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THÈSE

En vue de l'obtention du DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par l'Université Toulouse 3 - Paul Sabatier

Présentée et soutenue par

Lucie BERNARD

Le 13 janvier 2020

Utilisation de bactéries du microbiote pulmonaire pour moduler le système immunitaire local à l'état basal et pendant l'infection par *Mycobacterium tuberculosis* chez la souris

Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

Spécialité : **IMMUNOLOGIE**

Unité de recherche :

IPBS - Institut de Pharmacologie et Biologie Structurale

Thèse dirigée par

Geanncarlo LUGO

Jury

M. François LEULIER, Rapporteur

Mme Andrea COOPER, Rapporteur

M. Gérard EBERL, Examineur

M. Claude KNAUF, Examineur

M. Geanncarlo LUGO, Directeur de thèse

“My life amounts to no more than one drop in a limitless ocean.
Yet what is any ocean, but a multitude of drops?”

David Mitchell, *Cloud Atlas*

A mon grand-père, Camille,
qui éclaire ma voie.

Résumé

Utilisation de bactéries du microbiote pulmonaire pour moduler le système immunitaire local à l'état basal et pendant l'infection par *Mycobacterium tuberculosis* chez la souris

Les muqueuses du corps humain (notamment l'intestin) sont colonisées dès la naissance par des milliards de microorganismes formant le microbiote (ou flore commensale) et qui vivent en symbiose avec lui. La survie de notre organisme (l'hôte) et de son microbiote dépend de l'état d'activation de notre système immunitaire. Alors qu'une trop faible activation nous rend susceptibles aux infections, une trop forte activation ou inflammation altère nos tissus. Certaines bactéries du microbiote intestinal, interagissent avec les cellules du système immunitaire pour moduler cette balance. Leur administration en tant que probiotique améliore de nombreuses pathologies et est envisagée pour le traitement d'infections respiratoires. L'infection causant le plus de décès dans le monde est la tuberculose, une maladie respiratoire causée par *Mycobacterium tuberculosis* et dans laquelle une hyper-activation du système immunitaire détruit les tissus pulmonaires. Dans ce projet de thèse, j'ai cherché à savoir si des bactéries du microbiote peuvent influencer la réponse du système immunitaire à la tuberculose, évaluant ainsi le potentiel d'une stratégie probiotique pour améliorer les traitements de cette maladie. En particulier, j'ai fait l'hypothèse que des bactéries commensales isolées du microbiote pulmonaire (récemment décrit) pourraient, par un effet local, fortement modifier cette réponse comme c'est le cas dans l'asthme, chez la souris. Dans un premier temps, j'ai montré que certaines bactéries du microbiote pulmonaire, administrées à des souris saines par voie intranasale, ont une forte capacité à moduler les lymphocytes T CD4⁺ des poumons tels que les Th1 ou Th17, impliqués dans l'immunité pro-inflammatoire, et les T régulateurs (Treg) réduisant l'activation immunitaire. En particulier, elles induisent la prolifération d'un sous type de Treg exprimant ROR γ t (facteur de transcription caractéristique des Th17), nommé ROR γ t⁺ Treg, récemment identifié dans l'intestin où l'activation de ces cellules par le microbiote limite les maladies inflammatoires de l'intestin comme la colite. Nous avons montré pour la première fois que ces cellules sont induites dans les poumons de souris traitées avec des bactéries isolées du microbiote pulmonaire appartenant aux genres bactériens *Lactobacillus*, *Staphylococcus* et *Neisseria* et avons caractérisé leur phénotype. Comme dans l'intestin, ces cellules semblent avoir un fort potentiel anti-inflammatoire, soutenu par leur forte expression des molécules inhibitrices CTLA-4, ou PD-1, du marqueur d'activation ICOS et de la cytokine anti-inflammatoire TGF- β associée à une faible sécrétion de cytokines pro-inflammatoires telles que le TNF- α . De façon intéressante, j'ai observé que des souches de *Lactobacillus* pulmonaires induisent les mêmes populations leucocytaires dans le modèle murin de tuberculose que dans des souris naïves, et notamment les ROR γ t⁺ Treg. Tandis qu'aucune des souches testées ne diminuent la charge bactérienne de *M. tuberculosis* dans les poumons ou la rate des souris infectées, une souche de *Lactobacillus murinus* (CNCM I-5314) qui augmente fortement les Th17 et les ROR γ t⁺ Treg, réduit l'infiltration leucocytaire pulmonaire, suggérant sa capacité à réduire l'inflammation associée à cette infection. Bien que la détermination du rôle des Th17 et des ROR γ t⁺ Treg dans ce phénotype reste à élucider, nos résultats démontrent d'ores et déjà que l'administration de bactéries commensales pulmonaires peut fortement moduler l'immunité locale même au cours d'infections comme la tuberculose. Une meilleure caractérisation des composants du microbiote pulmonaire et des mécanismes par lesquels ils interagissent avec notre système immunitaire pour maintenir la santé respiratoire devrait donc permettre l'émergence d'une nouvelle génération de probiotiques, d'origine pulmonaire, pour prévenir et améliorer le traitement des maladies respiratoires.

Abstract

Use of lung microbiota strains to shape local immunity at steady state and during *Mycobacterium tuberculosis* infection in the mouse model

Human mucosal sites (such as the gut) are colonized from birth by trillions of microorganisms that form the microbiota (or commensal flora), living in symbiosis with our organism. Survival of the host and of its microbiota is dependent on the activation status of our immune system. While poor immune activation results in sensitivity to infections, its uncontrolled activation or inflammation compromises host tissue integrity. Bacteria from the gut microbiota naturally interact with cells of our immune system to preserve an equilibrium. Administration of such commensals as probiotics improve many disease outcomes and is currently studied to improve respiratory infection treatment. The primary infectious cause of death, tuberculosis, a respiratory disease caused by *Mycobacterium tuberculosis*, involves an over-activation of the immune system causing lung damages. In this thesis project, I investigated whether bacterial strains from the microbiota influence the immune response in tuberculosis to assess the potential of probiotics to improve tuberculosis treatment. In particular, we hypothesized that pulmonary commensal bacteria modify the local immune response to tuberculosis, as recently demonstrated in an asthma murine model. Through intranasal administration in mice, I first identified different lung commensal bacterial strains with a strong ability to modulate the lung CD4⁺T cell compartment at steady state. Indeed, these strains induced T-helper (Th) cells involved in pro-inflammatory immunity, such as Th1 and Th17, and regulatory T cells (Treg) involved in anti-inflammatory responses. In particular, they increase proliferation of a specific Treg subtype, expressing ROR γ t (a transcription factor characteristic of Th17). These ROR γ t⁺ Treg were recently described in the gut, where they are induced by the microbiota and are able to decrease inflammation occurring in the mouse model of colitis. We show for the first time that these cells are induced in the lungs of mice treated with pulmonary bacterial strains from the *Lactobacillus*, *Staphylococcus* and *Neisseria* genera, and characterize their phenotype. As in the gut, these cells seem to have a strong anti-inflammatory profile, supported by their high expression of the inhibitory molecules CTLA-4 and PD-1, activation marker ICOS, and suppressive cytokine TGF- β ; associated to a poor production of pro-inflammatory cytokines such as TNF- α . Interestingly, I demonstrate that pulmonary *Lactobacillus* strains induced the same lung leukocyte populations in the mouse model of *M. tuberculosis* infection as in naïve mice, including ROR γ t⁺ Treg. While none of the tested strains reduced *M. tuberculosis* burden in lung or spleen, the *Lactobacillus murinus* (CNCM I-5314) strain, which induce a high number of Th17 and ROR γ t⁺ Treg accompanied by a reduced leukocyte infiltration in the lung, suggesting a capacity to reduce lung inflammation associated with *M. tuberculosis* infection. The role of Th17 and ROR γ t⁺ Treg in this phenotype remains to be elucidated. Nevertheless, our results clearly indicate that the administration of pulmonary commensal bacteria strongly modulate the local immunity, even during chronic infections such as tuberculosis. Therefore, a better characterization of the lung microbiota components and of the mechanisms by which they interact with our immune system to maintain health in the respiratory system, might lead to the emergence of a new generation of probiotics, of lung origin, to better prevent and treat pulmonary diseases.

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Abbreviations

ADC	Albumin Dextrose Catalase	EAE	Experimental autoimmune encephalomyelitis
AHR	Aryl hydrocarbon receptor	EDTA	Ethylenediaminetetraacetic acid
AM	Alveolar macrophage	ELISA	Enzyme-Linked Immunosorbent Assay
AMP	Antimicrobial peptide	ESAT-6	6 kDa early secretory antigenic target
APC	Antigen presenting cell	FBS	Fetal Bovine Serum
BAL	Broncho-alveolar lavage	FMO	Fluorescence Minus One
BHI	Brain Heart Infusion	Foxp3	Forkhead box P3
BMDC	Bone marrow derived dendritic cell	FSC	Forward Scatter
BSL	BioSafety Level	GPR	G-protein coupled receptor
CD	Cluster of Differentiation	GRAS	Generally Recognized As Safe
cDC	conventional dendritic cell	h	Hour
CFSE	Carboxyfluorescein succinimidyl ester	HDM	House dust mite
CFU	Colony forming unit	HE	Hematoxylin and Eosin
CFU	Colony Forming Unit	i.g.	Intragatric
CNCM	National Collection of Micro-organism Culture	i.n.	Intranasal
CNRS	National Center for Scientific Research	i.p.	Intraperitoneal
CNRZ	National Collection of Zootechny Research	i.v.	Intravenous
COPD	Chronic obstructive pulmonary disease	IBD	Intestinal bowel diseases
CSB	Cell Staining Buffer	ICOS	Inducible T cell costimulator
CSF2 (GMCSF)		IDO	Indoleamine-2,3-dioxygenase
CTLA-4	Cytotoxic T-lymphocyte antigen-4	IFN	Interferon
CX3CR1	CX3C chemokine receptor 1	Ig	Immunoglobulin
DC	Dendritic cell	IL	Interleukine
DNA	Deoxyribonucleic acid	IM	Interstitial macrophages
DPBS	Dulbecco's Phosphate-Buffered Saline	INRA	National Institute of Agricultural Research
DSS	Dextran sulfated sodium	IPBS	Institute of Pharmacology and Structural Biology
		KLRG1	Killer cell lectin-like receptor G1

Ln	normal logarithm	PMN	Polymorphonuclear
LPS	Lipopolysaccharide	PPD	Purified protein derivative
LXA4	Lipoxin A4	RNA	Ribonucleic acid
MAMP	Microbe associated molecular pattern	ROR γ	RAR-Orphan Receptor gamma
MDR	Multi-drug resistant	ROS	Reactive oxygen species
MHC-II	Major histocompatibility complex type II	RPMI	Roswell Park Memorial Institute
min	Minute	RT	Room Temperature
MLN	Mesenteric lymph node	RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
M-MLV	Moloney Murine Leukemia Virus	SCFA	Short chain fatty acid
MMP-9	Matrix metalloproteinase 9	SFB	Segmented Filamented Bacteria
MRS	Man, Rogosa and Sharpe medium	SPF	Specific pathogen free
MUC2	Mucin 2	SPF	Specific Pathogen Free
NLR	NOD like receptor	SSC	Side Scatter
OADC	Oleic Albumin Dextrose Catalase	TCR	T-Cell Receptor
OD	Optical density	TGF	Tumor Growth Factor
PBS	Phosphate Buffered Saline	Th	Lymphocyte T helper
PD-1	Programmed cell death-1	TNF	Tumor Necrosis Factor
PFA	ParaFormAldehyde	Treg	Regulatory T cell
PGE2	Prostaglandin E2	TSLP	Thymic stromal lymphopoietin
PMA	Phorbol Myristate Acetate	WT	Wild type
		XDR	Extensively drug resistant

Introduction

Since the demonstration by Louis Pasteur that we are living in a world of microbes, and the discovery by Robert Koch that some of these, the pathogens, can cause human infectious diseases and epidemics, the understanding of how our immune system deals with this threat has been an intense area of research. A recent concept in immunology suggests that host defense mechanisms allowing the host survival to infection follow two strategies: resistance and disease tolerance (Schneider and Ayres, 2008; Medzhitov, Schneider and Soares, 2012). The former, and most described, comprises inflammation and microbicidal processes allowing the host to kill pathogens. However, pathogen elimination by resistance mechanisms can have deleterious consequences for the host (such as impairment of tissue structures or functions), known as immunopathology. For this reason, the latter strategy known as disease tolerance has a neutral or positive impact on the pathogen survival, but is essential to the host health because it prevents pathogen-mediated damages and immunopathology through tissue repair, neutralization of toxins, metabolic homeostasis and regulation of inflammation. Importantly, the concept of disease tolerance addressed in this manuscript is a different one from central immune tolerance (occurring in the thymus, where lymphocytes are generated), which grants unresponsiveness to self-antigens. Yet, disease tolerance may include mechanisms of peripheral immune tolerance that prevent over-reactivity of the immune system to allergens and some bacteria.

Usually, equilibrium between the two arms of immune defense is attained with relation of the microbes that inhabit in our bodies, a clear reflection that the majority of our interactions with microbes do not result in a pathogenic outcome. Indeed, even healthy individuals harbor at least as much bacteria than eukaryotic cells, for an estimate of around 10^{13} bacteria belonging to different phyla and families, along with viruses, fungi, archaea and protozoa, which altogether form a resident community called the microbiota (Lloyd-Price, Abu-Ali and Huttenhower, 2016; Sender, Fuchs and Milo, 2016). As long as these microorganisms remain contained within the established limits at mucosal sites, their cohabitation with our body is not only harmless, but also it appears essential for important physiological functions. Recent findings suggest that, similarly to pathogens, beneficial microorganisms can manipulate biological processes in the host, such as resistance and tolerance mechanisms, to improve their own fitness. Yet, while pathogen fitness has detrimental consequences for the host, beneficial microbe fitness is obtained through promotion of the host health and fitness (Ayres, 2016). Therefore, microbiota fitness is improved in absence of pathogens. Consequently, among behaviors acquired by beneficial microorganisms, some result in direct inhibition of pathogens growth, and others modulate resistance and tolerance mechanisms of the host, leading to better infection outcome. This opens new avenues to the development of microbiota-based therapies against infection. One of the best examples of a pathogen altering the resistance/tolerance balance to promote its own fitness is *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The main objective of this introduction is to provide a conceptual framework supporting the use of microbiota-targeting strategies to harness the immune response in respiratory infectious diseases, like tuberculosis, which was the central theme of this thesis.

