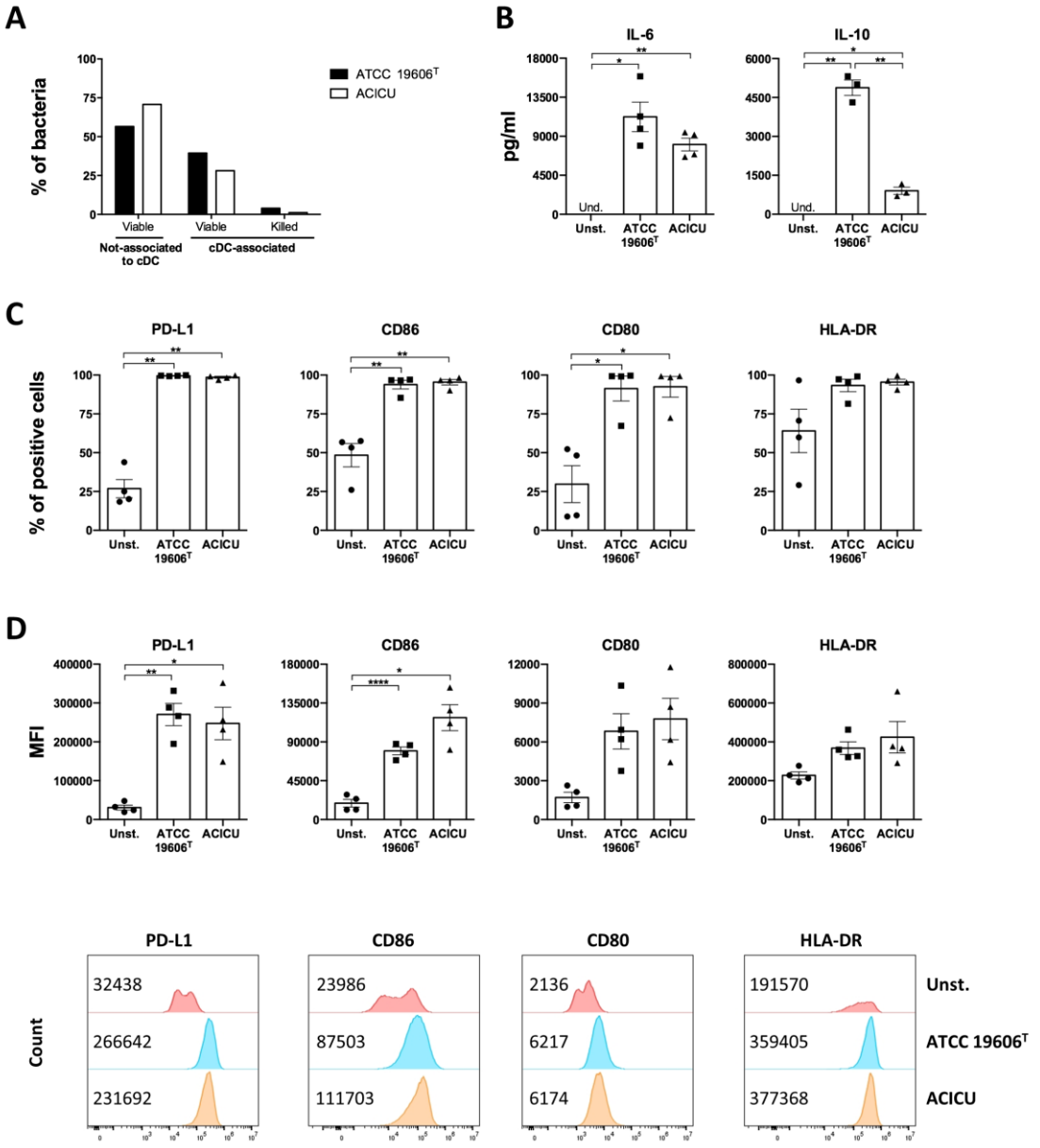

Figure 3. Interaction between human cDCs and Acinetobacter baumannii ATCC leads to DC activation

Human cDCs purified from peripheral blood of healthy donors were cultured for 1h without stimulation (Unst.), or with the strain of Acinetobacter baumannii ATCC 19606T or ACICU at MOI 100 (bacterial CFU/cDC). The percentage of viable bacteria obtained by lysed host cells (associated to macrophages) or in the culture supernatants (not-associated) after 1h of infection were determined by CFU assay, and compared to CFU derived from bacteria used for the inoculum to obtain the percentage of killed bacteria (A).

Human cDCs were infected for 24h with heat-killed bacteria at MOI 100, levels of IL-6 and IL-10 were measured in the culture supernatants by ELISA assays (B); expression of molecules PD-L1, CD86, CD80, HLA-DR analysed by flow cytometry was reported as percentage of positive cells (C) and median fluorescence intensity (MFI) (D). Data are the mean of 4 independent experiments, each from different donors. Error bars represent SEM. One-way ANOVA was used to compare different experimental conditions (p-value ≤ 0.05; ** p-value ≤ 0.01; **** p-value ≤ 0.0001).*

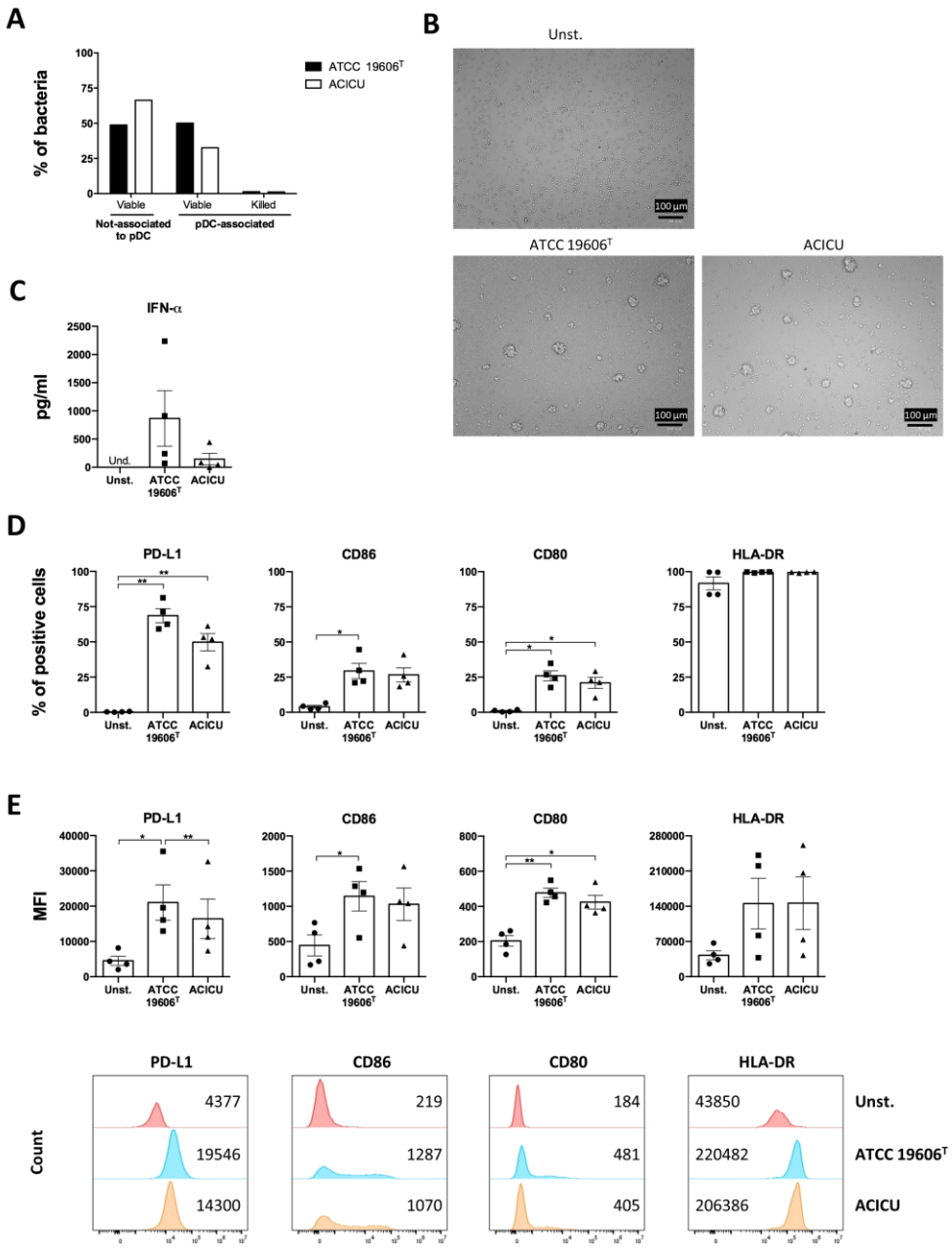

Figure 4. Interaction between human pDCs and Acinetobacter baumannii leads to DC activation

Human pDCs purified from peripheral blood of healthy donors were cultured for 1h without stimulation (Unst.), or with the strain of Acinetobacter baumannii ATCC 19606T or ACICU at MOI 100 (bacterial CFU/pDC). The percentage of viable bacteria obtained by lysed host cells (associated to macrophages) or in the culture supernatants (not-associated) after 1h of infection were determined by CFU assay, and compared to CFU derived from bacteria used for the inoculum to obtain the percentage of killed bacteria (A).

Human pDCs were infected for 24h with heat-killed bacteria at MOI 100. Pictures by optical microscopy showing cell clusters (B); levels of IFN-α measured in the culture supernatants by ELISA assays (C); expression of molecules PD-L1, CD86, CD80, HLA-DR analysed by flow cytometry was reported as percentage of positive cells (D) and median fluorescence intensity (MFI) (E). Data are the mean of 4 independent experiments, each from different donors. Error bars represent SEM. One-way ANOVA was used to compare different experimental conditions (p-value ≤ 0.05; ** p-value ≤ 0.01).*

3.3 Discussion

Previous studies on *A. baumannii* infections focused on the molecular epidemiology of the infections and the mechanisms of antibiotic resistance (Lee, Lee, Park, Park, Bae, Kim, et al., 2017; Morris, Dexter, Kostoulias, Uddin, and Peleg, 2019). Relatively limited data are available on the host innate immune response to *A. baumannii*. It is clear that recruitment and activation of innate immune cells to the site of infection is a hallmark of host defense against *A. baumannii* infection (Chen, 2020). *A. baumannii* interacts with innate immune cells mainly by engaging TLR2 and TLR4, with CD14 as co-receptor (Wong, Nielsen, Bonomo, Pantapalangkoor, Luna, and Spellberg, 2017; Morris, Dexter, Kostoulias, Uddin, and Peleg, 2019; Garcia-Patino, Garcia-Contreras, and Licona-Limon, 2017; Peng, Han, Ye, and Zhang, 2018). However, it has been reported that intracellular receptors, such as NOD2, NOD1, RIP2, and TLR9-are important for host defense against *A. baumannii* (Choi, Hyun, Lee, Lee, Lee, Kim, et al., 2008; Bist, Dikshit, Koh, Mortellaro, Tan, and Sukumaran, 2014). Monocytes, macrophages and dendritic cells express those pathogen pattern recognition receptors. Our current knowledge on the interaction between innate immune cells and *A. baumannii* is largely derived from *in vitro* studies in different macrophage-like cell lines, murine bone-marrow-derived macrophages and dendritic cells, and human monocytes-derived macrophages. Those *in vitro* studies consistently showed that both human and murine macrophages produced large amounts of pro-inflammatory cytokines and chemokines in response to *A. baumannii* infection (Bruhn, Pantapalangkoor, Nielsen, Tan, Junus, Hujer, et al., 2015; Sato, Unno, Miyazaki, Ubagai, and Ono, 2019; Qiu, KuoLee, Harris, Van Rooijen, Patel, and Chen, 2012; Kim, Jeong, Lee, Jeon, Park, Kang, et al., 2013; Bist, Dikshit, Koh, Mortellaro, Tan, and Sukumaran, 2014; Lee, Aslanyan, Vidyasagar, Brennan, Tauber, Carrillo-Sepulveda, et al., 2020).