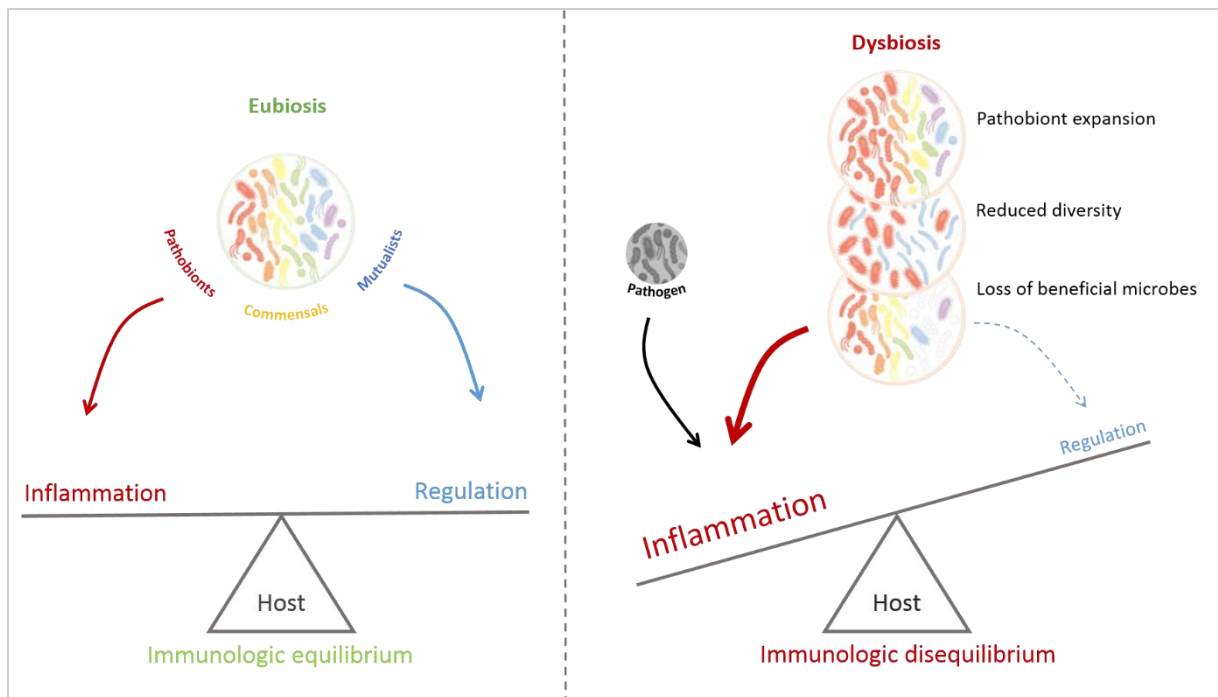


Figure 1: Equilibrium in the microbiota composition is required for the host health. At homeostasis, the balanced presence of symbionts or mutualists (with health promoting functions), commensals (with a neutral effect), and pathobionts (with the potential to induce pathology), is associated with protective functions benefiting the host and is controlled by a low level of inflammation. Genetic variations or environmental insults (*e.g.*, antibiotic use, diet) may perturb this microbiota composition, leading to dysbiosis, characterized by pathobiont expansion, loss of diversity and/or loss of beneficial microbes. Dysbiosis can favor pathogen invasion and both often result in inflammation, leading to further commensal microbe elimination along with host tissue-damage, if not controlled. Figure adapted from (Round and Mazmanian, 2009; Petersen and Round, 2014).

Section 1: The microbiota, a key factor in host health and disease

I. The human body, a super-host in symbiosis with its microbiota guest

1. Characteristics of the host-microbiota interaction

At homeostasis, the microbiota and the host have a relationship called mutualistic symbiosis (implying mutual benefit). On the one hand, the host provides the microbiota with a niche, including nutrient sources (such as non-digestible fibers), and controlled environmental parameters. On the other hand, the microbiota metabolism produces compounds important for the host such as B group vitamins or short chain fatty acids (SCFA) that can be transformed in glucose by the host cells (LeBlanc *et al.*, 2017). Microbiota is also essential for correct host growth, mucosa development and immune homeostasis. However, such beneficial interaction is fragile and require equilibrium of multiple factors. For example, within the microbiota a balance is necessary between mutualist symbionts (having a beneficial effect for the host), commensals (having a neutral effect) and pathobionts (usually harmless for the host but becoming pathogenic if over-represented) (Round and Mazmanian, 2009). Even if commensals and pathobionts are not directly beneficial to the host, they may be needed to obtain the global microbiota protective role due to the complex interactions among microbiota organisms. For instance, some bacteria sustain the growth of other species, which are directly beneficial for the host or produce substrates transformed by other species in host accessible compounds. Such cooperation has been observed in the conversion of fibers in metabolites (Dethlefsen, McFall-Ngai and Relman, 2007). In addition, the status of one microbe can vary depending on the environment. An equilibrated microbiota composition, in adequation with host health is called eubiosis. Perturbation of microbiota composition, so-called dysbiosis, is observed in many diseases (as exemplified in different sections of this chapter). Dysbiosis can be characterized by the loss of beneficial microbes, expansion of pathobionts, loss of microbiota diversity, which are all associated with bad outcomes, such as infection and over-inflammation (Figure 1) (Petersen and Round, 2014). Homeostasis between the host and its microbiota also relies on an adequate immune activation. While microbial growth limitation by the host immune system is necessary to inhibit bacterial translocation, too much reactivity of our immune system against the resident microbes may result in collateral damages, such as direct harm to tissue integrity. Interestingly, it seems that during time, both the human body and its microbiota settler evolved to achieve an equilibrium favoring health. Thus, analyses of the microbiota composition variability between individuals suggest co-evolution between the host and its microbiota (considered as a super-host or holobiont) (Dethlefsen, McFall-Ngai and Relman, 2007). This hypothesis is supported by the fact that phylogenetically close host species harbor more similar microbiota than distant ones. For instance, human microbiota composition is more similar to Old World monkeys and apes than from New World primates and lemurs (Moeller *et al.*, 2016; Davenport *et al.*, 2017). This suggest that, in a healthy symbiotic situation, the microbiota composition and niches provided by the host should be adapted to the needs of the super-host.

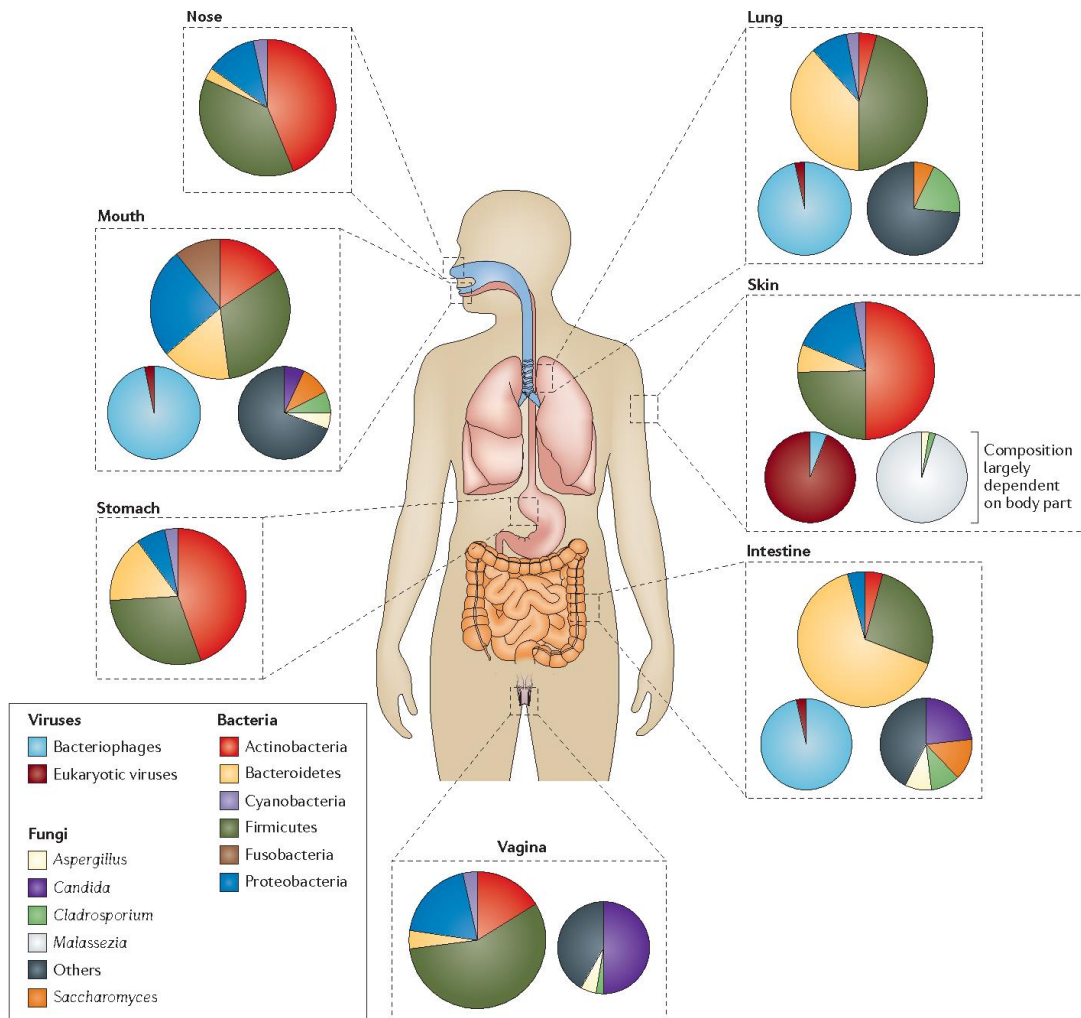


Figure 2: Microbiota composition at different mucosal sites in the human body. The microbiota is mainly composed of bacteria, viruses, and fungi. While the same microbial families are present in all mucosa, their relative proportion and composition (in term of species) is highly variable. For example, the human intestinal and lung bacteria are dominated by Bacteroidetes and Firmicutes, while Actinobacteria dominate the skin microbiota. Figure from (Marsland and Gollwitzer, 2014).

2. Structure of the microbiota

a) Components of the microbiota: beyond bacteria

Most microbes from the microbiota inhabit the gut mucosa. However, the skin, vagina, and even the lungs, are also the home of diverse microorganisms. Advances in sequencing and culture methods during the last decades improved knowledge about microbiota composition at various mucosal sites (Figure 2). They have also highlighted the presence of non-bacteria organisms such as viruses, fungi, archaea and protozoa in the microbiota. The virobiota (or virome) is composed of many bacteriophages (approximately 10^{15} in the human intestine) and some eukaryotic viruses (~ 8% of the sequences) (Neil and Cadwell, 2018). The mycobiota (or mycobiome) is less represented, accounting for up to 75 fungal genera, representing 0.1% of total gut microbiota (Hoffmann *et al.*, 2013). Viruses and fungi from the microbiota, as bacteria, directly modulate host physiology and the immune system. For instance, fungi are able to produce eukaryote metabolites such as prostaglandine (PGE)₂, an important immunomodulatory molecule; murine norovirus infection preserve intestinal morphology and immune system maturation impaired in absence of the whole microbiota, preventing susceptibility to different diseases (Underhill and Iliev, 2014; Neil and Cadwell, 2018). Viruses and fungi may also indirectly modulate host functions by altering bacteria composition of the microbiota. Indeed, bacteria can be killed through bacterial lysis by virus or by antibiotic produced by fungi. In addition, some fungi produce a matrix that can be used by certain bacteria to settle down (El-Jurdi and Ghannoum, 2017; Neil and Cadwell, 2018). The mycobiota and virobiota are highly susceptible to environmental changes, and their disruption can lead to disease caused by pathobiont (from bacterial, fungi or viral origin) outgrowth (Underhill and Iliev, 2014; El-Jurdi and Ghannoum, 2017). Archaea have also been detected, in particular *Methanobrevibacter*, as well as protozoa, but their role is not understood at the moment (Hoffmann *et al.*, 2013). Study of all these microorganisms is essential to better understand how the different compartments of the microbiota interact together and with the host. However, because their role in respiratory diseases is poorly described, the rest of this introduction is focused on the bacterial components of the microbiota.

b) Variation of the microbiota bacterial composition depending on body site

Predominant bacterial phyla are very similar in all body sites. They include Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Cyanobacteria. However, they are present in different abundance and are composed of different genera depending on body sites (Figure 2) (Cho and Blaser, 2012; Hoffmann *et al.*, 2013; Marsland and Gollwitzer, 2014). This is not surprising because microbial composition is largely dependent on constraints from the environment that comprise nutrient availability, peristalsis rate, pH, oxygen levels, immune system, and host derived molecules (Dethlefsen, McFall-Ngai and Relman, 2007; Tropini *et al.*, 2017). Similarly, bacteria are not randomly distributed but form different communities in each mucosa site. For instance, it is the case in the intestinal tract harboring the highest microbial density. Colonization of this mucosa mainly results from ingestion of bacteria present on food. During digestion, the bacteria will travel through different compartments with very distinct properties (Figure 3). In the small intestine, the pH is low and transit is fast, favoring facultative anaerobes with rapid growth based on simple sugar metabolism, such as Proteobacteria and *Lactobacilli*. By contrast, in the colon, the transit is slow and oxygen level low, favoring anaerobic bacteria that ferment host-indigestible plant polysaccharides or use host mucus to settle down. Of note, the colon and cecum contain the highest density and diversity of microbes (Ohland and Jobin, 2015; Tropini *et al.*, 2017). They are dominated by Firmicutes and Bacteroidetes, including a majority of *Clostridium*, *Bacteroides* and *Prevotella* species (Rinninella *et al.*, 2019).