We confirmed that human macrophages are the innate immune cells most effective in the clearance of *A. baumannii*. In fact, we found that human macrophages are able to completely kill either ATCC 19606^{T}, used as model strain of *A. baumannii* infection, than ACICU, the multidrug-resistant strain. In this context, we found that human macrophages do not produce large amount of pro-inflammatory cytokines, and do not increase expression of activation molecules, indicating that the immediate bacterial clearance mediated by macrophages prevents further host cell activation. Consistently, the infection of monocytes and dendritic cells, that are less effective in killing *A. baumannii*, leads to a strong activation and production of cytokines. In particular, monocytes and cDCs produce IL-6 and IL-10, while pDCs produce IFN-α. Moreover, monocytes increase expression of CD14, CD80, and PD-L1, while pDCs and cDCs increase expression of PD-L1, CD80, CD86, HLA-DR.

CD14 is co-receptor of TLR4 for the detection of bacterial lipopolysaccharide, thus its increase could amplify the recognition of *A. baumannii* by host cells. Importantly, CD14 is further increased by ACICU, compared to ATCC 19606^T strain. This result, together with the less efficacy of monocytes in the clearance of ACICU strain, indicates that viable ACICU persists in association with human monocytes, and that this persistence may lead to the increased expression of CD14 to further sense bacterial components and activate a proper immune response by monocytes. In contrast, CD80 expression and IL-10 production by monocytes is significantly lower in ACICU- compared to ATCC 19606^T -infected cells. Thus, lower expression of CD80 could be a mechanism adopted by ACICU to escape the immune system, by reducing the co-stimulatory molecule CD80, which is required to activate the adaptive immune response. The lower production of the anti-inflammatory cytokine IL-10 with ACICU compared to ATCC 19606^T -infected cells, that we observed in monocytes and especially in cDCs, could be related to a high level of inflammation in presence of the most pathogenic strain of *A. baumannii*. Consistently, we observed a minor increase of other two molecules associated to anti-inflammatory properties, IFN-α and PD-L1, in infected pDCs. Interestingly, the expression of PD-L1 has been already associated to production of IFN-α (Sabatini, Guerrera, Corsetti, Ruocco, De Bardi, Renzi, et al., 2022; Alculumbre, Saint-Andre, Di Domizio, Vargas, Sirven, Bost, et al., 2018) and both molecules mediate induction of the anti-inflammatory T lymphocytes (Alculumbre, Saint-Andre, Di Domizio, Vargas, Sirven, Bost, et al., 2018; Levings, Sangregorio, Galbiati, Squadrone, de Waal Malefyt, and Roncarolo, 2001). Moreover, the cytokines IL-10 and IFN-α that we observed reduced in ACICU compared to ATCC 19606^T -infected cells, are protective in pulmonary infection with *A. baumannii* (Li, Guo, Hu, Du, Guo, Di, et al., 2018; Kang, Jang, Park, Ahn, Lee, Kim, et al., 2020), indicating that low induction of these cytokines by the pathogenic strain ACICU, could represent another mechanism of escape of the immune response that could be pharmacologically targeted to improve immune response against *A. baumannii* infection.

Overall, this study confirmed that among innate immune cells, macrophages are the most effective in clearing pathogenic bacteria. We reported for the first time that human monocytes and dendritic cells sense and respond to *A. baumannii*. In particular, monocytes are able to kill bacteria, especially ATCC 19606^T, while dendritic cells associate with bacteria but they are not able to kill them. The recognition of bacteria by macrophages, monocytes, and cDCs is likely mediated by TLR2 and TLR4, while the recognition of *A. baumannii* by pDCs could be mediated by the intracellular TLR9, that is highly expressed by human pDCs, and binds to microbial DNA (Liu, Kanzler, Soumelis, and Gilliet, 2001).

Comparison between two strains of *A. baumannii* with different pathogenicity allows to identify potential mechanisms of immune escape adopted by bacteria, such as induction of low levels of CD80 on human monocytes, induction of low levels of IL-10 by monocytes and cDCs, and induction of low levels of PD-L1 and IFN-α by pDCs. Thus, these mechanisms could be therapeutically targeted to improve the effective immune response and to inhibit the bacterial immune evasion.

However, further analysis investigating the production of other inflammatory cytokines, such as IL-1β and IL12 (Lee, Lee, Lee, Jung, Jeong, Seong, Chung, and Park, 2007; Kang, Jo, Kim, and Park, 2017) could be important to better characterize the innate immune response to *A.baumannii*, and to potentially discover new mechanisms of immune escape exerted by the antibiotic-resistant strain.

Moreover, future studies on innate immune cells from infected patients supporting these data could help translation of these knowledge into clinical applications.

3.4 Material and methods

Purification of monocytes from blood and *in vitro* differentiation of macrophages

Peripheral blood mononuclear cells (PBMC) were purified from buffy coats of healthy adult (range 20-65 years of age) volunteer blood donors (independently of sex) by density gradient over Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Approval by the ethics committee of the Fondazione Santa Lucia and written informed consent in accordance with the Declaration of Helsinki from all participants were obtained before study initiation.

After isolation, cells were stained with the anti-CD14 APC (Miltenyi Biotec) and monocytes were purified by immunomagnetic positive selection using Anti-Mouse IgG MicroBeads (Miltenyi Biotec). Purified monocytes $CD14^+$ showed purity > 96%, as revealed by flow cytometry analysis (data not shown). Monocytes were cultured at a density of $1x10^6$ cells/ml in 24-well plates (Falcon) in RPMI 1640 with 10% of Fetal Bovine Serum (FBS) 1% of Hepes (Lonza), and induced to differentiate toward macrophages for 3 days in presence of M-CSF 50 ng/ml. At day 3 and 6, half of the medium was changed with fresh medium and M-CSF 25 ng/ml, as previously described (Donninelli, Saraf-Sinik, Mazziotti, Capone, Grasso, Battistini, et al., 2020).

Purification of DC subpopulations (cDC and pDC) from adult blood

PBMC ($400x10^6$) were enriched using the Human Pan-DC Pre-Enrichment Kit (Stemcell Technologies), specific for the purification of all dendritic cell (DC) type, by negative selection, as previously described (Sabatini, Guerrera, Corsetti, Ruocco, De Bardi, Renzi, et al., 2022). Briefly, after isolation, cells were stained with the anti-CD11c PEF594 (BD Biosciences), anti-CD4 PC7 (Sony Biotechnology), anti-CD3 PE (Sony Biotechnology), anti-CD14 PE (Sony Biotechnology), anti-CD16 PE (Miltenyi Biotec), anti-CD56 PE (Sony Biotechnology), anti-CD19 PE (Miltenyi Biotec), anti-CD235α (Sony Biotechnology), and sorted by 6-way sorting MoFlo Astrios (Beckman Coulter) as Lineage $(CD3,CD14,CD56,CD19,CD235\alpha)^-$, $CD4^+$, $CD11c^-$ for the pDC and Lineage $(CD3,CD14,CD56,CD19,CD235\alpha)^-$, $CD4^+$, $CD11c^+$ for the cDC. Sorted cells had a purity of over 95% and 94% respectively for pDCs and cDCs, as revealed by flow cytometry analysis.

Bacterial strains and culture media

Acinetobacter baumannii strains employed in this work are ATCC 19606^T (Artuso, Lucidi, Visaggio, Capecchi, Lugli, Ventura, et al., 2022) and ACICU (Iacono, Villa, Fortini, Bordoni, Imperi, Bonnal, et al., 2008). All bacterial strains were routinely grown in Luria-Bertani broth (LB, Sambrook et al., 1989) or LB supplemented with 1.5% (w/v)

agar (LA) at 37°C. When required, gentamicin (Gm) was added at the following concentrations: 10 μg/ml for *Escherichia coli* DH5α, 100 μg/ml for *A. baumannii* ATCC 19606^T, and 200 μg/ml for *A. baumannii* ACICU.

Before infection *A. baumannii* ATCC 19606^T and ACICU were grown in LB for 18 hours at 37°C in shaking. After incubation, bacterial cells were washed with PBS and diluted to a final concentration corresponding to a multiplicity of infection (MOI) of 100. In some experiments, heat-killed *A. baumannii* cells were prepared by exposure of bacteria to 80°C for 90 minutes, then diluted to a final concentration corresponding to a multiplicity of infection (MOI) of 100.

DNA manipulation.

Plasmid DNA was purified from bacterial cultures grown to stationary phase using the Wizard Plus SV Minipreps DNA Purification System (Promega Corporation), according to the manufacturer's instructions. FastDigest™ restriction enzymes were purchased from Thermo Fisher Scientific. DNA sequencing was performed using an ABI3730 Sequencer (service by Bio-fab Research, Rome, IT).

Plasmid constructs and preparation of competent cells.

The region encompassing the promoter of the *rrnB* gene, and the GFP-encoding gene were obtained by NotI digestion of the minitn7(Gm)P$_{rrnB\ P1}$*gfp*-a vector (Lambertsen, Sternberg, and Molin, 2004). The resulting fragment (*ca.* 2,000 bp) was cloned in the corresponding NotI unique restriction site of the pVRL1 vector (Lucidi, Runci, Rampioni, Frangipani, Leoni, and Visca, 2018), originating the pVRL1*gfp* plasmid. Competent *E. coli* cells were prepared by the rubidium-calcium chloride method and transformed according to the heat shock protocol. Electrocompetent cells of *A. baumannii* were prepared as previously described (Lucidi, Visaggio, Prencipe, Imperi, Rampioni, Cincotti, et al., 2019), and pVRL1*gfp* was introduced in the *A. baumannii* strains ATCC 19606^T and ACICU by electroporation (Lucidi, Visaggio, Prencipe, Imperi, Rampioni, Cincotti, et al., 2019).

Determination of CFUs in the infection assays.

Infection assays were performed as previously described with minor modifications (Volpe, Cappelli, Grassi, Martino, Serafino, Colizzi, et al., 2006). Briefly, *A. baumannii* ATCC 19606^T and ACICU were grown in LB for 18 hours at 37°C in shaking. After incubation, bacterial cells were washed with PBS and diluted to a final concentration corresponding to a MOI of 100. After 1 hour of incubation at 37°C with the indicated immunocytes in RPMI, cell suspensions were gently centrifuged (1,000 *g* x 5 min) and both pellets and supernatants were collected. Viable intracellular bacteria (VIB) were

estimated by lysing the cell pellets with sterile PBS containing 0.1% (v/v) saponin (Sigma, St Louis, MO) and bacteria were serially diluted in PBS before plating on LA. Viable bacteria not associated with the immunocytes (VBNAI) were quantified by serially diluting the collected supernatants in PBS before plating on LA. Bacteria inoculated at the same concentrations in the immunocytes-free RPMI and incubated for 1 hour at 37°C were used as a control. Colony-forming units (CFUs) were determined after 18 hours-incubation at 37°C. The percentages of VIB, VBNAI, and bacteria associated with immunocytes and killed (BAIK) were indirectly calculated as follows:

VIB (%) = $(CFU_{VIB}/CFU_{control}) \times 100$

VBNAI (%) = $(CFU_{VBNAI}/CFU_{control}) \times 100$

BAIK (%) = 100 - VIB (%) - VBNAI (%)

In vitro stimulation of innate immune cells

Monocytes, macrophages, pDCs and cDCs were cultured separately in plates (Corning) at a density of $1x10^6$/ml in RPMI 1640 with 10% of Fetal Bovine Serum (FBS) and 1% of Hepes for 1hour at 37°C without 5% of CO2 or 24 hours at 37°C with 5% of CO2, in absence of stimuli or in presence of *A. baumannii* or heat-killed *A. baumannii* (MOI 100 bacteria/immune cells), respectively. After infection, the cells were washed extensively with phosphate-buffered saline (PBS), to remove all extracellular bacilli.