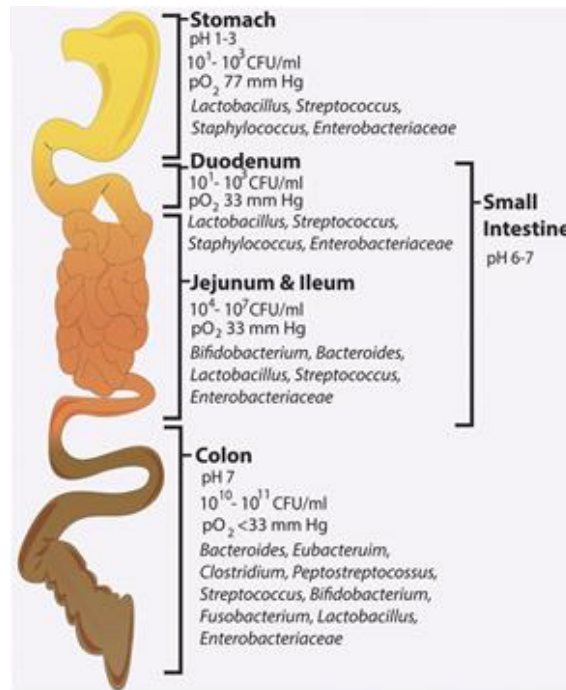


Figure 3: Bacterial microbiota gradient along the human digestive tract. The localization and spatial organization of the microbiota depends on parameters such as oxygen tension (pO₂) and pH among others, which vary along the gastrointestinal tract. Density and specie richness increase substantially in the colon and cecum. In these compartments, through fiber digestion, species will release SFCA having many immunomodulatory effects. Figure adapted from (Clarke *et al.*, 2019; Maynard, 2019).

c) Inter-individual variations in microbiota composition

Whereas rough microbiota composition is similar between individuals of the same species, inter-individual variability does exist. Indeed, among the approximately 1000 species that can be found in the gut microbiota, each individual carry a combination of “only” around 160 species in proportion as unique as a fingerprint (Qin *et al.*, 2010). This composition is dependent on genetic factors that may reflect differences in the habitat provided to the microbiota or in immune response components. In many diseases with a genetic component, such as intestinal bowel diseases (IBD), microbiota dysbiosis is often observed and exacerbates the pathologic outcome (Ley *et al.*, 2006; Belkaid and Hand, 2014). In healthy individuals, microbiota composition is more similar between family members than between unrelated individuals (Zoetendal *et al.*, 2001) and some members of the microbiota seem to be inheritable (Goodrich *et al.*, 2014). However, environmental factors are thought to play a more important role. For instance, microbiota similarity between family members may result from close contact and similar living habits rather than from genetic components. In this line, a recent study revealed that spouses (having close relationships) have a more similar microbiota than siblings (Song *et al.*, 2013; Dill-McFarland *et al.*, 2019). Furthermore, genetic-induced dysbiosis responsible for diseases can be counteracted by changing the environmental factors impacting the microbiota composition, ultimately improving patient health (L. Zhao *et al.*, 2018). Despite this high variation among the human population, some bacterial species are present in most healthy individuals forming the “core-microbiome” (Tap *et al.*, 2009). In addition, there is redundancy in functions exerted by different microbes that are important for the host health. These two mechanisms allow quite different microbiota at eubiosis to have similar functions promoting the host health while major variations leading to dysbiosis are deleterious.

3. How to study functions of the microbiota?

The functions of the microbiota promoting health at homeostasis are complex and so intricate with the host physiology that they are difficult to identify. A major approach used for the identification of microbiota functions has been to assess the consequences of microbiota dysbiosis to host health. Due to the high inter-individual variability and diverse impact of environmental factors on the microbiota composition, the study of the microbiota usually involves several steps and experimental models (Gilbert *et al.*, 2018). In general, the first step is the direct comparison of the microbiota of patients and healthy subjects, generating important correlations between dysbiosis and disease. A second approach is the use of laboratory animals having controlled genetic background and environment to prove the microbiota involvement in a given pathological disorder. Animal models are also needed to identify the microbiota species and the accompanying mechanisms involved in the process of question. These findings are then translated and validated in patients. Knowledge on the mechanisms and strains involved in health or disease can then provide the basis for the development of microbiota-based therapies to improve health (as detailed in Section 1.IV).

Different animal models harboring no or altered microbiota have been developed as tools for loss-of-function strategies. Germ-free (axenic) animals, born from Caesarian section under a sterile environment, can be maintained axenic if kept under such sterile environment (Smith, McCoy and Macpherson, 2007). The use of axenic mice has been critical to demonstrate the role of the microbiota in many biological processes. In particular, they were used to show that absence of microbes from birth results in defect in development of the host and of its immune system (as detailed in Section 1.II). However, these developmental consequences are pleiotropic and could affect the interpretation of

the consequences of a given biological process in adult mice. For this reason, axenic mice are sometimes replaced by other models to study the impact of the microbiota during particular challenges such as pathogenic infection. Alternatively, animals can be treated with antibiotics to deplete their microbiota for a specific period of time. A drawback of this method is that total depletion of microbiota is rarely achieved; however, a very useful advantage is that specific antibiotic regimens can be used to identify the type of microorganism involved in a given phenotype (Al Nabhani *et al.*, 2019). The gnotobiotic model provides a gain-of-function model used in the microbiota field. It consists in recolonization of germ-free animals either by co-housing germ-free with control microbiota-containing animals, or by oral gavage with fecal content from control animals, or with specific bacterial strains. This latter point is particularly useful to prove the involvement of specific bacterial species to a given biological process. This model also allows translational studies as germ-free animals can be colonized by human microbes (isolated from feces samples). In particular, recolonization with microbiota from patients *versus* healthy donors can demonstrate a specific causal effect in the development of a given disease (Charbonneau *et al.*, 2016).

Most studies in the field have been performed using Specific Pathogen Free (SPF)¹ mice as animal model. They do possess a complex gut microbiota, but somehow different from wild animals and with a reduced diversity. In particular, abundance of microbes in their respiratory tract is low (Yun *et al.*, 2014). In addition to mice, other animal models have also been developed. For example, studies in fruit flies, having a very simple microbiota (composed of approximately 10 species), can facilitate the characterization of mechanisms in host-microbiota interaction that are conserved throughout evolution of complex multi-cellular organisms before validation in species closer to humans (Schwarzer, Strigini and Leulier, 2018).

These different approaches have allowed researchers to unravel many essential functions of the microbiota and to decipher interactions between components of the microbiota and the host that lead to health or disease as exemplified in the following section.

4. Factors influencing microbiota composition and consequences for the host health

Microbiota acquisition is highly impacted by environmental factors. While microbiota dysbiosis is usually a reversible process, alteration of microbiota acquisition seems to have dramatic long-term consequences. This is because the microbiota have a key role in host development, as observed from studies in germ-free animals having impaired organ development and metabolic activities among other dysfunctions (Smith, McCoy and Macpherson, 2007). Likewise, the immune system development is also highly impacted by alteration of microbiota acquisition, but this concept will be addressed in part II of this section.

The presence of microbes in the placenta remains controversial (Noel T. Mueller *et al.*, 2015). Currently, most studies suggest that even if few microbes are present there, fetus do not possess a complex microbiota (Hansen *et al.*, 2015; Dominguez-Bello *et al.*, 2019). However, the gut and vaginal maternal microbiota undergo major changes during pregnancies (Aagaard *et al.*, 2012; Koren *et al.*, 2012) that may indirectly impact the fetus development. During labor and birth, newborns are highly exposed to microbes, in particular to the maternal vaginal and perineal fecal microbes. Thus, upon birth, babies are covered by microbes and have swallowed many of them coming from their mother

¹ The **SPF** sanitary status refers to laboratory animals kept in specific environmental conditions whereby a number of most of the known chronic and latent persistent pathogens are excluded.

microbiota, initiating their own colonization (Dominguez-Bello *et al.*, 2010). This first microbe colonization is very important. Indeed, children born by Cesarean section are at risk to develop celiac disease, asthma, type 1 diabetes or obesity, later in life (Kero *et al.*, 2002; Algert *et al.*, 2009; Decker *et al.*, 2010; Huh *et al.*, 2012; Mårild *et al.*, 2012; Blustein *et al.*, 2013; Noel T. Mueller *et al.*, 2015). Unlike naturally born babies, these newborns are first colonized by microbes closer to their mother skin microbiota with lower *Lactobacillus* species abundance compared to naturally born infants (Biasucci *et al.*, 2010; Dominguez-Bello *et al.*, 2010; Nagpal *et al.*, 2016). They also displayed an important delay and contrasting microbiota establishment at different sites such as the gut or lung (Grönlund *et al.*, 1999; Bosch *et al.*, 2016). Even if these differences are mainly transient, they may impair host function development occurring early after birth (Salminen *et al.*, 2004). Apart from birth delivery, altered mother microbiota composition (e.g., in the case of IBD) also leads to deficient microbiota colonization in newborns, which is directly associated with abnormal imprinting of the immune system (Torres *et al.*, 2019).

Following this first colonization, microbes (mainly *Enterobacteria* in naturally born child) will settle down in different places depending on site-specific environment, and they will influence future colonization by additional waves of microbes acquired from different sources. One of them comes from the alimentation. Contrary to formula milk, maternal milk contains many bacteria including *Lactobacillus* and *Bifidobacteria* species as well as oligosaccharides favoring the growth of these bacterial species, and will therefore shape the progressive colonization of the infant mucosa (Coppa *et al.*, 2004; Knol *et al.*, 2005; Palmer *et al.*, 2007; Perez *et al.*, 2007; Solís *et al.*, 2010; Khodayar-Pardo *et al.*, 2014). The first waves of microbe settlers may be important for essential organ development processes. For instance, presence of *lactobacillus* species in the respiratory tract were found to be required for alveoli structuration occurring during microbiota colonization in the mouse model (Yun *et al.*, 2014; Man, De Steenhuijsen Pijters and Bogaert, 2017). Conversely, malnutrition in young children is associated with impaired colonization by strains essential for the host correct growth (Schwarzer, Strigini and Leulier, 2018). Administration of *lactobacillus plantarum* WJL strain restores the normal growth of malnourished germ free fruitflies and mice, highlighting the importance of certain strains of the microbiota in host development (Storelli *et al.*, 2011; Blanton *et al.*, 2016; Schwarzer *et al.*, 2016).

The weaning period is also a crucial step, where the start of consumption of solid food by young children will progressively alter their microbiota composition shifting towards that of adults (Palmer *et al.*, 2007) (Figure 4). Expansion of the microbiota composition at this age is, for example, crucial to shape the immune system and intestinal maturation (Hooper *et al.*, 2001). Presence of indigestible carbohydrates favors the presence of *Bacteroides*, *Parabacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium* species and of *Faecalibacterium prausnitzii*, which are main producers of SCFA (Lloyd-Price, Abu-Ali and Huttenhower, 2016). The type of solid food introduced, depending on cultural determinants, will be a major factor determining microbiota composition. Increased fiber associated with decreased sugar, meat and fat, seems to promote bacterial richness in the human gut. For instance, the metagenome of some non-Western human populations (with an alimentation based on hunting and gathering, and sometimes agriculture) has different bacterial phyla proportion and is composed of more species than those of Western populations (Obregon-Tito *et al.*, 2015; Davenport *et al.*, 2017; Tanaka and Nakayama, 2017; Rinninella *et al.*, 2019). A shift in microbiota composition is also observed in vegetarians, suggesting that diet highly affects the microbiota composition even in adulthood (David *et al.*, 2014).

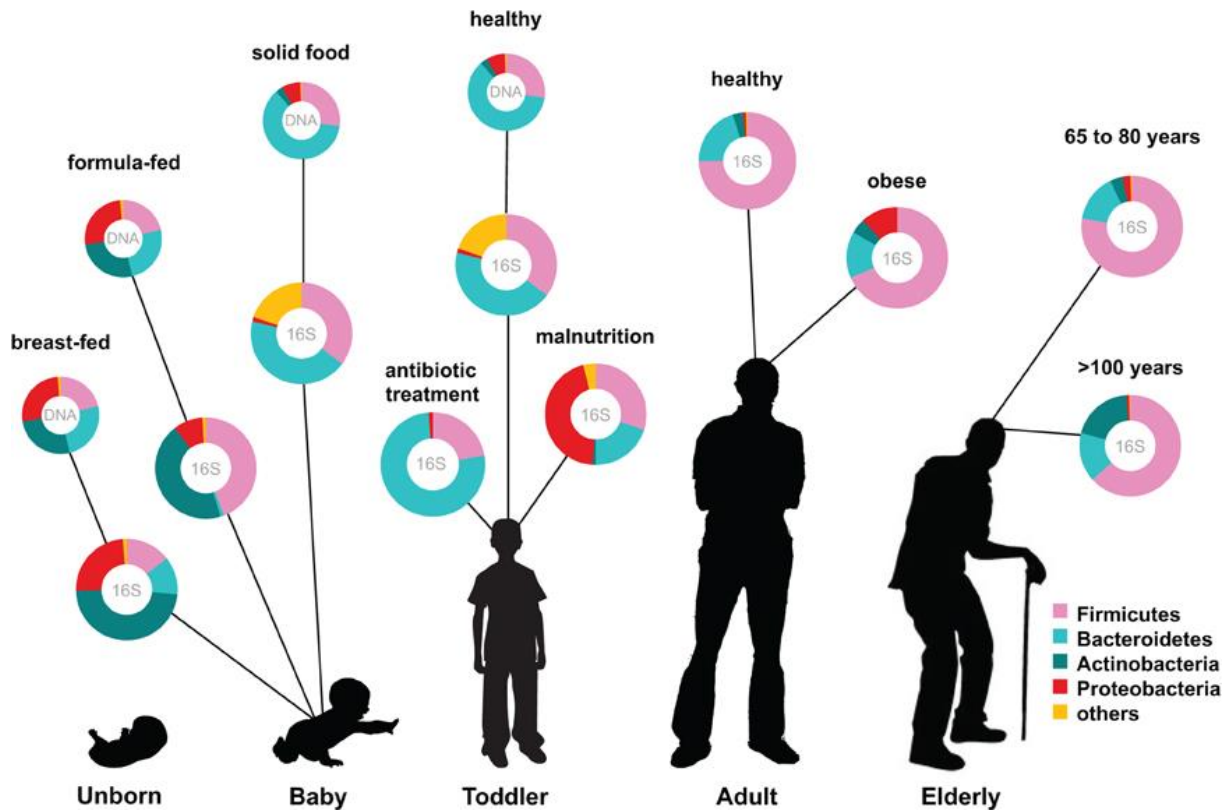


Figure 4: Microbiota composition and alteration during lifespan. Since acquisition of the first commensals at birth, the microbiota composition is enriched during infancy, soon resembling to the adult one, depending on environmental factors such as alimentation and presence of microbes in the environment. Thereafter, the microbiota composition globally remains stable until old-age, where its richness decreases. The graphs provide a global overview of the relative abundance of key phyla of the human microbiota in different stages of life measured by either 16S RNA or metagenomic approaches (DNA sequencing). Figure from (Ottman *et al.*, 2012).

Other environmental factors can explain differences between populations around the world and in particular the reduced diversity of microbiota observed in Western populations. The “hygiene hypothesis” suggests that individuals in rich-industrialized countries will experience a lower exposure to microbes due to the highly urbanized and sterile environment, easy access to antibiotic treatment and strong likelihood of vaccination during infancy, which will in turn alter immune responses (Strachan, 1989; Jernberg *et al.*, 2007; Budden *et al.*, 2017). In line with this hypothesis, antibiotic consumption before and after birth alters colonization by the microbiota, resulting in decreased proportions of *Lactobacilli* and *Bifidobacteria* in neonates. This has then long-term consequences, such as increased propensity to obesity and allergy (N T Mueller *et al.*, 2015). On the contrary, living in a microorganism-rich, environment (*e.g.*, farms), correlates with an increased diversity of microbial exposure and a decreased incidence of allergies (Riedler *et al.*, 2001; Ege *et al.*, 2011).

Low microbiota density is also critical in case of infection. Indeed, apart from their immunomodulatory role (see part II of this section), microbiota species directly compete with pathogens (Croswell *et al.*, 2009; Buffie *et al.*, 2012; Kamada *et al.*, 2013). The presence of microbiota in their niche limits space and nutrients available for pathogens, a phenomenon known as colonization resistance (Buffie and Pamer, 2013). Some species of the microbiota also produce toxic compounds, such as bacteriocins, which may directly impair pathogen growth (Schamberger and Diez-Gonzalez, 2002; Hammami *et al.*, 2013; Kommineni *et al.*, 2015; Sahoo *et al.*, 2015). Eventually some microbiota members can modify the environment; for instance, *Lactobacillus species* produce lactic acids that reduce pH to limit vagina colonization by pathogens (Turovskiy, Sutyak Noll and Chikindas, 2011).

However, interactions between microorganisms at mucosal sites are not limited to competition. Indeed, some microbiota species are known to release products used as nutrients by other species, including pathogens, favoring their colonization. Antibiotic treatment may have drastic consequences by altering such interactions. Indeed, dysbiosis of the microbiota induced by antibiotics may promote growth of bacterial species favoring pathogen colonization, or eliminate bacteria involved in colonization resistance allowing antibiotic-resistant pathogens to persist for years (Dethlefsen, McFall-Ngai and Relman, 2007; Pacheco *et al.*, 2012; Ng *et al.*, 2013; Ferreyra *et al.*, 2014; Theriot *et al.*, 2014; Sassone-Corsi *et al.*, 2016). Such mechanisms must be at play in *Clostridium difficile* infection as this bacteria is well known to colonize mainly individuals that have followed antibiotic treatment causing an alteration of the microbiota composition (Buffie *et al.*, 2012; Theriot *et al.*, 2014; Jenior *et al.*, 2017).

Some lifestyles known to be beneficial for the host are also linked with microbiota composition. Living with pets or practicing exercise are often associated with a healthy microbiota composition. Conversely, high levels of stress, sleep deprivation and smoking, for example, correlate with dysbiosis (Gilbert *et al.*, 2018). During the last centuries, dramatic changes in our living habits, including diet change and lower microbial exposure, have changed the environment we provide to our microbe settlers. This disturb the microbiota composition selected through millenaries of co-evolution for optimal immune functions, leading to the rise of pathologies resulting from misdirected immune response against self, microbiota components or environmental antigens (Dethlefsen, McFall-Ngai and Relman, 2007; Chow *et al.*, 2010; Belkaid and Hand, 2014). Not only microbiota beneficial functions may be absent, but this may even transform these allies into enemies. Therefore, better understanding the interaction between the host immune system and the microbiota in health and disease is key to reduce disease incidence and improve disease diagnosis and treatment.

II. Gut microbiota interaction with the local immune system

Because mucosal sites are exposed to many microbes and particles, an adequate balance between immune resistance and tolerance mechanisms is critical to maintain homeostasis. In the intestine, this is achieved by the containment of the microbiota at distance from tissue, the presence of efficient phagocytes that can kill invading bacteria, and the activation of B and T cells in secondary lymphoid organs draining the intestine, promoting restriction and tolerance toward these bacteria. As detailed in this section, this efficient mucosal immunity largely depends on the microbiota.

1. The mucosal firewall: containment of the microbiota for intestinal homeostasis

Intestinal homeostasis relies on the limitation of host tissue invasion (by commensals and pathogens) and of immune activation against harmless bacteria. Interestingly, as detailed in this section, several mechanisms involved in mucosal immune response fulfill both functions.

a) Physical barrier, the mucus layer

The intestinal mucosa structure favors intestinal homeostasis. Indeed, in the intestine and particularly in the colon, a stratified layer of mucus containing the MUC2 mucin produced by goblet cells separates most bacteria from host cells. It is composed of the outer mucus layer in contact with the lumen, containing many bacteria, and of the inner layer, which is thick and dense, limiting bacterial access to the tissue. This physical separation limits invasion of the lamina propria by the microbiota or by pathogens, and over-reaction of the immune system (Van der Sluis *et al.*, 2006; McGuckin *et al.*, 2011; Earle *et al.*, 2015; Tropini *et al.*, 2017). Disruption of the mucus layer, in MUC2-deficient mice or in dextran sulfate sodium (DSS) colitis model², results in an over-reaction of the immune system to microbiota members, causing colitis (Van der Sluis *et al.*, 2006). While most bacteria are kept away by this mechanism, other bacteria, such as *Bacteroides fragilis*, *Bacteroidetes thetaiotaomicron* or *Akkermansia muciniphila* are able to bind mucins of the mucus and thus benefit from a specific niche (Derrien *et al.*, 2004; Sonnenburg, 2005; Huang, Lee and Mazmanian, 2011). Some bacteria, such as Segmented Filamented Bacteria (SFB), are even able to adhere to epithelial cells (Hedblom *et al.*, 2018). As exemplified later, this proximity gives this bacterial family a unique opportunity to modulate immune functions.

b) Killing at proximity of the epithelium by AMP

AMP are produced by all type of intestinal epithelial cells and, in particular, by Paneth cells (Vaishnava *et al.*, 2008). AMP are composed of different type of proteins able to kill bacteria mainly through disruption of the bacterial cell wall or membrane. They include defensins, cathelicidins and specific members of the family of C-type lectins. Most of them, including many α -defensins, are produced constitutively, even in germ-free mice. Others, such as the C-type lectin regenerating islet-derived protein 3 γ (RegIII γ), are produced in response to microbial antigen recognition by Pattern Recognition Receptors (PRR)³ expressed by Paneth cells (Putsep *et al.*, 2000; Kobayashi *et al.*, 2005; Brandl *et al.*,

² **DSS** is a chemical agent inducing epithelial damages. Its administration in mice is one of the most widely used experimental models of IBD.

³ **PRR** are a wide family of receptors comprising Toll like Receptors (TLR), among other numerous families, present on epithelial and innate immune cells. Their combination with adaptor molecules, such as the myeloid differentiation primary response 88 (Myd88), allow sensing of pathogen, microbial or danger associated molecular pattern (PAMP, MAMP or DAMP) molecules, such as bacterial lipopolysaccharide (LPS). This signal

2007; Vaishnava *et al.*, 2008). AMP are mainly retained in the dense mucus layer, allowing bacterial persistence in the outer mucus layer and intestinal lumen, while restraining their presence near the epithelium (Meyer-Hoffert *et al.*, 2008). AMP production is a key mechanism in intestinal homeostasis as genetic polymorphisms in genes associated with Paneth cell development, bacteria recognition or AMP secretion, are risk factors for IBD (Hugot *et al.*, 2001; Ogura *et al.*, 2001; Wehkamp *et al.*, 2005; Cadwell *et al.*, 2008; Kaser *et al.*, 2008). In addition to AMP, proteolytic enzymes such as trypsin or cell wall degrading enzymes such as lysozyme are secreted by Paneth cells that all contribute to limitation of bacterial invasion (Chassaing *et al.*, 2014).

c) Efficient local innate immune response

Macrophages are also present in close contact with epithelial cells, and in the case of invasion, they will rapidly phagocytose bacteria crossing the epithelial barrier (Lee, Starkey and Gordon, 1985). These macrophages have high bactericidal capacity and are able to kill the captured bacteria through efficient production of reactive oxygen species (ROS)⁴ and AMP (Smythies *et al.*, 2005). Bacterial invasion is often allowed by epithelium disruption. In addition to bacterial killing, macrophages are also mediating efficient epithelium repair following injury through production of growth factor that enhance the proliferation of epithelial progenitors (Pull *et al.*, 2005).

Containment of the microbiota and pathogens also rely on the activation of T and B cells by antigen-presenting cells (APC). Professional APC comprise dendritic cells (DC), macrophages and B cells⁵, which are able to process foreign antigens and to present them to B and T cells on major histocompatibility complexes II (MHC-II). Lamina propria DC are mainly composed of two subtypes. CD103⁺ CD11b⁺ DC deriving from the common DC progenitor, exhibit a low antigen sampling capacity combined with high migratory properties and are involved in tolerance induction. CX3CR1⁺ CD103⁻ CD11b⁺ DC deriving from inflammatory blood-circulating monocytes are involved in effector T cell priming. Most macrophages highly express CX3CR1, have poor migratory properties and, during homeostatic conditions, display an anti-inflammatory profile (Rescigno *et al.*, 2001; Chieppa *et al.*, 2006; Rivollier *et al.*, 2012). Bacterial sampling in the gut mainly occurs in the Peyer's patch⁶ in the small intestine and isolated lymphoid follicles, located in both the small and large intestine, where bacteria can translocate through microfold cells (or M cells)⁷ and are then captured by lamina propria DC and macrophages (Figure 5) (Macpherson and Uhr, 2004; Kelsall, 2008).

transduction induces in general the activation of these cells toward an inflammatory profile that will promote pathogen killing and a global inflammatory response through secretion of chemokines and pro-inflammatory cytokines, which will recruit and activate other immune cells.

⁴ Oxygen radicals, such as **ROS**, are part of the molecules generated by innate immune cells after binding of PAMP through PRR in order to kill pathogens internalized through phagocytosis.

⁵ Unlike T cells, **B cells** can bind soluble antigens using their B cell receptor. They can then internalize and process the antigen that will be presented to helper T cells on MHC-II molecules. This interaction can result in the activation of cognate T cells. However, the role of B cells as APC in the context of the microbiota is less described than DC.

⁶ **Peyer's patches** are lymphoid follicles present in the small intestine lamina propria for the generation of immune response within the lamina propria. They consist of a dome area, B cell follicles and interfollicular T cells. In Peyer's patches the intestinal epithelium is characterized by few goblet cells (allowing bacteria to come closer) and the presence of microfold cells.

⁷ **Microfold cells** are particular epithelial cells present in mucosal-associated lymphoid tissue specialized in endocytosis, phagocytosis, and transcytosis of microbes from the lumen to the lamina propria, where macrophages or DC will uptake them to initiate a local immune response.

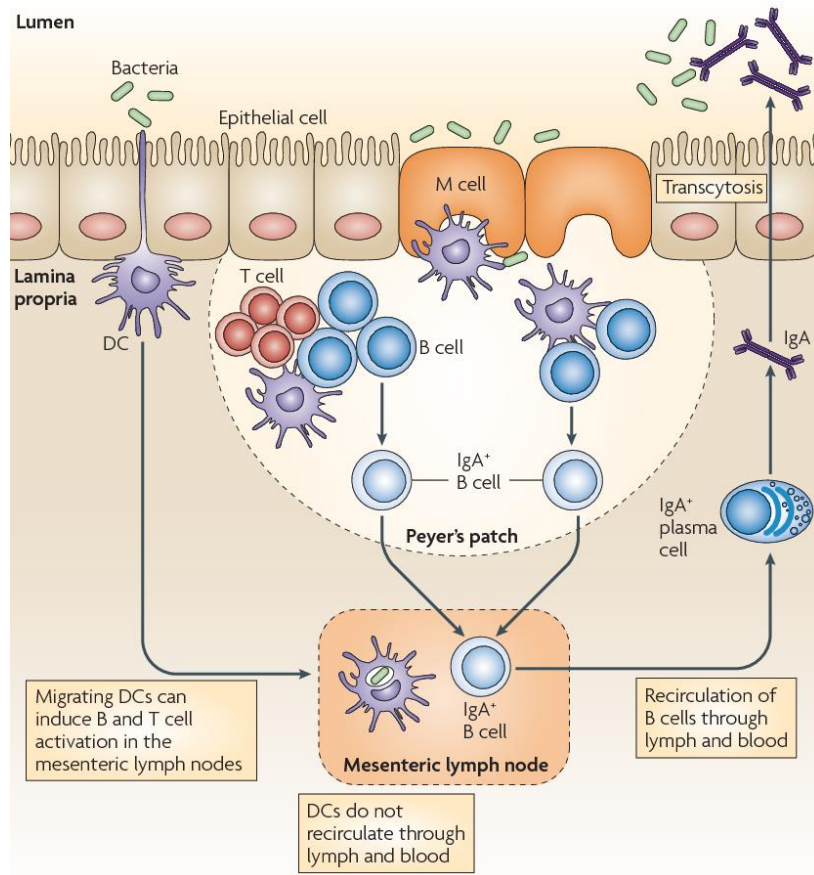


Figure 5: Establishing the physical limits and containment of intestinal microbiota by IgA. Commensal antigens can be captured by macrophages and DCs extending dendrites between epithelial cells or near M cells in Peyer's Patches. In Peyer's patches or mesenteric lymph nodes, presentation of commensal antigens by these DCs leads to the differentiation of commensal specific $CD4^+$ T cells, and induce B cells to differentiate into IgA-producing plasma cells. After homing to the lamina propria, these plasma cells secrete IgA that will be exported by transcytosis across epithelial cells to the lumen. Binding of these IgA to their targets prevents bacterial penetration of the lamina propria. Figure from (Hooper and Macpherson, 2010).

Alternatively, beyond these sites, CX3CR1^{high} APC are able to modify the tight junctions between epithelial cells, and squeeze their dendrites through these junctions to reach outside the epithelium and sample bacteria present at the epithelial cell surface (*e.g.*, SFB) (Rescigno *et al.*, 2001; Suzuki *et al.*, 2004; Chieppa *et al.*, 2006; Mazzini *et al.*, 2014).

Unlike other tissues, APC found in these sites will not induce systemic response after bacterial sensing. This is first explained by their poor capacity to produce pro-inflammatory signals (*e.g.*, Tumor Necrosis Factor (TNF- α)), contrasted by their generation of anti-inflammatory mediators (*e.g.*, PGE2) limiting the activation of neutrophils⁸ (Smythies *et al.*, 2005; Grainger *et al.*, 2013). Induction of systemic inflammation is also avoided by the limited migration capacities of lamina propria DC that are able to migrate to local mesenteric lymph nodes but not further, a mechanism also indirectly limiting pathogen dissemination *via* DC, which have poor bactericidal properties (Macpherson and Uhr, 2004). Nevertheless, these APC are able to induce a local adaptive immune response, as B and T cell follicles are present in the Peyer's patches and mesenteric lymph nodes. Macrophage and DC of the lamina propria having the capacity to rapidly eliminate invasive microbes, promote repair of epithelium damages, and induce adaptive immunity are therefore key mediators of resistance and tolerance mechanisms needed for mucosal homeostasis.

d) Restricted bacterial invasion by IgA

Upon antigen capture, some DC produce retinoic acid (a derivative of vitamin A) and the pro-inflammatory cytokine IL-6 or IL-5, to activate specific B cell (present in the Peyer's patch and isolated lymphoid follicles or mesenteric lymph node) Immunoglobulin (Ig) class-switch towards IgA-producing plasma cells⁹ (Macpherson and Uhr, 2004; Mora *et al.*, 2006). IgA are the main antibody class present in mucosa. Secreted dimeric IgA will be transferred to the lumen by transcytosis through epithelial cells. In the mucus layer, IgA will bind to both invading pathogen and commensal bacteria, limiting their presence at the cell surface and preventing lamina propria invasion (Figure 5). Interestingly, it seems that while DC will also activate cognate T cells favoring B cell differentiation, the induction of IgA specific for microbiota antigens is active even in the absence of T cells. This may allow a rapid IgA response to dysbiosis without inducing too much inflammation (Macpherson *et al.*, 2000; Hooper and Gordon, 2001; Hapfelmeier *et al.*, 2010). In addition, IgA may have an anti-inflammatory role on cells phagocytosing an opsonized bacteria, limiting microbial stimulation and preserving intestinal barrier integrity (Fernandez *et al.*, 2003; Peterson *et al.*, 2007; Boullier *et al.*, 2009).