Immunofluorescence

Monocytes and macrophages stained with Concanavalin A (ConA) 50microg/ml by incubation at room temperature for 30 minutes. Cells were plated in chamber-slides and infected with *A. baumannii* carrying the plasmid containing GFP. Cells were fixed with paraformaldeide/PBS 4% for 30 minutes at 4°C, permeabilized in 0.5% Triton/PBS for 10 minutes at room temperature, then washed. Nuclei were counterstained in DAPI 0.5 µg/ml.

Samples were visualized using a NikonA1+ confocal laser scanning microscope equipped with an Apo TIRF 100× oil immersion objective (NA 1.49). The 405, 488, and 561 nm laser lines were employed for DAPI, GFP, and ConA excitation, respectively. The emission bandwidths at 415–460 nm, 485–520 nm, and 600–720 nm were used for DAPI, GFP, and ConA detection, respectively. The images acquired at a sampling dimension of 512 x 512 pixels were deconvoluted using the NIS-Elements software (Nikon, Amsterdam, The Netherlands), using default parameters.

Scansion electron microscopy

Ultrastructural analysis of monocytes and macrophages infected with *A. baumannii* was performed by Field Emission Scanning Electron Microscopy (FESEM). Cells adhered

on 10-mm -diameter glass coverslips were fixed with 2.5% v/v glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer (pH 7.4) at room temperature for 1 hour, washed in cacodylate buffer and dehydrated through graded ethanols (30, 50, 70, 85, 95, 100%- 10 minutes each). After 100% ethanol, 1:1 ethanol:hexamethyldisilazane (HMDS) was added for 4 minutes at RT followed by a final step of 4 minutes incubation in HMDS. Air dried glass coverslips were glued on SEM pin stub and gold coated (V150R quorum). Finally, cells were examined by FESEM (Sigma-Zeiss).

Flow cytometry analysis

Innate immune cells were harvested after infection and resuspended in an EDTA-containing medium, then stained for 15 minutes at 4°C with the following antibodies: anti-human CD4 PC7 (Sony Biotechnology), anti-human PD-L1 PE (Sony Biotechnology), anti-human CD86 APC (Miltenyi Biotec) and anti-human CD80 BV650 (BD Bioscience), anti-human CD14 PE (Sony Biotechnology), anti-human CX3CR1 FITC (Sony Biotechnology), anti-human CD206 PE (Sony Biotechnology), anti-human HLA-DR BrilliantViolet 785 (BD Bioscience), anti-human CD163 VioBlue (Miltenyi Biotec), anti-human CD11b PerCP-Cy5.5 (Sony Biotechnology), anti-human CD45 PC7 (Beckman Coulter), anti-human CD68 APC (Miltenyi Biotec), anti-human CD14 APC-Vio770 (Miltenyi Biotec), and Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific).

Samples were washed in EDTA-containing medium, acquired using a Cytoflex cytometer (Beckman Coulter) and analyzed using FlowJo-10 software (version 10.3.0).

Analysis of Cytokine Production

IL-6 and IL-10 were measured by ELISA kit (RnD Biosystems), according to manufacturer's instructions. IFN-α was measured by ELISA kit (Invitrogen, Human IFN alpha Antibody Pair Kit) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using one-way ANOVA. We used GraphPad Prism software (version 6.01, GraphPad Software). Data were presented as the mean ± standard error (SEM). The p values (p) of 0.05 or less were considered statistically significant.

4. ORAL ADMINISTRATION OF *LACTOBACILLUS REUTERI* IN AUTISTIC CHILDREN MODULATES INFLAMMATORY PROPERTIES OF FECAL MICROBIOTA AND BLOOD IMMUNE RESPONSE

Results of Section 4 address the Aim 3, and have been included in:

Oral administration of *Lactobacillus reuteri* in autistic children modulates inflammatory properties of fecal microbiota and blood immune response

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4.1 Introduction

Autism spectrum disorder (ASD) refers to a group of neurodevelopmental disorders that affect social interactions, communication, and repetitive behavior. The global prevalence of ASD is estimated to range from 0.1% to 1.8% (Fombonne, 2009; Elsabbagh, Divan, Koh, Kim, Kauchali, Marcin, et al., 2012). As the prevalence of ASD appears to be increasing, and the pharmacological treatments for ASD are missing, the need for a better understanding of the disorder and a way to treat its symptoms is urgent. Although the main problems associated with autism are cognitive and social skills deficits, it has been shown that gastrointestinal symptoms are widely reported in ASD subjects (Wang, Tancredi, and Thomas, 2011). Moreover, gut microbiota disorders in ASD have been reported by numerous studies (Moos, Faller, Harpp, Kanara, Pernokas, Powers, et al., 2016; De Angelis, Francavilla, Piccolo, De Giacomo, and Gobbetti, 2015). The correlation between gastrointestinal disturbs and autistic behavior suggests that gut microbiota contributes to neurodevelopmental dysfunction in ASD, and provides a promising way to tackle this disorder through correcting the gut dysbiosis.

The use of probiotics represents a low-risk potential therapeutic solution to sustain a healthy microbiota or to restore a microbiological balance (Quigley, 2019; Doenyas, 2018). Probiotics are defined as "live micro-organisms that can provide health benefits on the host when administered in adequate amounts" (Abdelazez, Abdelmotaal, Evivie, Melak, Jia, Khoso, et al., 2018; Calcinaro, Dionisi, Marinaro, Candeloro, Bonato, Marzotti, et al., 2005). The most common microbes used as probiotics include lactobacilli and bifidobacteria or some nonpathogenic species from the genera *Saccharomyces* and *Streptococcus* (Plaza-Diaz, Ruiz-Ojeda, Gil-Campos, and Gil, 2019). However, the majority of studies use lactobacilli as probiotic interventions. It has been shown that probiotic supplements improve irritable bowel syndrome (IBS) and gastrointestinal disorder symptoms through manipulation of the gut microbiota (Zhang, Zhang, Zhang, Sun, and Duan, 2022; Mazzawi, 2022). Different studies using animal

models of ASD, as well as clinical studies in children with ASD, examined the use of probiotics as a potential safe therapeutic option.

In this regard, it has been demonstrated that *Lactobacillus reuteri* ATCC PTA 6475 (MM4-1A) is able to improves social behaviour of several ASD mouse models (Buffington, Di Prisco, Auchtung, Ajami, Petrosino, and Costa-Mattioli, 2016; Sgritta, Dooling, Buffington, Momin, Francis, Britton, et al., 2019).

Importantly, our collaborators performed a double-blinded, randomized, placebo-controlled clinical trial, and they demonstrated that microbial strain *Lactobacillus reuteri* ATCC PTA 6475 (MM4-1A) treatment had significant positive effects in both reducing severity of social impairments and improving adaptive social skills in ASD children, as measured by standardized parental questionnaires (submitted manuscript).

Preclinical studies revealed that *L. reuteri* interacts with vagus nerve, which promotes social reward by targeting the oxytocin-dopaminergic reward circuit (Sgritta, Dooling, Buffington, Momin, Francis, Britton, et al., 2019). However, probiotics may also indirectly affect the brain through the modulation of gut microbiota and immune system (Fioramonti, Theodorou, and Bueno, 2003). In fact, the use of probiotics may alter composition of gut microbiota, production of microbial metabolites, and activation of gut resident immune cells (Cristofori, Dargenio, Dargenio, Miniello, Barone, and Francavilla, 2021). Moreover, alteration of gut microbiota influences systemic immunity in humans (Schluter, Peled, Taylor, Markey, Smith, Taur, et al., 2020), indicating that immune cells of the gut associated lymphoid tissues (GALT), such as B and T lymphocytes, innate lymphoid cells, macrophages, and dendritic cells, sense microorganisms residing in the digestive tract (Rescigno, Urbano, Valzasina, Francolini, Rotta, Bonasio, et al., 2001) and migrate to the spleen and draining lymph nodes for the induction of a systemic immune response (Vazquez-Torres, Jones-Carson, Baumler, Falkow, Valdivia, Brown, et al., 1999).

In this context, we contributed to the clinical trial, which evaluated the effect of *Lactobacillus reuteri* ATCC PTA 6475 (MM4-1A) in autistic children, by the analysis of intestinal microbiota and systemic immune response induced by *L. reuteri* supplementation. Currently, completed studies and clinical trials involving probiotics treatment in ASD children, did not analyse the potential modulatory effect of immune system and microbiota. Here, we analyzed the intestinal microbiota and the systemic immune response, using stool and blood, respectively, of ASD children treated with placebo or *L. reuteri*.

4.2 Results

Probiotic *L. reuteri* alter fecal composition of *Firmicutes* phyla in ASD children

In order to examine the effect of probiotic *L. reuteri* in modulating gut microbiota in ASD children, we analysed fecal samples collected before probiotic supplementation (T0) and during 3 months and 6 months of probiotic consumption (T3 and T6, respectively). Hierarchal clustering of OTUs was completed at the phylum level to examine changes in relative abundance of specific bacteria at all time points. The taxonomic profile at phylum level, showed that *Firmicutes* were the most abundant phyla for the probiotic and control group at all time points, approximately 60% of the total community. We found that relative abundance of bacteria belonging to the phylum *Firmicutes* significantly increases in the probiotic compared to placebo group (Figure 1A). Since, *L. reuteri* included in the probiotic belongs to the *Firmicutes* phylum, we analysed whether the probiotic supplementation was able to increase the relative abundance of *L. reuteri* in the fecal microbiota. We observed that fecal samples from probiotic-treated ASD children were enriched of *L. reuteri* at T3 and T6 (Figure 1B), indicating that probiotic *L. reuteri* is able to survive the gastric acid of the stomach and the bile of the upper digestive tract.

To evaluate the *Firmicutes* composition at species level, we analyzed the relative abundance of all species discriminating those from *Clostridia* class and the others. We found that consumption of probiotic significantly increases the relative abundance of *Faecalibacterium prausnitzii* and *Eubacterium rectale* among *Clostridia* bacteria (Figure 1C), and *L. reuteri* among other classes of *Firmicutes* (Figure 1D).

Conversely, no statistically significant change between placebo and probiotic groups in the relative abundance of *Bacteroidetes* (p = 0,94 T3; 0,99 T6), *Actinobacteria* (p = 0,90 T3; 0,98 T6), *Proteobacteria* (p = 0,98 T3; 0,99 T6), *Verrucomicrobia* (p = 0,99 T3; 0,99 T6), and *Lentisphaerae* (p = 0,99 T3; p= 0,99 T6) (Figure 1A). Consistently with these results, we did not observe significant changes in the frequency of single species from *Bacteroidetes* (Figure 2), *Actinobacteria* (Figure 3), *Proteobacteria, Lentispherae* and *Verrucomicrobia* (Figure 4) phyla between placebo and probiotic treatment at T3 and T6 (Figure 2-3).