Therefore, the mucus, AMP, and IgA and B cells, are part of the recently proposed type-4 immune response, dedicated to the exclusion of microorganisms and protection of tissue from potentially destructive type-1 or -3 inflammation (that are described in the following paragraph), thus participating both in resistance and tolerance (Eberl, 2016).

⁸ **Neutrophils** are main actors of the anti-microbial response, recruited from the blood to the tissue upon infection. They are highly potent for bacterial clearance after phagocytosis, and are able to form Neutrophil Extracellular Traps (NET). In this particular cell death, neutrophils release their DNA along with anti-microbial peptides that will form a scaffold that will immobilize and neutralize many pathogens (Boeltz *et al.*, 2019).

⁹ Binding of antigen on B cell receptor and interaction between antigen presented on MHC-II and specific T cell activates B cells. Activated B cells, called **plasma cells**, will produce IgM and IgD. Further binding of T cell CD40L on CD40 and cytokine production will lead to recombination mechanisms called **class switching**, allowing the plasma cells to produce either IgG, IgA or IgE depending on the cytokines secreted.

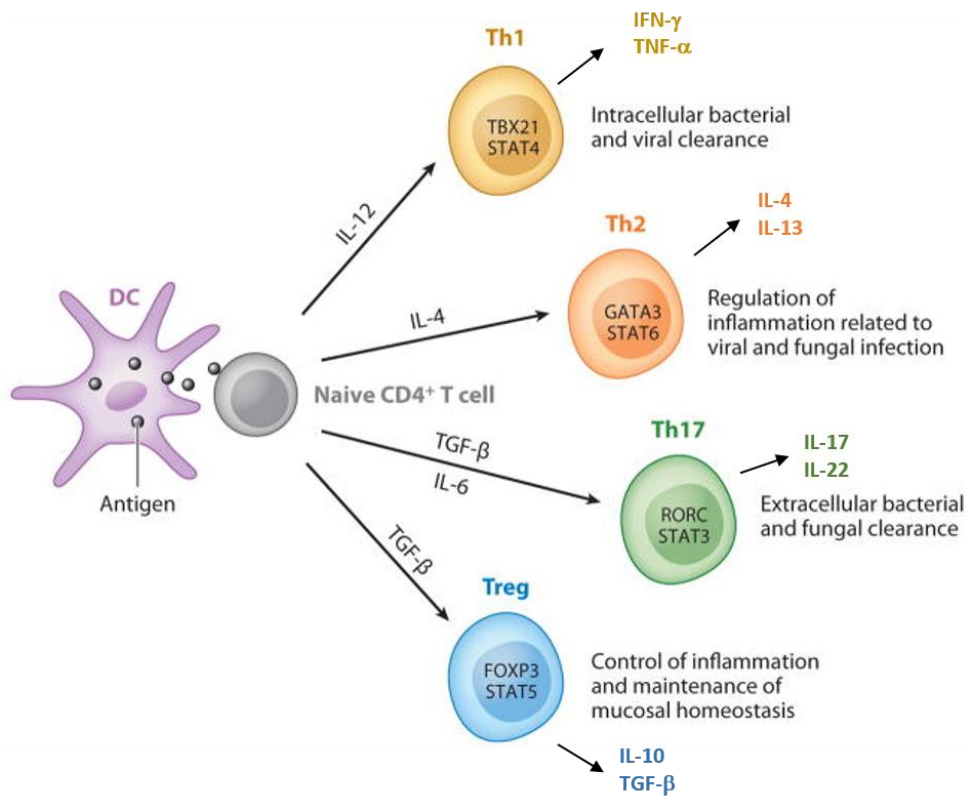


Figure 6: Priming and differentiation of the main CD4⁺ T helper (Th) subtypes. Naive CD4⁺ T cells differentiate into Th1, Th2, Th17 and Treg, after antigen encounter presented by dendritic cell (DC), and followed by lineage commitment that is controlled by specific cytokine environments (IL-12, IL-4, TGF- β /IL-6, or TGF- β , respectively). These signals regulate the expression of lineage-specific transcription factors (TBX21 encodes for the transcription factor T-bet, and RORC for ROR γ t). These effector T cells play specific roles in mucosal defenses, as denoted on the right side of each T cell illustration. Figure adapted from (Chen and Kolls, 2013).

2. Effector T cell present in mucosal tissue and controlling microbiota

While innate functions are efficient, they are not sufficient to preserve homeostasis. The induction of a dedicated T cell response ensures homeostasis with resident microbiota (Loschko *et al.*, 2016). Most effector and memory T cells reside in mucosal sites and are induced in response to the microbiota. The CD4⁺ T cells, which orchestrate the global immune response by producing cytokines and expressing co-stimulatory molecules, have a prominent role in the maintenance of homeostasis at mucosal sites. Different subsets of CD4⁺ T cells or helper T cells (Th) exist, having different properties that confer specific functions: Th1, Th2, Th17 and Treg are discussed here (but other subtypes also exist) (Figure 6). Th1 cells are characterized by the expression of the transcription factor T-box expressed in T cells (T-bet) and the production of interferon gamma (IFN- γ) and TNF α , which grant the host the capacity to fight and eliminate intracellular pathogen and cancer cells; Th1 cells are the hallmark characteristic of the type-1 immune response and, when uncontrolled, they are responsible for tissue immunopathology (Figure 6). Th2 cells express the transcription factors GATA-binding protein 3 (GATA3) and Interferon Regulatory Factor 4 (IRF4), produce IL-4 and IL-13, and are involved in extracellular pathogen killing; Th2 cells are the hallmark characteristic of the type-2 immune response and, when uncontrolled, they provoke inflammatory asthmatic and allergic disorders leading to potential tissue damage. Th17 express the transcription factors retinoic acid receptor-related orphan nuclear receptor gamma (ROR γ t) and Signal transducer and activator of transcription 3 (STAT3), confer the host anti-bacterial and anti-fungal properties, and are involved in neutrophil recruitment to mucosal tissue (Hirahara and Nakayama, 2016); Th17 cells are the hallmark characteristic of type-3 or mucosal immunity response and when, uncontrolled, they are involved in multiple autoimmune and mucosal inflammatory disorders. Type-1, -2, -3 and -4 responses antagonize each other, and there is an equilibrium between these subsets until one of them will be favored to face a particular insult (Eberl, 2016). Tregs are mainly responsible for the control of all effector and inflammatory T cells, granting the host an insurance mechanism against tissue immunopathology; Tregs are the hallmark characteristic of immune tolerance and, when uncontrolled, they are involved in pathogen susceptibility and tumor developmental and metastasis (generation and functions of these cells are more detailed in the following subsection).

In general, T cell subsets are determined during their priming by APC. This is dependent on the information delivered by i) antigen in the context of MHC molecules and specific for their T Cell Receptor (TCR), ii) co-stimulatory molecules such as B7 molecules CD80 and CD86 (that bind to CD28 expressed by T cells), and iii) secretion of soluble signals in the form of cytokines and chemokines¹⁰ that reflects the microenvironment and type of pathogen activating originally the APC. For example, the production of transforming growth factor (TGF)- β by APC is required both for differentiation of Th17 and Treg. At steady-state, additional production of retinoic acid lead to the differentiation of Treg, while production of IL-6 upon infection will favor Th17 priming (Weaver and Hatton, 2009). Cytokines involved in the priming and functions of Th subsets, including Th1 and Th2, are summarized in Figure 6.

¹⁰ **Cytokines** are proteins secreted by many cells including immune cells and are involved in cell signaling. They bind specific receptors expressed at the cell surface of many or specific target cells. They have various immunomodulatory functions such as maturation or responsiveness of immune cells that can differ depending on the target cell type, and expression level of their receptor. Chemokines are specific cytokines involved in cell recruitment. Upon production, they will diffuse in the organism and attract target cells that will migrate to the chemokine source following concentration gradient, a mechanism called chemotaxis.

In the intestinal mucosa, only a few Th2 cells are present; the main pro-inflammatory CD4⁺ T cell subset is Th17 followed by Th1, which are both essential to limit invasion by pathogen or misguided commensal bacteria. Hence, IL-17 blockade in patients, or T cell decrease during HIV infection, results in microbial translocation, increasing susceptibility to viral and fungal infections, and aggravating Crohn's disease (Hueber *et al.*, 2012; Klatt, Funderburg and Brenchley, 2013). Due to the antigen sampling mechanism that is performed only at the epithelial surface, and not further in the lumen, most T cells present in the lamina propria are Th17 specific for bacteria present at the epithelial surfaces, induced by CD103⁺ CD11b⁺ DC or macrophages producing IL-1 β (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009; Shaw *et al.*, 2012; Persson *et al.*, 2013; Panea *et al.*, 2015). Th17 are mainly specific for commensals (*e.g.*, SFB) rather than pathogens, which represent in comparison an occasional threat (Asseman, Read and Powrie, 2003; Niess *et al.*, 2008; Ivanov *et al.*, 2009; Furusawa *et al.*, 2013; Atarashi *et al.*, 2015; Hegazy *et al.*, 2017). Upon invasion of lamina propria, more T cells are generated including Th1 following recruitment of CX3CR1⁺ DC that produce high level of IL-12 (Rivollier *et al.*, 2012). While Th1 secretion of IFN- γ enhances bacterial killing properties of macrophages, Th17 produce IL-17 to promote neutrophil recruitment and activation, and IL-22 to enhance anti-microbial peptide production. Altogether this type of cytokine production improves killing of the invading bacteria (Belkaid and Hand, 2014).

The dynamics of the four CD4⁺ T cell subsets, and their corresponding mechanisms, help to monitor the status/composition of the local microbial residents or the presence of pathogens likely to invade the mucosal tissue, thus fomenting a homeostatic equilibrium and readiness to implement any required inflammatory response. This is well exemplified by a study showing that commensal-specific CD4⁺ T cells are highly proliferative and have high pro-inflammatory functions during infection, but they also display the specific capacity to decrease in number after bacteria elimination from the lamina propria (even though they remain alive in the intestinal lumen), demonstrating an intrinsic mechanism to prevent unnecessary inflammation (Hand *et al.*, 2012). As for pathogen-specific T cells, they will also form memory cells, improving the efficiency of the response by increasing its speed (Hand *et al.*, 2012). As already mentioned, prevention of unnecessary inflammation is crucial to avoid development of inflammatory diseases such as IBD (Asseman *et al.*, 1999; Asseman, Read and Powrie, 2003). Most of this prevention is achieved thanks to immune cells specialized in tolerance, described in the next section.

While not detailed here, unconventional T cells like invariant natural killer T cells (iNKT)¹¹, which induce the release of lysozyme from Paneth cells, are also important in the defense against invading bacteria from commensal of pathogenic origin (Nieuwenhuis *et al.*, 2009).

¹¹ **iNKT** are a subset of unconventional T cells. All unconventional T cells are highly present in mucosal sites, harboring low variant TCR allowing a rapid activation compared to classical lymphocytes. While less specific their rapid response seems key for an early activation of myeloid cells in case of infection. In particular, iNKT are activated by lipid or glycolipid antigens, presented on CD1 by myeloid cells. They can produce many cytokines such as Csf2, IFN- γ , IL-2, TNF- α , or IL-17 activating many pro-inflammatory immune cells and mediate lysis of infected cells.

3. Tolerance to the microbiota

The repertoire of leukocytes responsible to establish tolerance to the local microbiota are diverse. These include resident macrophages, CD103⁺ DC, Treg and Innate Lymphoid Cell¹² (ILC)3. In addition, some CD8⁺ T cells have regulatory properties, but this leukocyte will not be discussed in this section (Ligocki and Niederkorn, 2015). While intestinal inflammation is characterized by leukocyte infiltration and increased mucus production to control invading bacteria, intestinal tolerance to the resident microbiota is induced by different mechanisms with the goal to preserve the intestine integrity and functions (Hooper and Macpherson, 2010).

a) Treg, the main promoters of tolerance

In the gut, different type of Treg exist and most of these are characterized by expression of the transcription factor forkhead box P3 (Foxp3). Foxp3⁺ Treg found at mucosal site originate either from the thymus or are induced locally, and the presence of both subtypes seems to be needed to preserve gut homeostasis (Haribhai *et al.*, 2011; Cebula *et al.*, 2013). Thymic or natural Treg (nTreg) generated during CD4⁺ T cell selection are characterized by the expression of the transcription factor Helios¹³ and the receptor Neuropilin-1 (Nrp-1)¹⁴; they are specific for self-antigens and migrate to peripheral tissues to reduce auto-immunity (Haribhai *et al.*, 2011). In mucosal sites, there is another Treg subtype that is called peripheral or induced Treg (iTreg), which is essential to avoid immune activation toward local commensal micro-organisms (Haribhai *et al.*, 2011; Lathrop *et al.*, 2011; Kim *et al.*, 2016). iTreg are generated in the spleen, lymph nodes or mucosal-associated lymphoid tissue, and they are specific for microbiota and food antigens. Interestingly, it seems that their priming involves food- or microbiota-derived antigen sampling by CX3CR1⁺ macrophages that are able to transfer them to CD103⁺ DC (having poor antigen sampling but high priming capacities) in a process involving a rapid membrane transfer from macrophages to DC (Mazzini *et al.*, 2014). The conversion of naïve T cells to iTreg involves the production of retinoic acid and TGF- β by APC (Benson *et al.*, 2007; Janine L. Coombes *et al.*, 2007; Sun *et al.*, 2007; Nutsch *et al.*, 2016). After migration to the lamina propria dependent on the chemokine receptor CCR-9, iTreg will be locally re-stimulated by macrophages producing high quantity of IL-10 and induced to proliferate (Hadis *et al.*, 2011). Of note, retinoic acid and TGF- β are also produced by epithelial cells that promote a tolerogenic phenotype in DC, further improving their induction of iTreg (Iliev, Mileti, *et al.*, 2009; Iliev, Spadoni, *et al.*, 2009).

Treg are able to suppress proliferation and activation of immune cells through different mechanisms (Figure 7) (Parkash, Agrawal and Madhan Kumar, 2015). They first down-modulate the activation of effector T cells by impairing DC functions.

¹² ILC derive from the common lymphoid progenitor, but unlike T or B cells, they do not express antigen-specific receptors. They are considered innate leukocytes due to their presence at mucosal sites, where they engage in homeostatic functions and response to infection by rapidly secreting immunomodulatory cytokines to orchestrate the early immune response. They are comprised of i) ILC1 and cytotoxic natural killer cells, which mirror Th1 for their capacity to produce IFN- γ /TNF- α ; ii) ILC2 mimicking Th2 for producing type-2 cytokines (e.g.,IL-4); and iii) ILC3, which produce IL-17/IL-22 just like Th17 and are involved in mucosal immunity.

¹³ Helios is a transcription factor mainly expressed in Treg that may promote Foxp3 expression, stabilizing Treg suppressive functions (Getnet *et al.*, 2010)

¹⁴ Nrp-1 is a co-receptor for semaphorins and vascular endothelial growth factor that seems involved in Treg suppression of anti-tumoral immunity and in Treg stability at inflammatory sites (Delgoffe *et al.*, 2013)

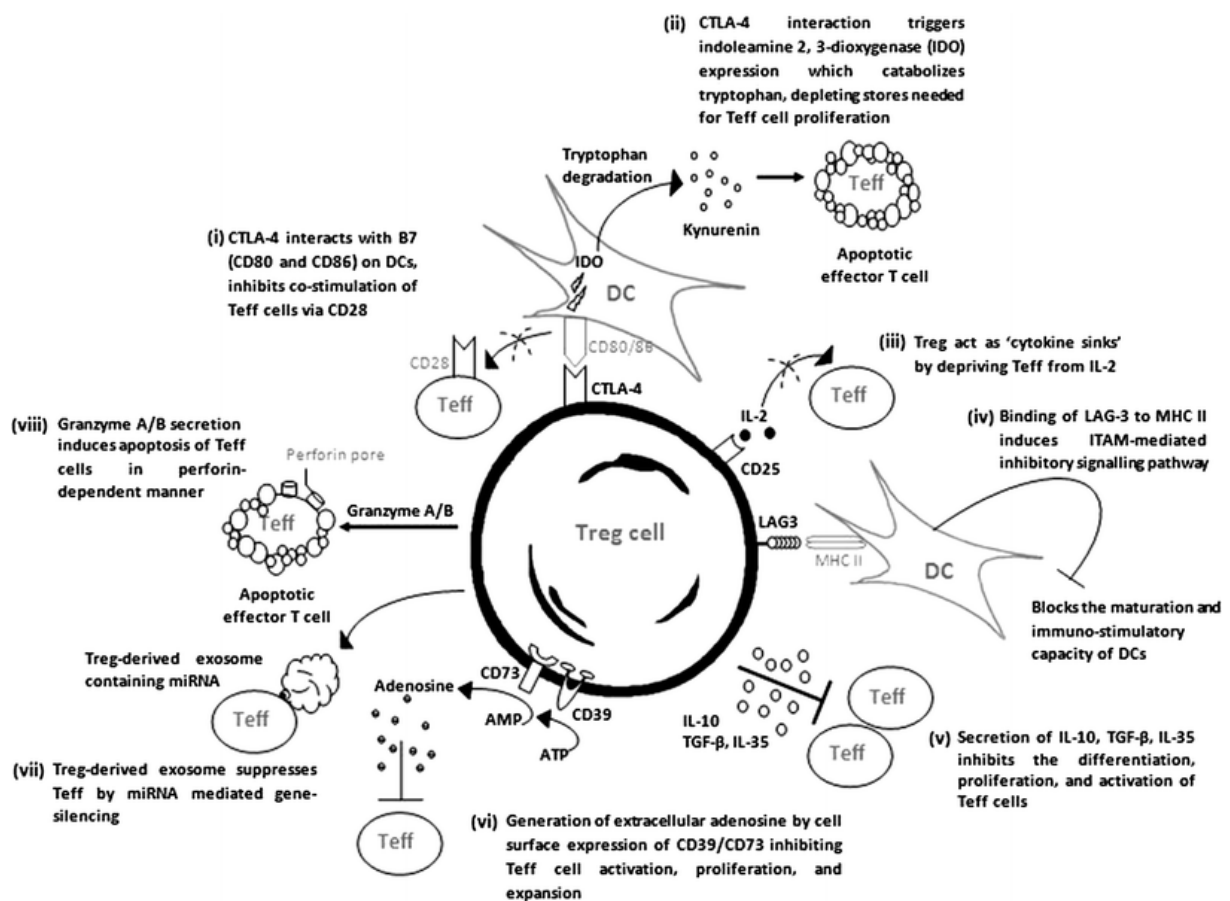


Figure 7: Mechanisms involved in Treg suppressive functions. Treg suppression involves the inhibition of T cell priming by DC as well as the production of soluble factors (*e.g.*, IL-10, TGF- β , granzyme A/B, or miRNA) that target directly effector T cell (Teff) proliferation/activation. Treg may also compete for IL-2 that is crucial for the clonal expansion of Teff. Figure from (Parkash, Agrawal and Madhan Kumar, 2015).

LAG-3 and CTLA-4 are also important to inhibit T cell proliferation (Huang *et al.*, 2004). Indeed, CTLA-4 induces the expression of indoleamine 2,3-dioxygenase (IDO) in DC, thus triggering tryptophan catabolism. In fact, the amino acid tryptophan is needed for effector T cell proliferation, but the tryptophan catabolites induce conversion of peripheral naïve T cell into Treg (Fallarino *et al.*, 2006). In addition, Treg highly express CD39, an enzyme able to degrade Adenosine Triphosphate (ATP) released by damaged cells and acting as a pro-inflammatory signal activating effector T cells (Borsellino *et al.*, 2007). They also produce vesicles called exosomes containing miRNA¹⁵ that upon delivery inside effector T cell will mediate gene silencing inhibiting T cell proliferation or function (Okoye *et al.*, 2014). Treg can also induce effector cell death through cytolysis by producing perforin/granzymes as CD8⁺ T cells or NK cells that will create pores in target cells dying from osmotic choc (Grossman *et al.*, 2004; Borsellino *et al.*, 2007; Okoye *et al.*, 2014). Treg also modulate effector T cells through production of TGF- β and IL-10 that inhibit their differentiation, proliferation and activation (Rubtsov *et al.*, 2008).

In the gut, another type of Treg named Tr1¹⁶, which does not express Foxp3, may also be crucial for tolerance since they produce more IL-10 than conventional Foxp3⁺ Treg (Barnes and Powrie, 2009). One function of IL-10 is the ability to change macrophage metabolism from glycolysis (needed for pro-inflammatory functions) to oxidative phosphorylation (needed for tissue repair and homeostatic functions) (Shouval *et al.*, 2014; Zigmund *et al.*, 2014; Ip *et al.*, 2017). Together with TGF- β , IL-10 limits Th1 differentiation (Davidson *et al.*, 1996; Asseman *et al.*, 1999; Asseman, Read and Powrie, 2003), and on its own that of Th17 (Zhou and Sonnenberg, 2018).

The crucial role of Treg is highlighted in mice deficient in Foxp3 that develop spontaneous colitis, which is also reproduced in mice deficient in IL-10 or TGF- β (Davidson *et al.*, 1996; Li, Wan and Flavell, 2007). In mouse models with T cell-deficiency, such as the RAG¹⁷ or SCID¹⁸, it is well-established that upon adoptive transfer of CD4⁺ T cells (that differentiate into pro-inflammatory Th1), the animals develop colitis; this process is inhibited when iTreg or Tr1 are co-transferred with CD4⁺ T cells (Cong *et al.*, 2002; Haribhai *et al.*, 2009). Likewise, simultaneous administration of anti-IFN- γ (or anti-TNF- α) antibodies and recombinant IL-10 inhibits colitis development in this mouse model, highlighting the importance of cytokines as mediators of inflammation or tolerance (Powrie *et al.*, 1994).

¹⁵ MicroRNA or **miRNA** are small non-coding RNA that inhibit expression of messenger RNA by binding on complementary sequences.

¹⁶ Type 1 Treg (**Tr1**) are a subset of Treg involved in peripheral tolerance, specific of antigens from self and non-self. They highly express CD49b and LAG3, but not Foxp3. Their suppressive functions are mediated by rapid production of high levels of IL-10 and TGF- β , expression of CTLA-4, CD39 and granzymes/perforins. They are induced in the periphery in presence of IL-27 and TGF- β .

¹⁷ Recombination-activating gene (**RAG**) 1 and 2 are needed for gene rearrangement allowing the expression of antigen receptor on T and B cells. Mice deficient in RAG1 or RAG2 do not possess T and B lymphocytes.

¹⁸ Severe combined immunodeficiency (**SCID**) is caused by a mutation in the gene coding for an enzyme necessary for double-stranded DNA breaks reparation, a process also implicating in T and B cell receptor gene rearrangement. Similarly, to RAG mice, SCID mice are devoid of T and B cells. b

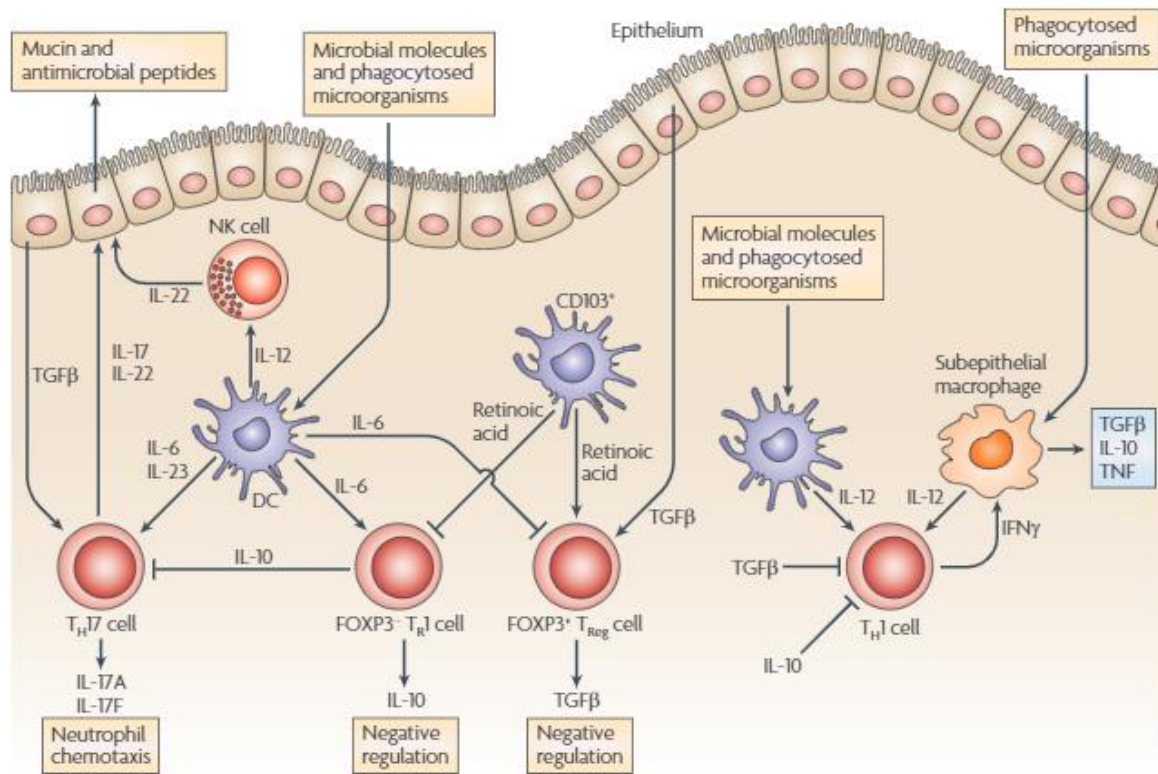


Figure 8: Establishment of physical limitations and tolerance mechanisms for the resident intestinal microbiota. The presence of NK cells as well as the generation of pro-inflammatory T cells, such as T helper 1 (Th1) that improve macrophage killing properties, or Th17 that augment the recruitment of neutrophils and production of antimicrobial peptides (AMP), is essential to prevent lamina propria invasion of pathogen or misguided commensal bacteria. Yet, too much of these pro-inflammatory functions may result in tissue damage. For this reason, acute inflammation is prevented by the induction of Foxp3⁺ Treg *via* CD103⁺ dendritic cells (DC) producing retinoic acid in presence of TGF-β, and by Foxp3⁺ Tr1 cells in the presence of IL-6 that will limit effector T cells functions. In addition, ILC3 also participate in tolerance to the microbiota, but are not illustrated in this figure. Figure from (Hooper and Macpherson, 2010).

Treg not only prevent inflammation in absence of invasion, but also promote its resolution post-insult (e.g., after pathogen clearance) through production of IL-10 and TGF- β (Maloy *et al.*, 2003) (Figure 8). Finally, Treg promote Ig class switching to improve IgA production by B cells, limiting bacterial invasion of epithelial tissue (Cong *et al.*, 2009). The role of Treg in this mechanism has been shown to improve microbiota diversity and may be implicated in selection of microbes (Kawamoto *et al.*, 2014).

b) ROR γ t⁺ Treg, a Treg subset specialized in tolerance to the microbiota

Growing evidence support the hypothesis that all immune cells including T cells can display some degree of plasticity to adapt to a given environment. In particular, in the case of Treg, it seems that co-expression of master transcription factors governing all types of immunity will increase their ability to control other leukocytes expressing the same transcription factors (Figure 6). Thus, T-bet co-expression in Foxp3⁺ Treg occurs locally at sites where type-1 inflammation is engaged and requiring Treg surveillance to prevent tissue damage. Likewise, Foxp3⁺ Treg can also co-express GATA3/IRF4 to potentially regulate the type-2 immune response, and ROR γ t/STAT3 to control type-3 mucosal immunity (Chaudhry *et al.*, 2009; Koch *et al.*, 2009; Zheng *et al.*, 2009). In the latter case, more and more studies suggest that many of the protective functions in intestinal homeostasis are in fact promoted by Foxp3⁺ Treg co-expressing ROR γ t named either “ROR γ t⁺ Treg”, “Type 3 Treg” or “bifunctional Treg (biTreg)”. In fact, most iTreg in the gut express ROR γ t, representing around half of all colonic Treg (Ohnmacht *et al.*, 2015; Sefik *et al.*, 2015). Interestingly, analysis of the Treg TCR reveals that all commensal-specific Treg express ROR γ t while the other iTreg seem specific for food antigens (Kim *et al.*, 2016; Solomon and Hsieh, 2016). ROR γ t⁺ Treg are also present in other mucosal sites, such as the lungs, but are present at low levels in the spleen or lymph nodes (Lochner *et al.*, 2008; Yang *et al.*, 2016). The ROR γ t⁺ Treg generation requires the presentation of microbiota antigens on MHC-II molecules by DC, stimulation with IL-6 and IL-23 along with STAT3 activity (necessary for ROR γ t expression), and the presence of retinoic acid (favoring ROR γ t⁺ Treg generation over Th17) (Lochner *et al.*, 2011; Ohnmacht *et al.*, 2015; Yang *et al.*, 2016).