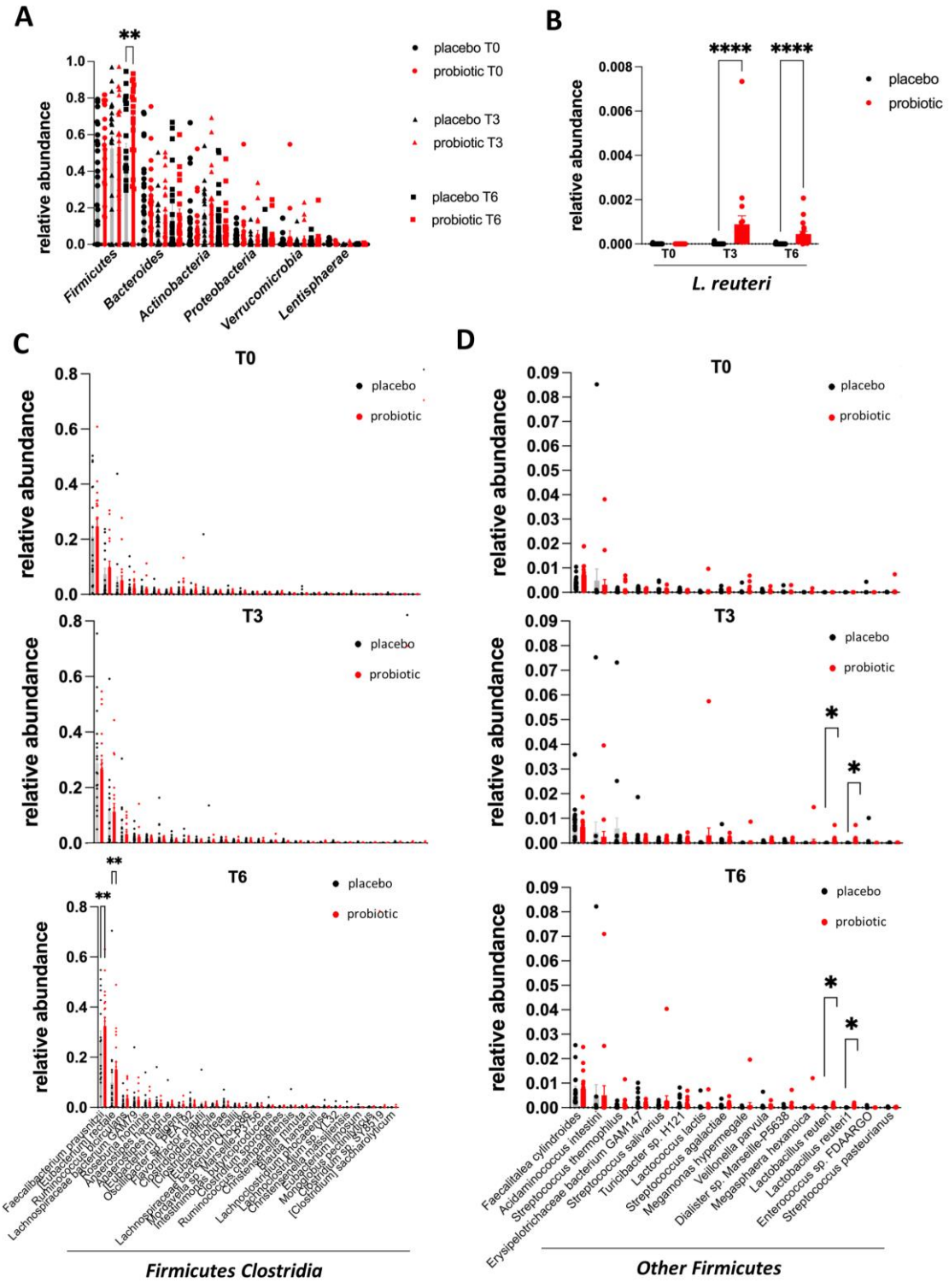

Figure 1. Bacteria from Firmicutes phyla increase in stool from probiotic-compared to placebo-treated ASD children

*DNA extracted from fecal samples of ASD children treated with probiotic reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) were analysed. Relative abundance of fecal bacterial taxa at the phylum level (A), of L. reuteri (B), and single species from Clostridia class (C) or other class of Firmicutes (D) was reported. Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition (*p-value ≤ 0.05; **p-value ≤ 0.01).*

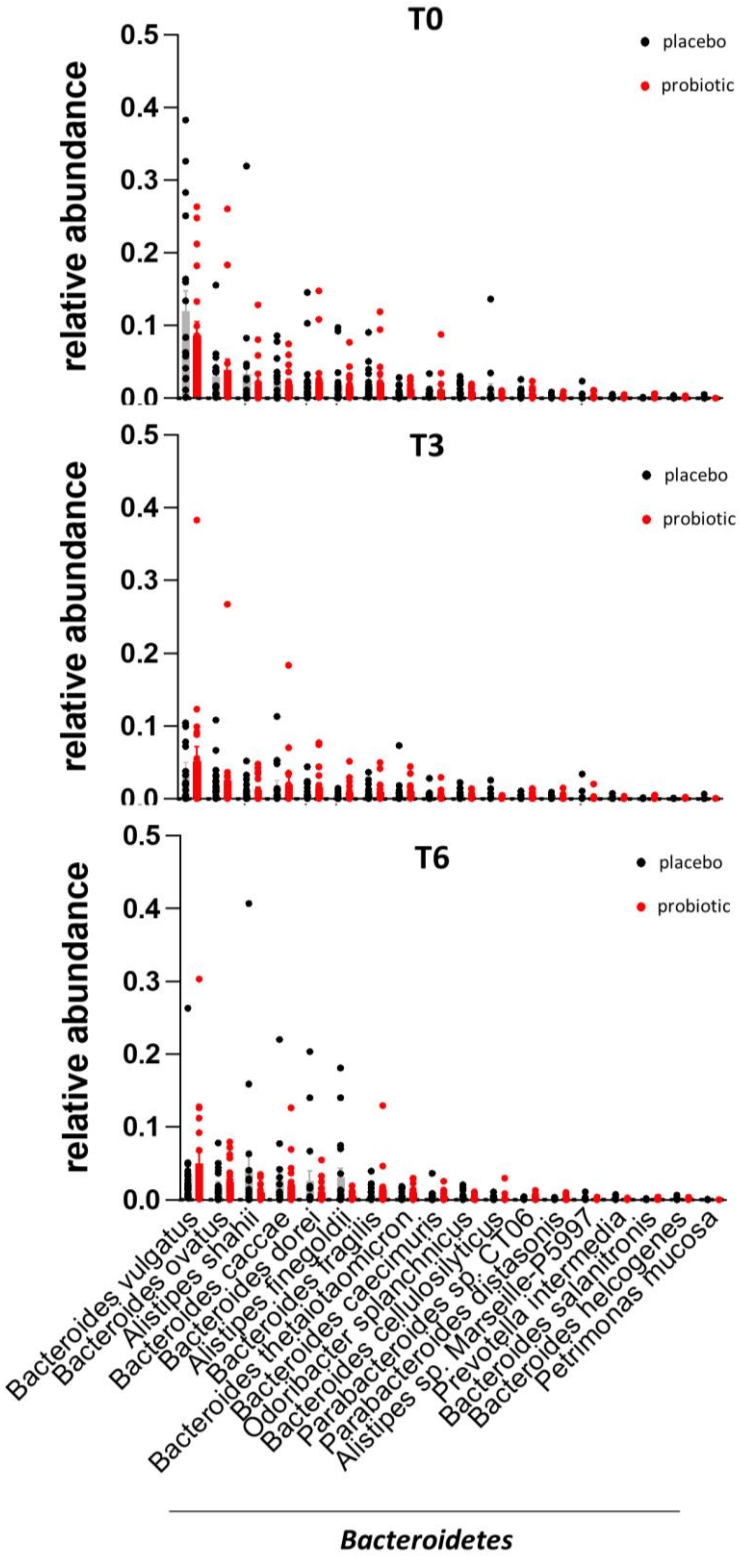

Figure 2. Probiotic L. reuteri administration does not modulate relative abundance of Bacteroidetes in stool from ASD children

DNA extracted from fecal samples of ASD children treated with probiotic L. reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) were analysed. Relative abundance of single species from Bacteroidetes phylum was reported. Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition

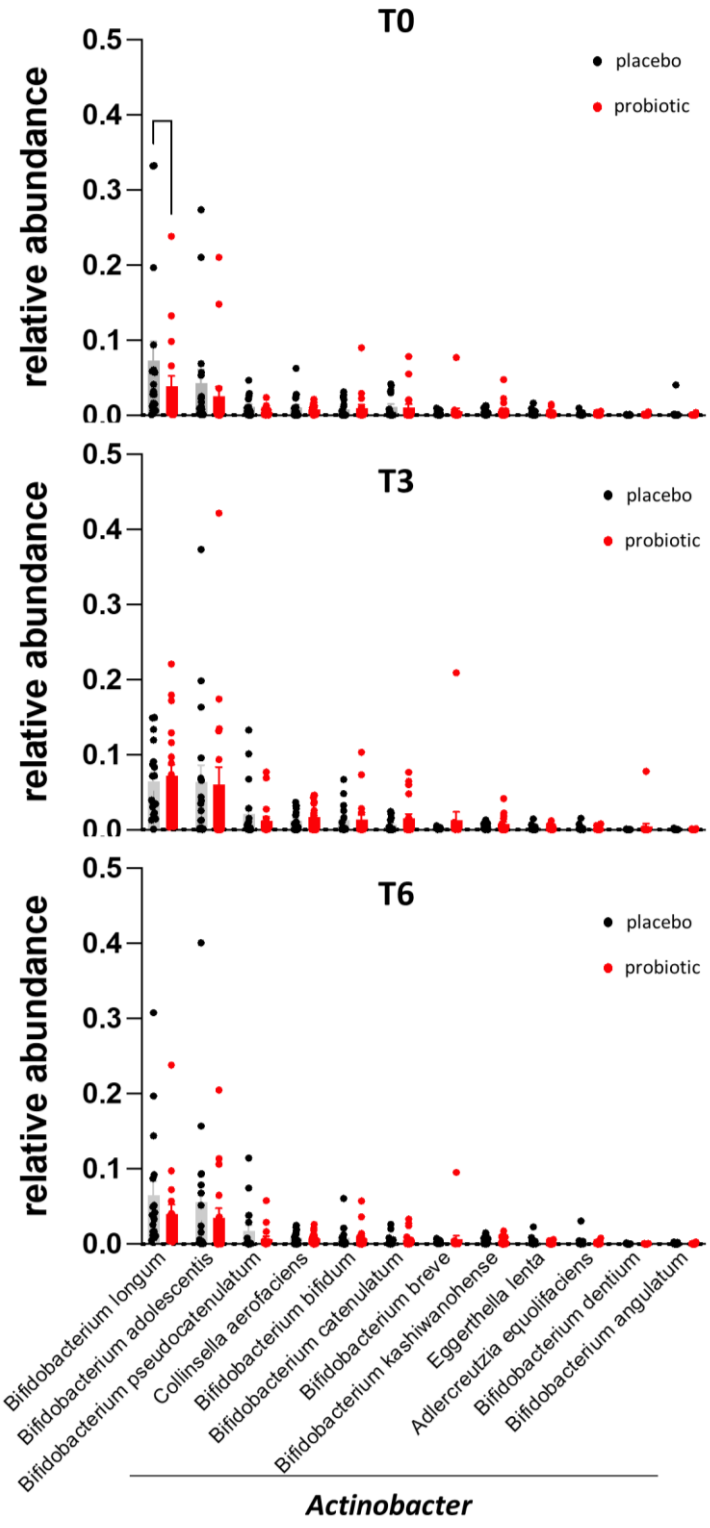

Figure 3. Probiotic L. reuteri administration does not modulate relative abundance of Actinobacteria in stool from ASD children

*DNA extracted from fecal samples of ASD children treated with probiotic L. reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) were analysed. Relative abundance of single species from Actinobacteria phylum was reported. Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition (*p-value ≤ 0.05).*

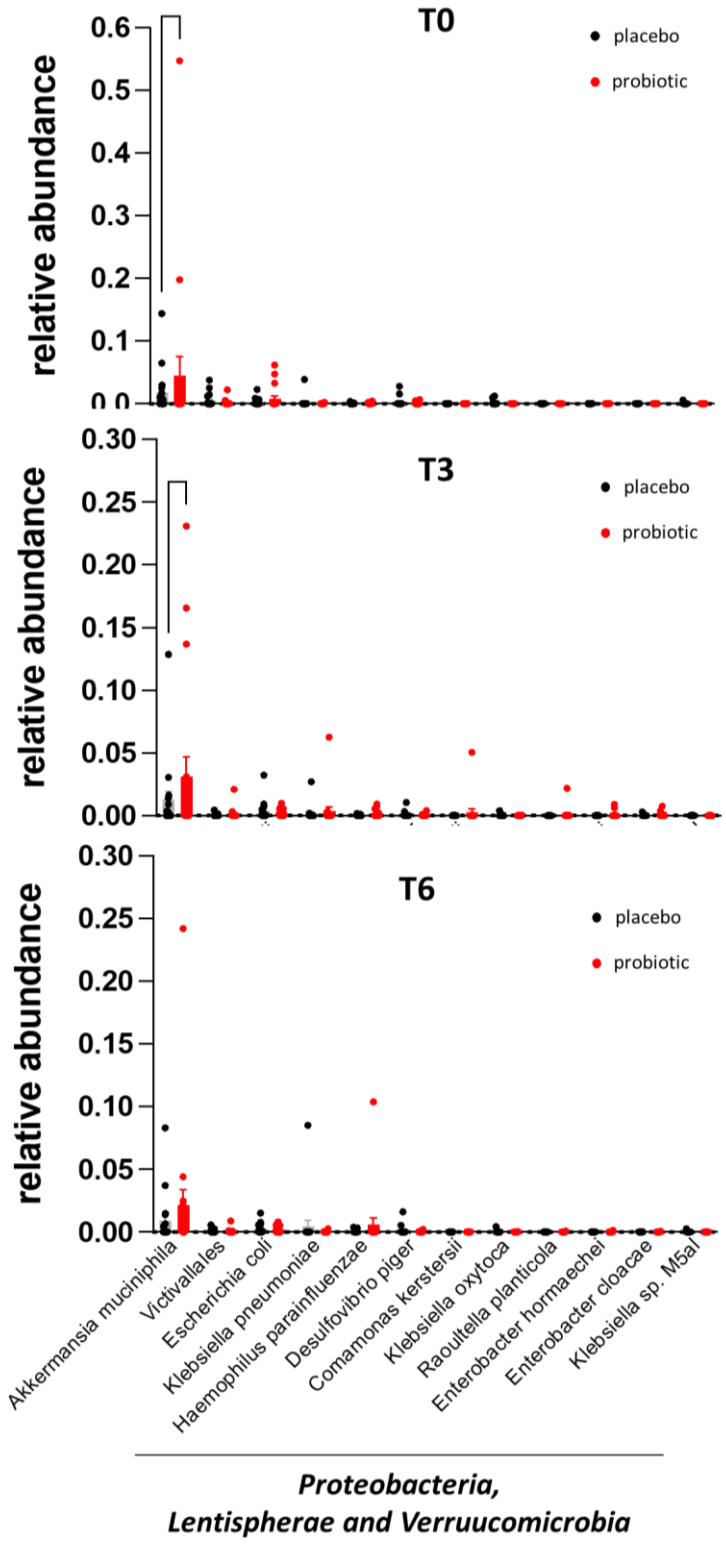

Figure 4. Probiotic L. reuteri administration does not modulate the relative abundance of Proteobacteria, Lentispherae and Verrucomicrobia in stool from ASD children

DNA extracted from fecal samples of ASD children treated with probiotic L. reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) were analysed. Relative abundance of single species from Proteobacteria, Lentispherae and Verrucomicrobia phyla was reported. Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition.

Probiotic *L. reuteri* administration does not modulate systemic immune response in ASD children

Given the strong relationship between microbiota and immune system, we investigated whether the alteration of *L. reuteri, F. prausnitzii* and *E. rectale* frequencies, observed in probiotic-treated ASD children, were associated to a modulation of the systemic immune response.

First, we analyzed the innate immune response, which is characterized by monocytes, dendritic cells (Figure 5A), natural killer cells innate lymphoid cells (Figure 5B), and molecules related to innate immune cell functions, such as GM-CSF, Fraktalkine, IFN-α2, MCP-3, MDC, IP-10, MCP-1, MIP-1α, MIP-1β Figure 5C), and molecules related to inflammation and neuroinflammation, such as IL-12, IL-1α, IL-1β, IL-6, IL-8 TNFα, IL-1Ra, and TGF- (Figure 5D). We observed significant differences between placebo and probiotic at baseline (Figure 5), likely related to interindividual variability. However, we did not observe significant modulation mediated by *L. reuteri* supplementation at 3 months and 6 months after probiotic treatment.

Then, we analyzed the adaptive immune response, which consists of conventional and unconventional T lymphocytes, and B lymphocytes. Among T lymphocytes, we analyzed the blood frequency of distinct CD4 T helper (Th) cells, such as Th1, Th2, Th1/17, Th17 and Tregulatory cells (Treg) (Figure 6A), CD8 T cells, mucosal invariant T cells (MAIT), and γδT cells (Figure 6B). In the same setting we analysed plasma factors derived from cells of the adaptive immune response, such as IFN-γ, IL-10, IL-13, IL-9, IL-4, IL-5, IL-17, and soluble CD40L (sCD40L) (Figure 6C).

We observed that probiotic *L. reuteri* supplementation does not affect persistently the blood frequency of adaptive immune cells (Figure 6A,B). In fact, we found a significant probiotic-mediated reduction of Th1 cells at 3 months of treatment, that is not confirmed at the endpoint of the study (6 month of treatment) (Figure 6A). Among soluble plasma factors related to the adaptive immune cells, we found a significant reduction of sCD40L mediated by *L .reuteri* at the endpoint of the study, that could be associated to a reduced activation of T lymphocytes or platelets, which are the main sources of sCD40L in the blood.

Moreover, we measured the plasma levels of neuropeptides and neurotransmitters derived from either microbiota or enteric nervous system that could contribute to the regulation of gut-brain axis. However, we did not observe modulation of oxytocin, serotonin, dopamin, adrenalin, and noradrenalin in ASD children treated with *L. reuteri* compared to placebo (Figure S4).

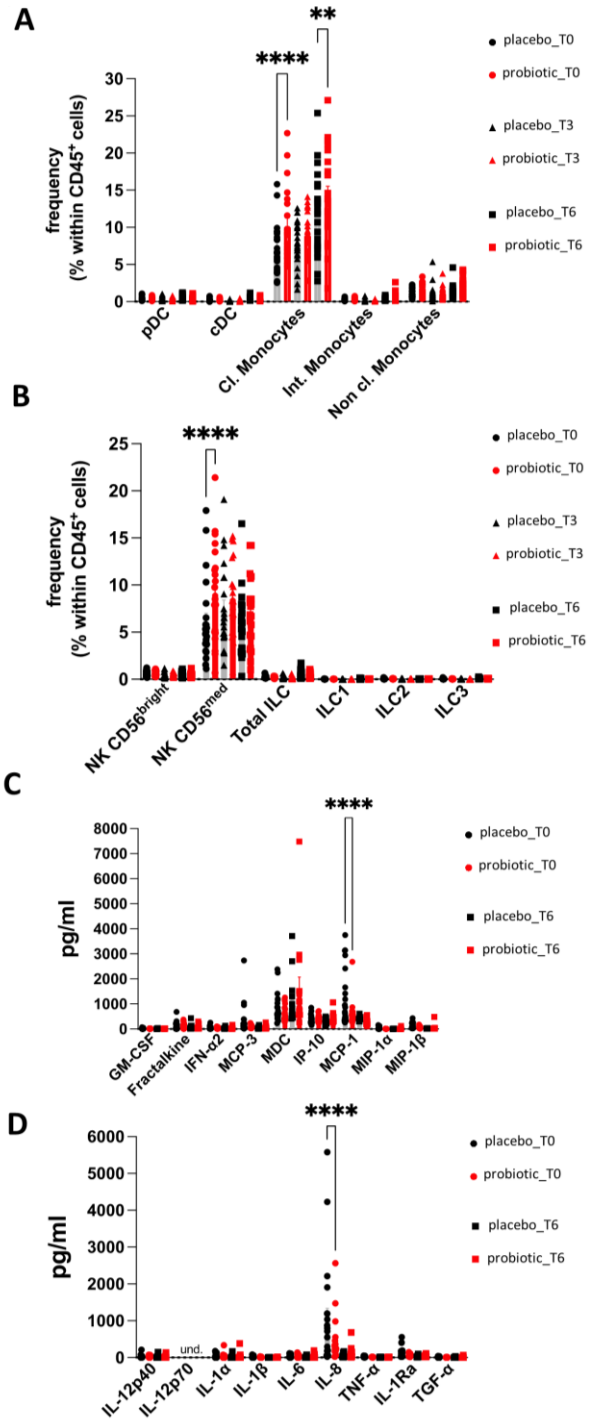

Figure 5. Probiotic L. reuteri administration does not modulate peripheral innate immune response in ASD children

*Peripheral blood mononuclear cells were isolated from ASD children treated with probiotic L. reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) after treatment. Cells were stained with specific antibodies to analyse the frequency of innate immune cells and analysed by flow cytometry. Graphs show mean frequency of each cell subset within the whole leukocyte population ($CD45^+$ cells) (A, B). The levels of indicated factors were measured, by multiplex assay, in the plasma of ASD subjects. Each plasma sample was analyzed in duplicates and means are represented (C,D). Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition (**p-value ≤ 0.01; **** p≤ 0.0001).*

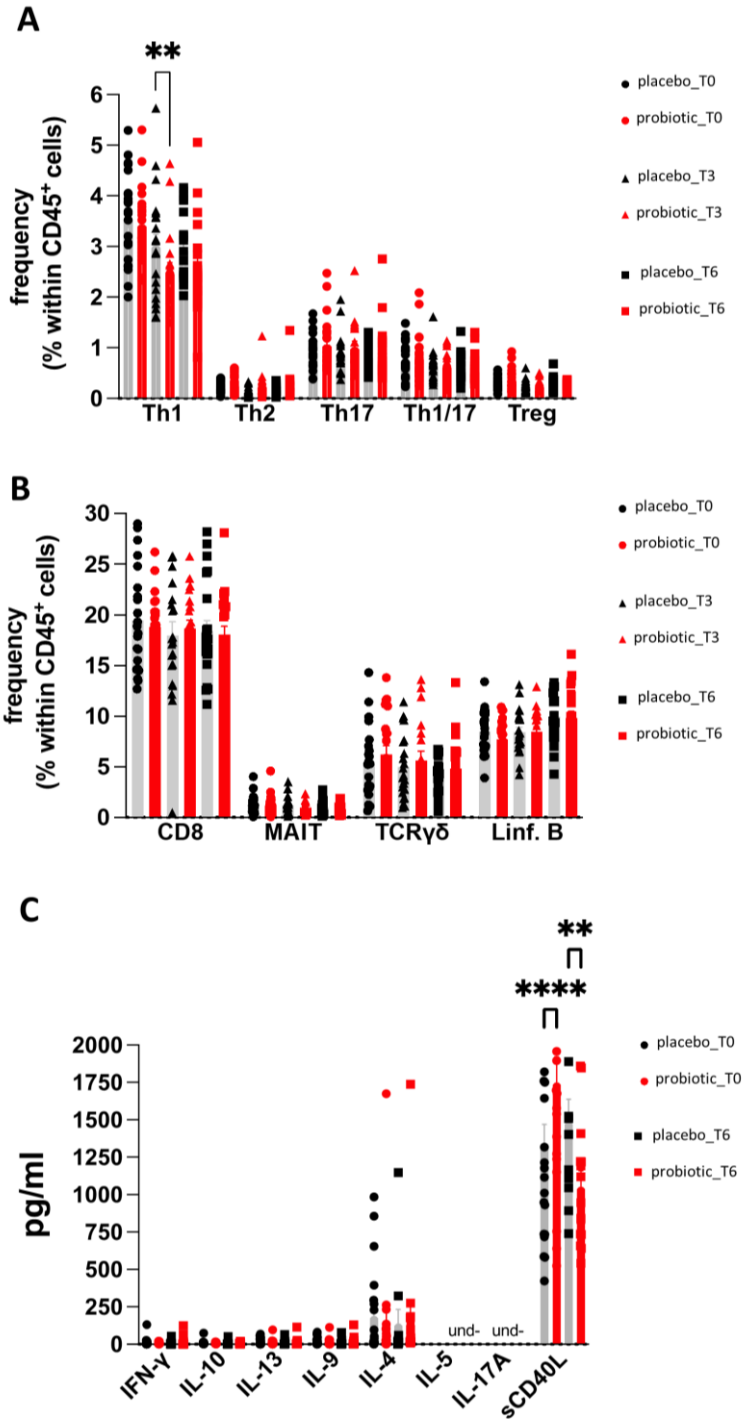

Figure 6. Probiotic L. reuteri administration does not modulate peripheral adaptive immune response in ASD children

Peripheral blood mononuclear cells were isolated from ASD children treated with probiotic L. reuteri (n=21) or placebo (n=22) at baseline (T0), 3 months (T3) and 6 months (T6) after treatment. Cells were stained with specific antibodies to analyse the frequency of adaptive immune cells and analysed by flow cytometry. Graphs show frequency of each cell subset within the whole leukocyte population ($CD45^+$ cells) (A, B).