Different approaches indicate that these Foxp3⁺ROR γ t⁺ population is a Treg subset rather than Th17 effector cells. First, while there is a plasticity allowing conventional Foxp3⁺Treg to acquire ROR γ t expression, Th17 are unable to co-express Foxp3 (Osorio *et al.*, 2008; Yang *et al.*, 2016). However, once Foxp3⁺ Treg acquires ROR γ t expression, it becomes a stable cell lineage as ROR γ t⁺ Treg (Yang *et al.*, 2016). Second, ROR γ t⁺ Treg have a specific transcriptomic signature and genetic profile sharing Treg and Th17 signatures, but remaining more similar to that of Treg (Koenen *et al.*, 2008; Sefik *et al.*, 2015; Yang *et al.*, 2016). Accordingly, ROR γ t⁺ Treg highly express the marker of activation Inducible T-cell COStimulator (ICOS)¹⁹ and CTLA-4, and produce IL-10 (but no IL-17) at steady state; it is thought that Foxp3 represses the IL-17 production usually induced by ROR γ t (Lochner *et al.*, 2008; Zhou *et al.*, 2008). ROR γ t⁺ Treg also express CCL20 and its receptor CCR6, which are involved in attraction of DC and lymphocytes to mucosal-associated lymphoid tissues (Ohnmacht *et al.*, 2015; Chellappa *et al.*, 2016). Based on this phenotype, ROR γ t⁺ Treg are capable to suppress proliferation of intestinal CD4⁺ T cells (Lochner *et al.*, 2008). Yet, their physiological function seems complex and highly dependent on the microenvironment. During immune development, presence of these cells prevents eventual susceptibility to cancer, allergy and colitis (Al Nabhani *et al.*, 2019). ROR γ t⁺ Treg increase is also highly

¹⁹ ICOS or CD278 is a costimulatory molecule, mainly expressed at the surface of activated CD4⁺ T cells. Its binding to the ICOS ligand (ICOSL) expressed on phagocytes induces T-cell proliferation and cytokine secretion. ICOS can also be expressed on Treg, facilitating Foxp3 transcription to favor their suppressive functions.

protective in different models of colitis through various mechanisms. For example, in a model of trinitrobenzene-sulfonic acid (TNBS) induced colitis, depletion of ROR γ ⁺ Treg aggravates the inflammation mediated by excessive IL-17 and IFN- γ production (Sefik *et al.*, 2015). Similarly, ROR γ ⁺ Treg production of IL-10, is needed to avoid colitis mediated by Th17 cells specific for the pathobiont *Helicobacter hepaticus* (Xu *et al.*, 2018). In the colitis model mediated by the adoptive transfer of CD4⁺ T cells in RAG2-deficient mice, the co-transfer of ROR γ ⁺ Treg also prevents colitis and provides even better protection than ROR γ ⁻ Treg (Yang *et al.*, 2016). However, in this model, IL-17 and IFN- γ production was not impacted by the increase in ROR γ ⁺ Treg. Instead, total leukocyte infiltration in the lamina propria was reduced, suggesting that ROR γ ⁺ Treg may suppress the induction of other immune cells, such as Th2 cells. Indeed, mice depleted of ROR γ ⁺ Treg are more susceptible to colitis induced by oxazolone (an inducer of excessive type-2 inflammation) and more resistant to helminth infection, which require a Th2-mediated response (Ohnmacht *et al.*, 2015). As such, at steady state, mice depleted of ROR γ ⁺ Treg also have increased number of Th2 (Josefowicz *et al.*, 2012; Ohnmacht *et al.*, 2015). This anti-Th2 suppressive properties may be linked to their expression of IRF4, an important transcription factor that allows Treg differentiation to the detriment of that of Th2 cells (Zheng *et al.*, 2009; Ohnmacht *et al.*, 2015). Interestingly, germ-free mice colonized with microbiota from IBD patients, have an altered CD4⁺ T cell compartment that is characterized by low ROR γ ⁺ Treg and high levels of Th17 and Th2, which correlates with increased susceptibility to colitis (Britton *et al.*, 2019). Therefore, different mechanisms allow ROR γ ⁺ Treg to prevent inflammation by reducing pro-inflammatory CD4⁺ T cells specific for commensals.

Notwithstanding, ROR γ ⁺ Treg acquire pathogenic Th17 features in some conditions, such as high IL-17 production along with their suppressive properties (being bifunctional), which aggravate cancer or chronic inflammation (Esposito *et al.*, 2010; Kryczek *et al.*, 2011; Blatner *et al.*, 2012; Chellappa *et al.*, 2016; Kluger *et al.*, 2016, 2017). IL-17 production by ROR γ ⁺ Treg seems to be dependent on the presence of pro-inflammatory cytokines, such as IL-6 or IL-1, and the activation of Mincle or Dectin-1 in DC (factors that drive Th17 activation) (LeibundGut-Landmann *et al.*, 2007; Osorio *et al.*, 2008; Yang *et al.*, 2008; Martínez-López *et al.*, 2019). (LeibundGut-Landmann *et al.*, 2007). Therefore, like other immune cells, the protective role of ROR γ ⁺ Treg can be diverted by changes in the environment and may be linked to the pathologic role of the microbiota in different diseases. Importantly, other Treg subtypes, expressing T-bet or GATA3, can similarly acquire pathogenic Th1 or Th2 properties, respectively (Wohlfert *et al.*, 2011; Van Gool *et al.*, 2019). It appears that GATA3 and ROR γ expressions are mutually exclusive and antagonistic (Wohlfert *et al.*, 2011; Ohnmacht *et al.*, 2015). GATA3⁺ Treg are also important to reduce tissue damage. Hence, protection of host tissue integrity may be dependent on the type of inflammation causing damage and mediated by an equilibrium between the different Treg subtypes (Lochner *et al.*, 2008; Park and Eberl, 2018). It is possible that the differences observed in Th1 and Th17 in absence of ROR γ ⁺ Treg are the consequence of deregulation of another Treg subset, such as GATA3⁺ Treg. Another argument in favor of a role of ROR γ ⁺ Treg different from Th17 inhibition comes from the observation that in different models they evolved with similar tendencies. Indeed, both Th17 and ROR γ ⁺ Treg increase after SFB administration, or in colitis or influenza infection (but not in cancer), and Th17 are decreased in ROR γ ⁺ Treg deficient mice (Lochner *et al.*, 2008; Ohnmacht *et al.*, 2015). Also, ROR γ ⁺ Treg protective effect in the *H. hepaticus* model seems independent of their ROR γ expression (Xu *et al.*, 2018). Further studies may help to decipher the protective role of these cells in homeostasis that seems key mediators of tolerance to the microbiota.

c) ILC3 also participate in tolerance establishment

Another important immune cell type involved in microbiota tolerance are the ILC3, an ILC subtype that also expresses ROR γ t. ILC3 express MHC-II molecules that carry and present commensal antigens to T cells. Upon binding of the MHC-II/antigen complex to commensal-specific TCR, ILC3 are able to induce cell death of the recognized T cell. This process is known as “intestinal selection”, because it mimics thymic selection involved in suppression of self-specific T cells. For example, intestinal selection is responsible for the deletion of Th17 cells activated by commensal SFB (Hepworth *et al.*, 2013; Qiu *et al.*, 2013). Apart from T cell suppression, ILC3 are the main producers of IL-22, a cytokine that induces the early production of AMP, such as REGIII γ , preventing tissue invasion by *Citrobacter rodentium* or *Alcaligenes* species from the microbiota (Zheng *et al.*, 2008; Sawa *et al.*, 2011; Sonnenberg *et al.*, 2012). Production of IL-22 is also important to increase mucus production and wound repair, highlighting the essential role of this cytokine for mucosal homeostasis (Zenewicz and Flavell, 2011). ILC3 also promote tolerance by enhancing Treg generation and suppressive functions in combination with myeloid cells (Zhou *et al.*, 2019). For instance, macrophage sensing of bacterial compounds through the PRR family of Toll-like receptors (TLR) does not lead to the production of conventional TNF- α , but instead they will generate mature IL-1 β . This is important because the activation of IL-1R in ILC3 induces the production of colony-stimulating factor 2 (Csf2)²⁰ and IL-2. Csf2 importantly modulate myeloid functions and will induce the production of retinoic acid and IL-10, which favor Treg generation (Mortha *et al.*, 2014). IL-2 is needed for proliferation of Th1, Th2 and Treg, but it inhibits Th17 differentiation (Boyman and Sprent, 2012). Importantly, Treg express high levels of CD25, the IL-2 receptor, allowing these cells to sequester IL-2 (as a sink), inhibiting Th1 and Th2 proliferation. This mechanism is crucial for homeostasis as CD25 depletion on Treg, or IL-2 depletion, result in inflammatory diseases (Waidmann *et al.*, 2003; Schuppler *et al.*, 2004; Chinen *et al.*, 2016).

As discussed in the past sections, Th17, ILC3 and ROR γ t⁺ Treg that regulate type-3 immunity have a key role in maintenance of a symbiotic relationship between the host and the microbiota in the gastro-intestinal track (Park and Eberl, 2018). Therefore, a better understanding of their role in other mucosal sites like the lungs, and of how they interact together and with local microbiota, is needed to improve the development of new prophylactic and therapeutic approaches to treat infectious and inflammatory diseases.

4. Natural modulation of immunity by the microbiota

a) Immune system development following microbial colonization

The basal level of tolerance of our immune system toward the microbiota, which develops during infancy, is a key parameter for health. This initial tolerance level determines the lifetime relationship our immune system will have toward the microbiota and environmental antigens but also upon infection (Al Nabhani *et al.*, 2019). Microbiota colonization is first possible because the newborn's immune system is immature; at this stage, the protection of the mucosa is ensured by IgA and immune cells present in the maternal milk, and by the first microbial settlers that rapidly induce tolerance at mucosal sites (Belkaid and Hand, 2014). This tolerance is induced through different mechanisms. First,

²⁰ **Csf2**, previously known as granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine promoting proliferation and maturation of hematopoietic precursors differentiating into macrophages, DC or granulocytes. It can also activates some of these cells functions such as ROS production.

there is a reduced accumulation of effector immune cells. For example, *Bacteroides fragilis* is known to reduce the accumulation of iNKT observed in the gut and lungs of germ-free neonates, which are susceptible to IBD and asthma (Olszak *et al.*, 2012; An *et al.*, 2014). Second, the repeated stimulation of PRR by MAMP (microbial associated molecular pattern) leads to hypo-responsiveness of sentinel cells. In neonates, intestinal epithelial cells develop a decreased ability to respond to TLR activation by LPS (lipopolysaccharides) and remain hypo-responsive during life, demonstrating this is a key mechanism to avoid hyper-inflammation later in life; neonates born by C-section fail to activate such mechanisms and become highly susceptible to different inflammatory disorders in adult life (Rakoff-Nahoum *et al.*, 2004; Lotz *et al.*, 2006). Another example of this mechanism is that of macrophages, since stimulation of TLR signaling results in low oxygen radical activation and induction of the anti-inflammatory IL-10, which then leads to an hypo-activating environment against microbiota (Kollmann *et al.*, 2012). Finally, there is the induction of specific tolerogenic leukocytes. In mice, the increase in *Clostridia species* (e.g., SFB) during intake of solid food, along with factors present in the weaning milk, induces a particular immune response called “weaning reaction”. This allows the set-up of a tolerogenic immune system governed by ROR γ t⁺ Treg; the inhibition of these cells increases the risk to develop colitis, allergic inflammation and cancer (Al Nabhani *et al.*, 2019).

The establishment of tolerance towards the microbiota by the mechanisms described above does not mean the immune system is impaired or unable to develop resistance for life-time. On the contrary, upon microbiota colonization, secondary lymphoid structures will develop including the Peyers’ patches that are directly driven by microbial compounds (Mazmanian *et al.*, 2005; Jijon *et al.*, 2018). Similarly, stimulation of TLR signaling does not only mediate IL-10 production, but also that of IL-6 and IL-23, resulting in robust Th17 differentiation and higher protection of the epithelial barrier (Kollmann *et al.*, 2012). Therefore, in order to protect themselves and their ecological niche, microbiota first settlers ensure a basic education of the newborn’s immune system establishing a basal level of tolerance accompanied by the reinforcement of homeostatic mechanisms to delineate critical physical boundaries between them and leukocytes.

b) Immunomodulatory properties of particular species of the microbiota

Symbiosis with the microbiota is so tightly intricate that it is difficult to decipher whether the immune compartment controls microbiota composition or vice-versa. In fact, both options may occur simultaneously, resulting in the complex crosstalk that we just begun to understand. Development of both compartments is dependent on the other. As previously mentioned, germ-free mice have an altered intestinal barrier and exhibit defects in the establishment of immunological peripheral tolerance. In particular, they possess fewer Treg (above all ROR γ t⁺ Treg) and Th17 (Belkaid and Hand, 2014; Ohnmacht *et al.*, 2015; Sefik *et al.*, 2015; Kim *et al.*, 2016). Germ-free mice also have impaired epithelium tissue repair following injury, suggesting the important role of the microbiota in the maintenance of epithelial barrier integrity (Pull *et al.*, 2005).

Among the microbiota, there are individual microbes with particular strong immunomodulatory properties. These properties are dependent on two mechanisms that are the presence of a specific antigen repertoire or the production of soluble metabolites such as SCFA, both exerting varying immunomodulatory effects on different host cell types. In particular bacteria residing in proximity to host cells seem to have potent immunomodulatory properties. For instance, while *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii* influence mucus production, *Lactobacilli* species expressing an aryl hydrocarbon receptor (AhR)²¹ ligand enhance the ILC3 mediated AMP secretion and

²¹ The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor having a key role in immune cell development and functions including IL-22 production by ILC3.

reinforcement of epithelial tight junctions (P. D. Cani *et al.*, 2007; Lochner *et al.*, 2011; Kinnebrew *et al.*, 2012; Wrzosek *et al.*, 2013; Zelante *et al.*, 2013; Duranti *et al.*, 2016; Schroeder *et al.*, 2018). By contrast, avirulent *Salmonella* strains inhibit epithelial cell-derived production of inflammatory cytokines and promote tolerance (Neish *et al.*, 2000).