*The levels of indicated factors were measured, by multiplex assay, in the plasma of ASD subjects. Each plasma sample was analyzed in duplicates and means are represented (C). Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition (**p-value ≤ 0.01; **** p≤ 0.0001).*

4.3 Discussion

Preclinical studies demonstrate that gut-microbiota-brain axis regulates behavior in ASD (Vuong, and Hsiao, 2017; Borre, O'Keeffe, Clarke, Stanton, Dinan, and Cryan, 2014; Sharon, Sampson, Geschwind, and Mazmanian, 2016). Specifically, a pro-social probiotic treatment activates vagus nerve, which induces release of oxytocin by periventricular neurons, and synaptyic potentiation of dopaminergic neurons in the ventral tegmental area (Sgritta, Dooling, Buffington, Momin, Francis, Britton, et al., 2019), known to promote social interaction (Buffington, Di Prisco, Auchtung, Ajami, Petrosino, and Costa-Mattioli, 2016; Gunaydin, Grosenick, Finkelstein, Kauvar, Fenno, Adhikari, et al., 2014; Huang, and Hessler, 2008). In humans, the involvement of gut-brain axis in autism is supported by the high incidence of intestinal comorbidity (Vuong, and Hsiao, 2017). However, the studies of mechanisms regulating microbial-mediated changes in social behavior of ASD individuals are missing. In this context, we collaborated with neuropsychiatrists who demonstrated that supplementation of *L.reuteri* improves social behavior of autistic children. Our role was to investigate the potential involvement of gut microbiota and systemic immune response in ASD children treated with probiotic or placebo. This randomized controlled trial is the first study that examines the impact of probiotic treatment not only on clinical but also on microbiological and immunological patterns, thus providing new insights into the gut-brain connection in autism. In fact, immune dysfunction (Ashwood, Krakowiak, Hertz-Picciotto, Hansen, Pessah, and Van de Water, 2011; Li, Chauhan, Sheikh, Patil, Chauhan, Li, et al., 2009) and alteration in gut microbial composition (Tomova, Husarova, Lakatosova, Bakos, Vlkova, Babinska, et al., 2015; De Angelis, Francavilla, Piccolo, De Giacomo, Gobbetti, 2015; Zhang, Ma, Zhang, He, Wang, 2018) have been reported in ASD individuals.

Previous clinical trials evaluating the effect of the probiotic in the gut microbiota of ASD children revealed an increase in Bifidobacterial and Lactobacilli levels after 3 months supplementation with the probiotic containing *L. acidophilus*, *L. rhamnosus* and *B. longum* (Shaaban, El Gendy, Mehanna, El-Senousy, El-Feki, Saad, et al., 2018). Similarly, we found a significant increase of *L. reuteri* after 3 months and 6 months of supplementation, demonstrating that bacteria supplemented by oral administration survive in the acid environment of the digestive tract and persist in the intestinal microbiota. Moreover, we found an enrichment of the *Firmicutes* phyla, that could have a beneficial role in the light of previous studies reporting a decrease in the level of *Firmicutes* in ASD compared with neurotypical children (Tomova, Husarova, Lakatosova, Bakos, Vlkova, Babinska, et al., 2015; De Angelis, Francavilla, Piccolo, De Giacomo, Gobbetti, 2015; Zhang, Ma, Zhang, He, and Wang, 2018). In particular, we

found an increase of *Faecalibacterium prausnitzii* in the microbiota of ASD children treated with probiotic. Consistently with the hypothesis of the beneficial effect of probiotic in regulating gut microbiota, it has been reported a lower concentration of *F. prausnitzii* in autism cases versus neurotypical peers (Agarwala, Naik, and Ramachandra, 2021). Moreover, decreased levels of *F. prausnitzii* in the gut microbiota has been reported in other diseases associated to gastrointestinal disturbs (Lloyd-Price, Arze, Ananthakrishnan, Schirmer, Avila-Pacheco, Poon, et al., 2019; Frank, St Amand, Feldman, Boedeker, Harpaz, and Pace, 2007), suggesting a potential protective role of this bacteria in gut mucosal inflammation. In fact, *F. prausnitzii* is a main producer of butyrate that has anti-inflammatory properties (Zhang, Huang, Yoon, Kemmitt, Wright, Schneider, et al., 2021). Therefore, the increase of *F. prausnitzii* observed in our study could promote butyrate production and anti-inflammatory pathways in intestinal mucosa of autistic children, thus preventing gastrointestinal and behavioral disturbs (Rose, Yang, Serena, Sturgeon, Ma, Careaga, et al., 2018). In order to further insight into this mechanism, we will analyse the levels of butyrate in stool or plasma samples of ASD children treated with either probiotic or placebo.

In this regard, we observed reduced plasmatic levels of soluble CD40L (sCD40L), which is a molecule known to activate innate immune cells and B lymphocytes (Grewal, and Flavell, 1998). High levels of plasmatic CD40L in Crohn's disease and ulcerative colitis were proportional to the extent of their mucosal inflammation (Danese, Katz, Saibeni, Papa, Gasbarrini, Vecchi, et al., 2003). Therefore, our results indicate that decrease of sCD40L mediated by probiotic *reuteri* could reflect a reduced mucosal inflammation in ASD children. Previous evaluation of the plasmatic levels of immune factors revealed a reduction in IL-13 and TNF-α production in some participants of the study (Sanctuary, Kain, Chen, Kalanetra, Lemay, Rose, et al., 2019). Moreover, the reduction of inflammatory cytokines, such as TNF-α mediated by different strains of probiotics has been reported in inflammatory bowel disease, obesity, and neurological diseases (Cristofori, Dargenio, Dargenio, Miniello, Barone, and Francavilla, 2021). However, a global analysis of the systemic immune response in patients treated with probiotics was never been performed. Here, we found that the global immune response and microbiota are not significantly affected by probiotic. In fact, a scatter plot of the first two principal components from the PCA on the biological data at T0 and T6, shows no clear separation of the probiotic and placebo groups, convergently indicating no effect of the probiotic (Figure S5). Notwithstanding the absent global influence of the probiotic on fecal microbiota, systemic immune response, and plasmatic neurotransmitters, it has been shown a prosocial effect of *L. reuteri* in ASD children through parental measurements (submitted manuscript).

These results are not surprising given the large number of non-microbial and microbial stimuli that each individual receive from the environment and that affect both immune system and microbiota. Moreover, the inter-individual variability for both immune system and microbiota suggests that subsequent studies should use larger group of patients and/or defined sub-group of patients. Overall, the current study has highlighted the possibility to analyze global immune response and gut microbiota from blood and stools, respectively, that are the most accessible human specimens for this purpose. However, further studies investigating mucosal immune response and microbiota in *in vivo* experiments, using mouse models (Sgritta, Dooling, Buffington, Momin, Francis, Britton, et al., 2019), or *in vitro* experiments (Mileti, Matteoli, Iliev, and Rescigno, 2009) could implement these discoveries.

4.4 Material and methods

ASD subjects for blood and stool collection

Forty-three children with a diagnosis of ASD based on the criteria in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (First, 2013) were enrolled in the study registered on ClinicalTrials.gov (NCT04293783) (submitted manuscript). Diagnosis was confirmed by the administration of the gold standard measure Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) (Lord, Risi, Lambrecht, Cook, Leventhal, DiLavore, et al., 2000) by a licensed multidisciplinary team of child psychiatrists or clinical psychologists. The trial was a single-center randomized double-blind, parallel-group, placebo-controlled study to test the effect of *L. reuteri* supplementation (BioGaia Gastrus, Ñ $2x10^8$ CFU *L. reuteri*/tablet) on the behavioral profiles of children with ASD, as previously described (submitted manuscript). Demographic and clinical data of ASD subjects included in the study are described in Table 1. No differences were observed between the groups at baseline (Table 1). Approval by the ethics committee of the Policlinico Tor Vergata Foundation Hospital (PTV) Institutional Review Board (#244/19) and written informed consent in accordance with the Declaration of Helsinki from parents or legal guardians of all participants were obtained before study initiation. All children included in the blood study did not take immunomodulant, immunosuppressive compounds, they did not follow special diet and they did not suffer of coeliac disease, or other organic gastrointestinal disorders. At baseline (T0), and after 3-months (T3) and at 6-months (T6) of probiotic *L. reuteri* or placebo supplementation, blood and stool samples were collected.

Peripheral blood mononuclear cells purification and frequency analysis

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation (GE Healthcare), as previously described (Ruocco, Rossi, Motta, Macchiarulo, Barbieri, De Bardi, et al. 2015), from 5 ml of whole blood of ASD subjects, and stained with fluorochrome-conjugated antibodies for specific markers of each immune subpopulation. In particular: (panel 1) CD45, CRTH2, CD161, CD19, CD56, CXCR3, CD8, TCR-γδ, CCR6, Live Dead, CD3, CD4,CD69 T helper (Th)1, 2, 17, 1/17 lymphocytes, cytotoxic lymphocytes (CD8 T cells), γδT cells, MAIT cells, natural killer cells, B lymphocytes; (Panel 2) CD45, CD39, CD25, CD16, CD11c, CD86, CD127, CD14, CD3, CD19, CD56, Live Dead, CD123, CD80, CD4 for monocytes, plasmacytoid and myeloid dendritic cells, T regulatory cells; Panel 3: CD117, CD127, CRTH2, CD161, CD117, NKP44, Live Dead, CD3, CD14, CD16, CD19 for innate lymphoid cells (ILC) 1,2,3 (Panel 3). After staining, cells were acquired on a Cytoflex

(Beckman Coulter) flow cytometer. Data were analyzed with FlowJo v 10.8. Gating strategy is shown in Figure S1-S3.

Plasma cytokine and chemokine analysis

Plasma cytokine and chemokine profiles were analyzed using Millipore multiplex magnetic bead-based antibody detection kits, following the manufacturer's instructions. Specifically, Human Cytokine/Chemokine/Growth Factor Panel A (Millipore Cat. No. HCYTA-60K-PXBK38) was used for detection of the following analytes: interleukin (IL)-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-13, IL-15, IL-17A, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), interferon-gamma (IFN)-γ, IFN-α2, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein (MCP) 1, MCP3, macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage-derived chemokine (MDC), tumor necrosis factor-alpha (TNF)-α, TNF-β, and transforming growth factor (TGF)-β. Plasma aliquots (50 μl) were used for analysis, with a minimum of 50 beads per analyte acquired. Each sample was analyzed in duplicate. Median fluorescence intensities were measured using a Luminex 200 analyzer. Standard curves and values were calculated using xPONENT 4.2 software for MAGPIX®. Data are analyzed and reported as concentration readings (pg/ml).

Analysis of neuropeptides and neurotransmitters

Oxytocin, Serotonin, Dopamin, Adrenalin and Noradrenalin in plasma were measured by ELISA kits (Novus Biologicals), according to manufacturer's instructions.

Metagenomic analysis of gut microbiota

Fecal microbiota of ASD children were analysed by using a metagenomic approach based on the amplification and sequencing of a portion of the bacterial 16S rRNA gene. DNA were extracted from feces using well-established protocols. A panbacterial amplicon were produced by targeting the V3-V4 hypervariable regions of the 16S rRNA gene. Sequencing were finally be performed using a paired-end approach (2 x 300 bp) on a Illumina MiSeq platform. Raw sequences were analyzed using well-established bioinformatic pipelines, mostly based on the Mothur software. After quality control, the resulting highquality reads were clustered into Operational Taxonomic Units (OTUs) at 97% homology. Taxonomic attribution of OTUs will be obtained against reference databases.

Principal Component Analysis

A Principal Component Analysis (PCA) was performed at baseline (T0) and endpoint (T6) on biological data including fecal microbiota, systemic immune response, and plasmatic neurotransmitters to reduce their dimensionality. Data were normalized by centering and scaling to mean 0 and standard deviation 1. Data analysis and visualization was performed in R programming language using the package "Factoextra" (Kassambara, and Mundt, 2017).

Statistical analysis

Two-way ANOVAs were performed to analyze the main effects and interactions of two conditions on the dependent variables. The significance level was $p \leq 0.05$. Data were visualized using GraphPad PRISM v.9 and presented as mean ± standard error (SEM).

4.5 Supplementary materials

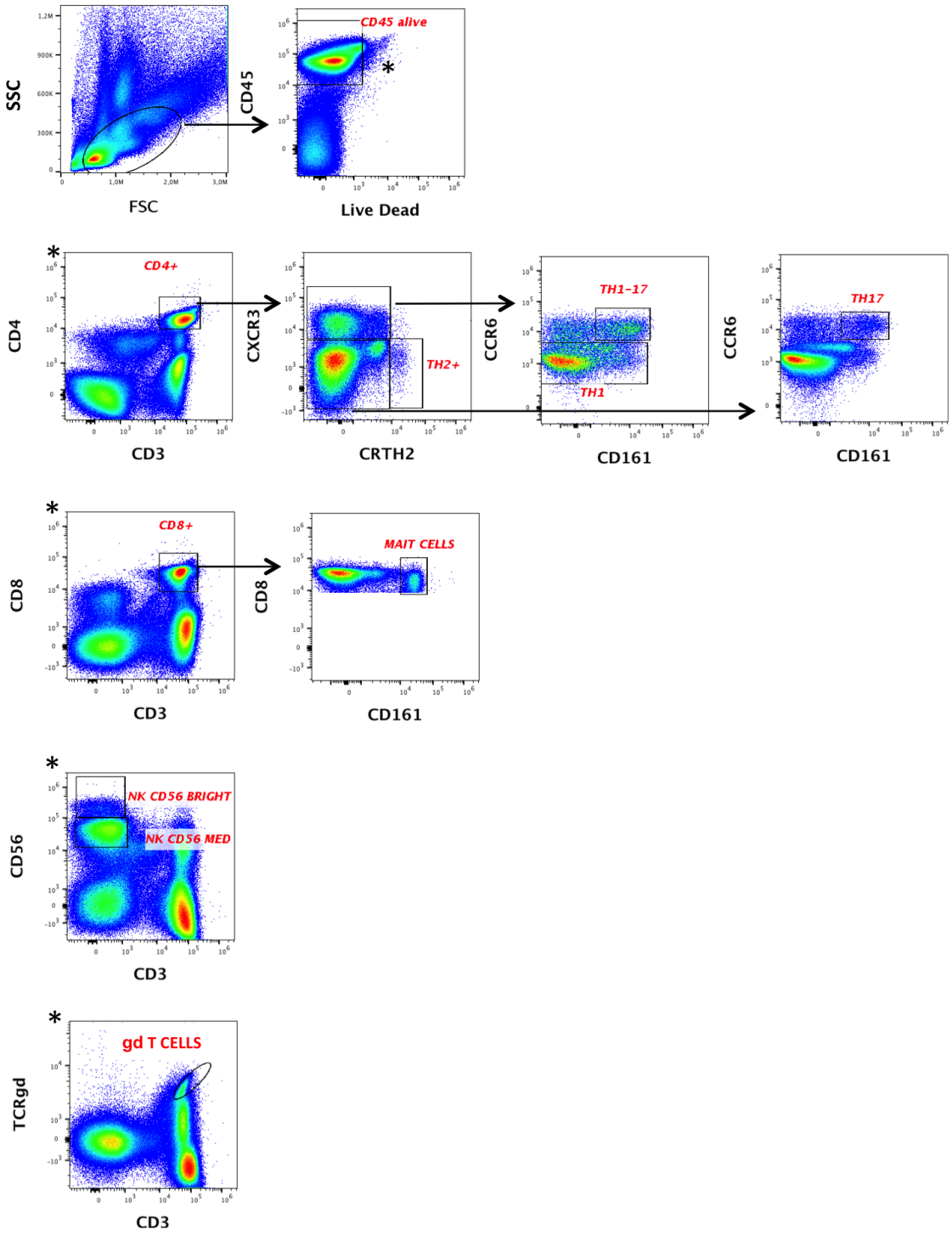

Figure S1. Gating strategy for the wide characterizaton of T lymphocytes by polychromatic flow cytometry

Peripheral blood mononuclear cells were stained with specific antibodies discriminating T cell subsets and analysed by flow cytometry. A representative experiment with gating strategy is shown. The frequency of each population within total immune cells ($CD45^+$ alive cells marked with asterisk) will be calculated and compared before and after probiotic treatment in each individual

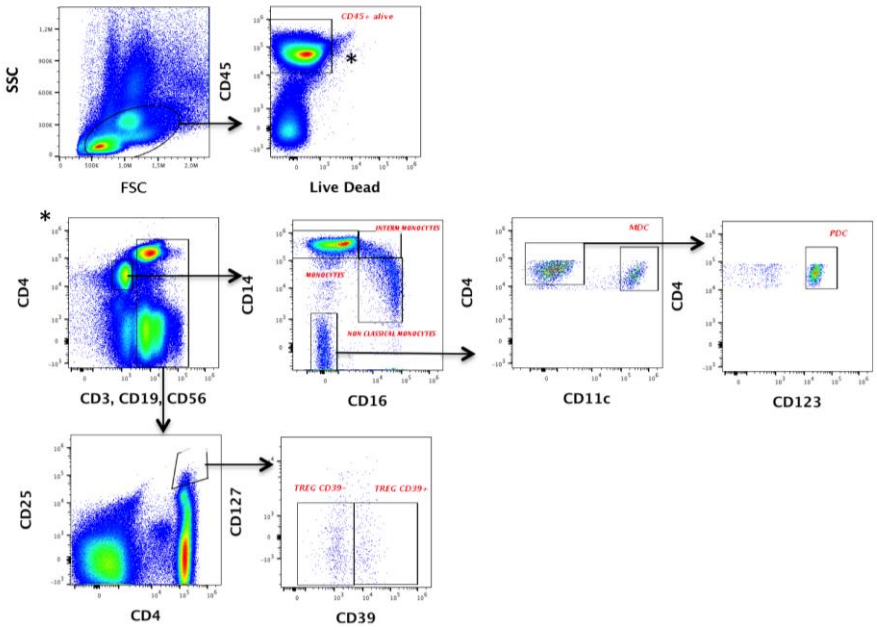

Figure S2. Gating strategy for the wide characterization of innate immune cells and T regulatory cells by polychromatic flow cytometry

Peripheral blood mononuclear cells were stained with specific antibodies discriminating innate immune cells and T regulatory cells, and then analysed by flow cytometry. A representative experiment with gating strategy is shown. The frequency of each population within total immune cells ($CD45^+$ alive cells marked with asterisk) will be calculated and compared before and after probiotic treatment in each individual.

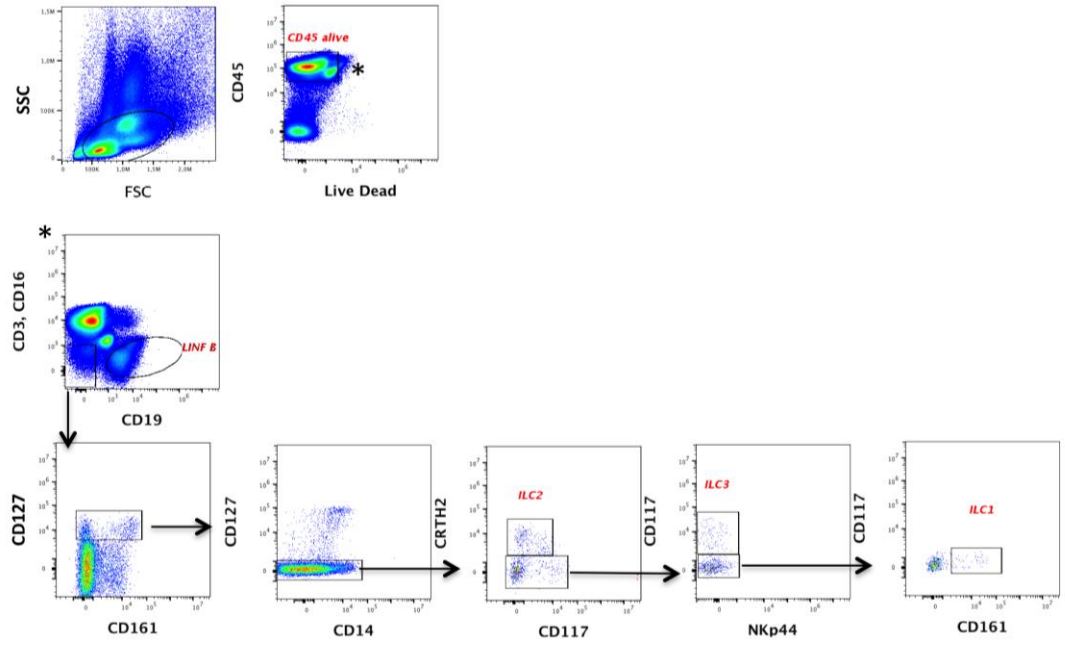

Figure S3. Gating strategy for the wide characterization of innate lymphoid cells and B lymphocytes by polychromatic flow cytometry

Peripheral blood mononuclear cells were stained with specific antibodies discriminating innate lymphoid cell subsets and B cells, and then analysed by flow cytometry. A representative experiment with gating strategy is shown. The frequency of each population within total immune cells ($CD45^+$ alive cells marked with asterisk) will be calculated and compared before and after probiotic treatment in each individual.

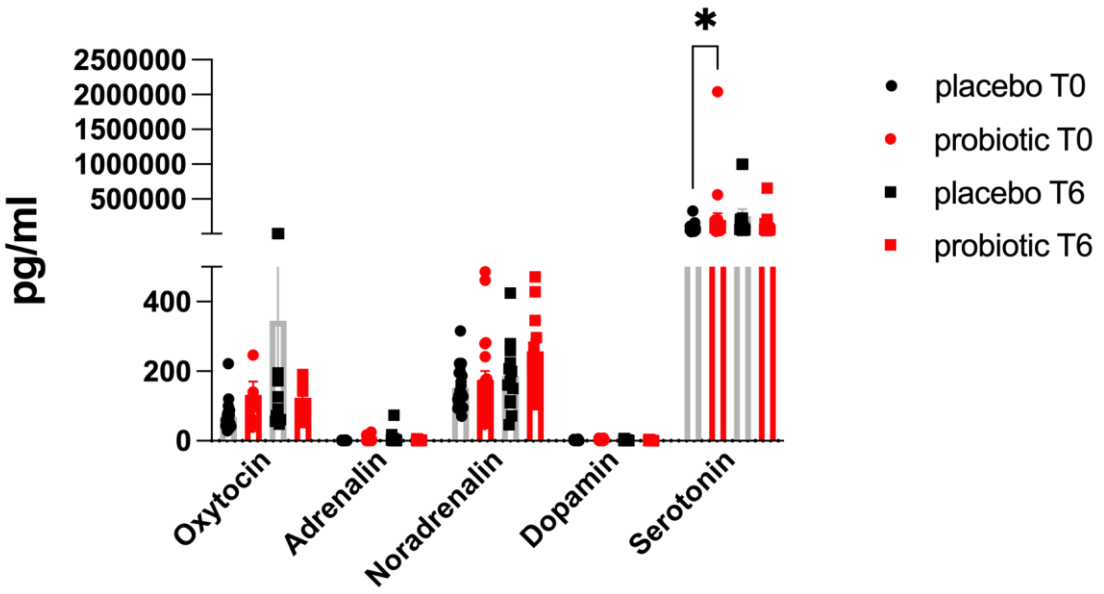

Figure S4. Probiotic L. reuteri administration does not modulate plasma levels of some neuropeptides and neurotransmitters in ASD children

*The levels of indicated neuropeptides or neurotransmitters were measured, by ELISA, in the plasma of ASD subjects. Each plasma sample was analyzed in duplicates and means are represented. Data are reported as mean ± SEM. Two-way ANOVA test was used to compare different experimental condition (*p-value ≤ 0.05).*

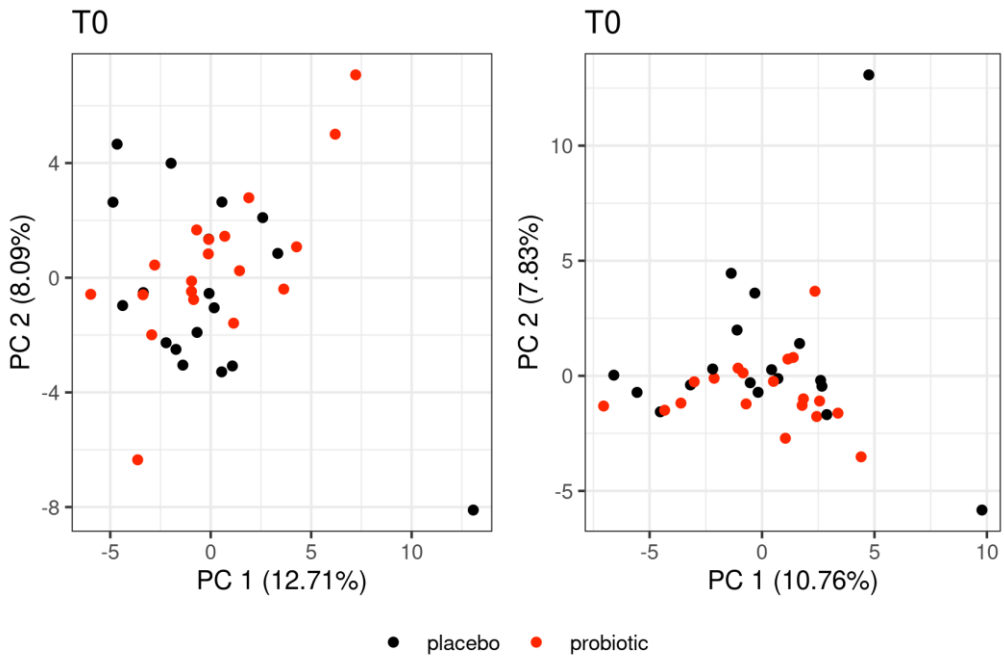

Figure S5. Variables measuring peripheral immune response and fecal microbiota do not separate placebo- and probiotic-treated ASD children
A Principal Component Analysis (PCA) of the data reported in previous figures was used to plot the first two dimensions for T0 and T6. Each point is an individual, which position is defined based on their value on the first two Principal Components (PC) of the analysis. PC1 and PC2 explain the most of the variance in the dataset, indicated between parentheses in the axis labels.

Table 1. Demografic and clinical characteristics of ASD subjects at the baseline

	Placebo group (n=22)	***L. reuteri* group (n=21)**	**p value**
gender (male/female)	16/6	19/2	0.24
age (years)	5.5 ± 1.2	5.8 ± 1.3	0.48
Leiter QI	88.9 ± 21.3	95.1 ± 25.0	0.27
ADOS_CSS	7.1 ± 0.8	6.7 ± 1.5	0.42

5. CONCLUSION, PERSPECTIVES, AND REFERENCES

5.1 Conclusion

Results from these studies allowed to better characterize the interaction between human innate immune cells and microorganisms. In particular:

1) we characterized the interaction of human blood DCs with *Saccharomyces cerevisiae*, fungal component of the intestinal microbiota. We discovered a differential activation of human blood pDCs and cDCs by *S. cerevisiae*. In fact, pDCs recognize single strand RNA, double and single strand DNA of *S. cerevisiae*, produce IFN-α and induce a Th polarization characterized by IL-10 production. On the other side, cDCs do not recognize fungal nucleic acids, and induce production of pro-inflammatory cytokines, likely due to their interaction with fungal cell wall components.

This information indicates that the opposite role of pDCs and cDCs in response to *S. cerevisiae* could mediate the equilibrium between pro-inflammatory and the anti-inflammatory immune response in the gut, which is important either for the gut homeostasis than during a *S. cerevisiae* opportunistic infection;

2) we characterized the interaction between human innate immune cells and two strain of the opportunistic pathogen *Acinetobacter baumannii*, ATCC19606^{T} and ACICU. We found that monocyte, macrophage, cDC and pDC sense and respond to both strains of *A. baumannii*. Specifically, macrophage exert a strong killing of bacteria and do not produce cytokines; monocytes are able to kill bacteria, especially the less virulent strain ATCC19606^{T}, produce the pro-inflammatory cytokine IL-6 and express co-stimulatory molecules in response to infection; cDC and pDC do not kill bacteria, however they are activated and produce cytokines upon infection. Importantly, we discovered that the multidrug-resistant *A. baumannii*, ACICU strain, induces lower expression of regulatory cytokines, such as IL-10 by monocytes and cDC, and IFN-α by pDC, in comparison with the less pathogenic strain. Therefore, higher virulence of ACICU than ATCC19606^{T}, could be related to the reduction of IL-10 and IFN-α that have beneficial role in the defense against *A. baumannii* infection;

3) we analysed the impact of *L. reuteri* supplementation in modulation of immune system and microbiota composition of children with ASD. We observed a significant increase of *L. reuteri* in stool of children treated with probiotic compared to placebo. Moreover, we found that consumption of probiotic significantly increases the relative abundance of *Faecalibacterium prausnitzii* and *Eubacterium rectale*, producers of short-chain fatty acids (SCFAs), which have anti-inflammatory properties. In addition, we found that probiotic induces a significant reduction of soluble CD40L, which may reflect a reduced platelet and lymphocyte activation.

Collectively, result from this thesis uncovered new mechanisms involved in the regulation of inflammatory versus anti-inflammatory immune response mediated by host/microbe interaction.

5.2 Perspectives

The elucidation of mechanisms regulating host/microbe interaction is important to respond to pathogenic infections, to prevent excessive inflammation, and to exploit the beneficial effect of microbiota and probiotics.

For instance, we observed an anti-inflammatory role of pDCs in response to *S. cerevisiae*. Thus, future studies aimed to investigate the molecular mechanisms leading to pDCs activation, such as the identification of the activating RNA or DNA sequence of *S. cerevisiae*, could open new perspectives towards therapeutic approaches for dysbiosis-related diseases, and/or probiotic intervention. On the other side, the identification of the molecular mechanisms leading to the pro-inflammatory activity of cDCs in response to *S. cerevisiae* could be useful for the therapeutic targeting of chronic inflammatory diseases, and for a better understanding of the mechanisms underlying immune response during fungal infections in immunocompromised patients.

In the context, of *A. baumannii* infection, the identification of IL-10 and IFN-α differentially modulated in innate immune cells by the most pathogenic strain ACICU, suggests that these molecules could be therapeutically modulated to improve the effective immune response during opportunistic infections in immunocompromised patients. Moreover, further insight into cytokines potentially modulated by the ACICU strain, including IL-1β and IL-12, could unveil new pathogenic mechanisms and new therapeutic target for *A. baumannii* infection.

The study of *L. reuteri* revealed that probiotic administration in ASD children increases *F. prausnitzii* and *E. rectale* in microbiota, and decreases sCD40L in plasma. Since *F. prausnitzii* and *E. rectale* are butyrate-producing bacteria, we plan to evaluate the butyrate levels in the fecal samples of probiotic/placebo-treated ASD children.

In fact, given the anti-inflammatory role of butyrate, this knowledge could be useful for future studies in ASD children, and other group of patients affected by dysbiosis-related diseases.

However, future studies investigating the effect of *L. reuteri* on mucosal immune response and microbiota in *in vivo* experiments, using mouse models, or *in vitro* experiments using complex culture systems, may further dissect the effect of the treatment on gut microbiota composition and on systemic inflammatory response.

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6. Ph.D. CAREER

6.1. List of publications

- Sacchi, A., Tumino, N., **Sabatini, A**., Cimini, E., Casetti, R., Bordoni, V., Grassi, G., & Agrati, C. (2018). Myeloid-Derived Suppressor Cells Specifically Suppress IFN-γ Production and Antitumor Cytotoxic Activity of Vδ2 T Cells. Frontiers in immunology, 9, 1271.

- Agrati, C., Tumino, N., Bordoni, V., Pinnetti, C., **Sabatini, A**., Amendola, A., Abbate, I., Lorenzini, P., Mondi, A., Casetti, R., Cimini, E., Grassi, G., Antinori, A., & Sacchi, A. (2019). Myeloid Derived Suppressor Cells Expansion Persists After Early ART and May Affect CD4 T Cell Recovery. Frontiers in immunology, 10, 1886.

- De Marco, L., D'Orso, S., Pirronello, M., Verdiani, A., Termine, A., Fabrizio, C., Capone, A., **Sabatini, A**., Guerrera, G., Placido, R., Sambucci, M., Angelini, D. F., Giannessi, F., Picozza, M., Caltagirone, C., Salvia, A., Volpe, E., Balice, M. P., Rossini, A., Rötzschke, O., … Borsellino, G. (2022). Assessment of T-cell Reactivity to the SARS-CoV-2 Omicron Variant by Immunized Individuals. JAMA network open, 5(4), e2210871.

- **Sabatini, A**., Guerrera, G., Corsetti, M., Ruocco, G., De Bardi, M., Renzi, S., Cavalieri, D., Battistini, L., Angelini, D. F., & Volpe, E. (2022). Human Conventional and Plasmacytoid Dendritic Cells Differ in Their Ability to Respond to Saccharomyces cerevisiae. Frontiers in immunology, 13, 850404.

6.2. Conference communications

- Poster - CFCD Annual meeting "DC-phering mononuclear phagocyte biology in health and disease" December 16th – 17th 2021, Paris, France.
  Andrea Sabatini, Gisella Guerrera, Marta Corsetti, Gabriella Ruocco, Marco De Bardi, Sonia Renzi, Duccio Cavalieri, Luca Battistini, Daniela Angelini, Elisabetta Volpe - Differential response of human myeloid and plasmacytoid dendritic cells to Saccharomyces cerevisiae.

6.3. Courses

- Regulation (EU) 2016/679 course – IRCSS Santa Lucia Foundation, Rome, Italy, March 2019
- Corso di formazione Sol Group "Utilizzo dei gas in sicurezza" - IRCSS Santa Lucia Foundation, Rome, Italy, 2021
- "The use of statistics in biomedical research - basic course" – Dr. Daniele Peluso, Dr. Alessandro Giuliani – Fondazione Santa Lucia, 20-22th June 2022

6.4. Additional information

- Qualification as Profession Biologist, Tor Vergata University, Roma, Italy –2019