Microbiota recognition by the immune system induces the generation of Th17 and Treg. Likewise, certain bacterial antigens are also capable to induce high proportion of Th17 or Treg, which has consequences for the host health. In the case of Th17 cells, epithelium adherent bacteria (*e.g.*, SFB) are able to induce specifically high levels of these effector T cells. For example, SFB mono-colonization of germ-free mice induces similar Th17 cell levels compared to those found in SPF mice (Ivanov *et al.*, 2009; Furusawa *et al.*, 2013; Atarashi *et al.*, 2015). In this context, Th17 differentiation is mediated by the high quantity of ATP released by this strain into the environment, which then induces epithelial cell-derived production of IL-6, IL-23 and TGF- β in the lamina propria; these cytokines favor Th17 differentiation during CD4⁺ T cell priming (Atarashi *et al.*, 2008). This induction is so strong that it occurs even during infection by *Listeria monocytogenes*, a strong Th1 inducer (Yang *et al.*, 2014). Moreover, SFB-induced Th17 appear to be important to prevent pathogen infections, such as *C. rodentium* infection (Heczko, Abe and Finlay, 2000; Ivanov *et al.*, 2009). Indeed, the presence of SFB at the epithelial surface makes it more complicated for this pathogen to access the lamina propria, and the specific induction of Th17 by this commensal participates in pathogen clearance. By contrast, in the case of Treg induction, many microbiota strains promote their differentiation and improve their suppressive functions, limiting specifically the differentiation of Th17. For instance, T cells specific for some *Clostridia* species, *Helicobacter hepaticus*, *B. thetaiotaomicron* or *B. fragilis*, acquire a Treg phenotype under homeostatic conditions (Round and Mazmanian, 2010; Lathrop *et al.*, 2011; Nutsch *et al.*, 2016; Xu *et al.*, 2018). Such tolerogenic cells allow these bacteria to colonize niches at the proximity of epithelial cells, which benefits the host. This is further exemplified in the case of *B. fragilis* administration, which prevents many immune development defects observed in germ-free mice (Mazmanian *et al.*, 2005), and reduces colitis and colitis-associated colorectal cancer developments (Mazmanian, Round and Kasper, 2008; Lee *et al.*, 2018). This protective effect is due to *B. fragilis*-derived polysaccharide A (PSA) binding to T cell TLR2²², favoring Treg induction and their production of IL-10, while also suppressing the development of specific IL-17-producing Th17 (Round and Mazmanian, 2010; Round *et al.*, 2011; Dasgupta *et al.*, 2014). For this reason, some commensal compounds are referred to as “symbiont-associated molecular patterns” owing to their capacity to trigger different PRR signaling pathway, resulting in the discrimination of immune response to pathogens from immune tolerance toward commensals (Round *et al.*, 2011).

Apart from effect of antigens, many bacteria modulate immune functions through their production of soluble molecules or metabolites. In particular, SCFA, produced during fiber fermentation, are used by the host as carbon source to produce ATP and also modulate immune functions (Cummings *et al.*, 1987). The most abundant (in the colon) and best characterized SCFA are acetate, propionate and butyrate. Their functions are mainly mediated by binding to G protein-coupled receptor (GPR) GPR43, GPR41, GPR109a and olfactory receptor Olfr-78, present on epithelial and immune cells (Corrêa-Oliveira *et al.*, 2016). Also, butyrate and propionate (but not acetate) are reported to inhibit histone deacetylase activity, regulating epigenetic changes in immune cells (Arpaia *et al.*, 2013). SCFA supplementation to immune cell culture medium *in vitro*, and administration in mice, have controversial effects depending on the context, SCFA concentration, and interaction with other factors

²² TLR are not only expressed on myeloid cells but also on T cells. Their activation on T cell have been shown to promote Th17 or Treg differentiation depending on the antigen (Reynolds *et al.*, 2010; Round *et al.*, 2011)

(Corrêa-Oliveira *et al.*, 2016). Also, their effect on a given cell type may be different depending on this cell differentiation stage (Schulthess *et al.*, 2019). However, there are clear beneficial effects enacted by SFCA. First, SCFA have a strong potential to increase AMP production and modify cytokine/chemokine produced by epithelial cells (Kim *et al.*, 2013; Kelly *et al.*, 2015; Y. Zhao *et al.*, 2018). Second, they modulate macrophage activation, and may be linked to their LPS hyporesponsiveness (Cox *et al.*, 2009; Chang *et al.*, 2014; Ciarlo *et al.*, 2016; Lee *et al.*, 2017). Interestingly, SCFA may promote macrophage antimicrobial activity without increasing inflammatory cytokine response, resulting in better resistance to pathogens (Schulthess *et al.*, 2019). Third, SCFA modify the T cell compartment and are involved in Th17-mediated protection against *C. rodentium* infection, which is distinguished by an accelerated T cell memory response (Kim *et al.*, 2013; Balmer *et al.*, 2016). Fourth, they directly inhibit pathogen virulence through modulation of virulence genes expression (Gantois *et al.*, 2006; Hung *et al.*, 2013; Hryckowian *et al.*, 2018). Last, SFCA production by some bacteria, such as Clostridia, strongly participate to immune tolerance. For instance, they modulate the DC priming capacity to enhance iTreg accumulation in mucosal tissues, reducing susceptibility to colitis and colorectal cancer (Liu *et al.*, 2012; Arpaia *et al.*, 2013; Furusawa *et al.*, 2013; Singh *et al.*, 2014; Gurav *et al.*, 2015; Qiang *et al.*, 2017). They also increase directly the Treg suppressive functions by establishing the acetylation of Foxp3 promoter (Thorburn *et al.*, 2015).

Therefore, through two different mechanisms, bacteria from the microbiota are key players in gut mucosal development and homeostasis. Yet, this role is not limited to the gut. Even if mechanisms are less characterized outside of the gut, it is now clear that the gut microbiota (partly via SCFA) modulate homeostasis at distal mucosal sites such as the lungs.

III. Role of the microbiota in respiratory health and disease

Respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, acute lower respiratory tract infections and tuberculosis, are among the most common causes of death and severe illness (*Forum of International Respiratory Societies The Global Impact of Respiratory Disease*, 2012). The microbiota is altered in many of these diseases and this alteration may aggravate the disease outcome. Germ-free mice are susceptible to a wide range of respiratory diseases, including asthma, viral and bacterial infections, leading to pneumonia (Olszak et al., 2012; An et al., 2014; Brown, Sequeira and Clarke, 2017). For the past few years, there has been an intense focus in deciphering if the gut and/or pulmonary microbiota modulate lung immunity, and whether this knowledge could shed light on respiratory health and the prevention/treatment of disease.

1. The lung immune system at a glance

a) Entry limitation to the alveoli

Although mucosal sites such as the gut and lungs share many basic features, they also have different functions, structure and exposure to the external environment, which overall shape their immune response. Being in direct contact with the external environment and highly vascularized, the lungs are potentially the portal of entry of many harmless particles and, unfortunately, dangerous pathogens. Therefore, the immune system at this site must be potent but also highly regulated. The first line of defense is composed of epithelial cells, which prevent microbe invasion throughout the respiratory tract. The respiratory tract is divided into the upper respiratory tract, ranging from the nares to the vocal cords (including the nasopharynx, oropharynx and part of the larynx), and the lower respiratory tract, going from the vocal cords to the alveoli (comprising part of the larynx, bronchi and bronchioles). Excepting in the alveoli, epithelial cells produce high levels of mucus and AMP, and they promote the constant elimination of bacteria and particles trapped in the mucus through mucociliary clearance, which acts like a brush expelling materials towards the respiratory tract (Grubor, Meyerholz and Ackermann, 2006). Like in the gut, IgA are produced in the lungs and limit bacterial invasion. In the alveoli, different epithelial cells are present, namely Type I and II pneumocytes, ensuring the gas exchange in the absence of mucus, which is replaced by less viscous alveoli surfactant. Contrary to the bronchi or bronchioles, pathogens reaching the alveoli are, therefore, highly prone to invade the interstitial tissue. As a compensatory mechanism, microbial invasion is usually prevented by the presence of alveolar macrophages.

b) Characteristics of lung macrophages

Two main subtypes of macrophages are found in the lungs: the alveolar and interstitial macrophages. Alveolar macrophages are present in the alveoli airspace, in direct contact with microbes. By contrast, interstitial macrophages (and other leukocytes) reside in the lung tissue, separated from direct microbial contact (Kopf, Schneider and Nobs, 2015). Both subtype express the macrophage markers F4/80, the receptor tyrosine kinase MerTK, the Fc receptor CD64 and the integrin CD11c. Alveolar macrophages can be discriminated as Siglec-F^{high}CD11b^{low}MHC-II^{low} cells, and interstitial macrophages by Siglec-F^{low}CD11b^{high}MHC-II^{high} expression (Misharin *et al.*, 2013). Alveolar macrophages arise from embryonic progenitors, differentiating in presence of Csf2 and mainly replenished by proliferation, while interstitial macrophages appear to derive from blood monocytes recruited in case of infection (Kopf, Schneider and Nobs, 2015; L. Huang *et al.*, 2018). Inflammatory monocytes expressing Ly6C can also be recruited in response to inflammation. Alveolar macrophages have anti-inflammatory profile

under homeostatic conditions and produce IL-10 and TGF- β to suppress activation of other immune cells. In addition, it seems that they can convert vitamin A into acid retinoic more efficiently than lung DC, which makes them the main inducers of iTreg in the lungs (Soroosh *et al.*, 2013). Importantly, this induction occurs in the lung tissue and not in draining lymph nodes, suggesting a key establishment of tolerance to allergens in the lung mucosa. In addition, along with epithelial cells, alveolar macrophages have an important role in tissue integrity protection, partly achieved by their tight control of neutrophil recruitment (Kopf, Schneider and Nobs, 2015). Despite this "peace-keeping" role, alveolar macrophages can acquire a pro-inflammatory phenotype upon PRR activation by certain antigens, leading to increased phagocytic capacities and high secretion of IL-1 β , IL-6, TNF- α and TGF- β . While interstitial macrophages may have higher intracellular microbicidal properties, alveolar macrophages are still required to orchestrate an immune response following infection. For these reasons, and because these cells are at the first contact, pathogens have developed various mechanisms to counteract alveolar macrophages functions.

c) Lung dendritic cells and the T cell response

In addition to macrophages, DC have important functions in the lungs. Different subtypes of DC are present in the lungs, the most abundant being conventional DC (cDC)(Chen and Kolls, 2013). Lung cDC are continually replenished from bone marrow progenitors, and their differentiation from blood monocytes depends on Fms-related tyrosine kinase 3 ligand (Flt3L) and Csf2. In general, there are two subtypes of DC present in the lungs: i) CD103⁺ DC, which are dependent on the basic leucine zipper transcriptional factor ATF-like (BATF)3, and ii) CD11b⁺ DC, which rely on IRF4 expression (Kopf, Schneider and Nobs, 2015). Resident conventional DC present directly below the epithelium are able to sample many antigens either directly, through the extension of dendrites across alveolar epithelial cells into the alveoli, or indirectly, when delivered by M cells (Jahnsen *et al.*, 2006). Upon antigen sampling and acquisition, they will prime naïve T cells present in the lung-draining lymph nodes or in bronchus-associated lymphoid tissue. Noticeably, it seems that CD103⁺ DC are more prone to induce Th1 or Th2, while CD11b⁺ DC have a tendency for differentiating Th17 and Th2, but these modes of action depend on environmental factors (Kopf, Schneider and Nobs, 2015).

Similar to the gut, CD4⁺ T cell subtypes in the lung are important for clearance of different types of pathogens. Their short lifespan usually allows high activation of immune cells by T cell pro-inflammatory cytokine secretion without damaging the lung tissue. However, dysregulated T cell responses have been associated with many chronic lung diseases. For example, allergic induction of Th2 leads to asthma development. In COPD and cystic fibrosis, impaired elimination of pathogens, due to altered production of surfactant and AMP by epithelial cell, lead to persistent activation of T cells and prolonged inflammation resulting in parenchymal lung damage (Chen and Kolls, 2013).

DCs mainly determine the status of the lung T cell compartment. Even if they are less efficient than alveolar macrophages, CD103⁺ DC are also able to induce iTreg along with their protective effects against inflammatory disease. This involves TGF- β production by lung stromal cells, driving DC toward a regulatory phenotype and inducing iTreg, which then reduce the Th2 response in allergic asthma models (Li *et al.*, 2008). CD103⁺ DC are also able to induce effector CD8⁺ T cells during infections, while CD11b⁺ DC are important for the generation of central memory CD8⁺ T cells (Kopf, Schneider and Nobs, 2015).

It is known that APC from the gut or skin can induce T cells that specifically home to the gut or skin. Recently, it has been demonstrated that DC and macrophages activated in the lungs are similarly able to imprint T cell lung homing, which is mediated through induction of CCR4 expression (Mikhak,

Strassner and Luster, 2013). The success of the adaptive immune response largely depends on the rapidity of T cell homing during infection, which is accelerated by CCR4 expression. Therefore, lung APC play a key role in T cell-mediated protection against respiratory infections. As such, their interaction in the lungs with microbiota components may play a crucial role in maintenance of respiratory health (Mikhak, Strassner and Luster, 2013).

2. The gut-lung axis

As previously mentioned, dysbiosis of the gut microbiota alters the intestinal homeostasis, including the immune cell compartments. In the last years, however, evidence accumulated to demonstrate that the gut microbiota is also implicated in homeostatic functions at distal sites, such as the brain, liver, kidney, adipose tissue or the lungs (Schroeder and Bäckhed, 2016; Dinan and Cryan, 2017). Additionally, gut microbiota dysbiosis is often observed in patients with respiratory diseases, such as asthma or cystic fibrosis (Abrahamsson *et al.*, 2014; Bruzzese *et al.*, 2014). Mechanisms linked to this distal effect of the gut microbiota are diverse and include direct modulation of immune cells at peripheral sites by microbiota-derived compounds, or indirectly by the modulation of the gut leukocytes creating a systemic effect.

Multiple studies demonstrated that production of soluble mediators, such as SCFA, which can reach the blood stream and bind immune cell receptors in distant organs, is highly involved. In particular, this mechanism contributes to the effect of gut microbiota on pulmonary immunity by the so-called “gut-lung axis”. Indeed, mice deficient for the SCFA receptor GPR43, or under low-fiber diet (decreasing microbial SCFA-producers), developed aggravated inflammation in models of allergic asthma or infection with *Klebsiella pneumoniae* compared to WT mice (Maslowski *et al.*, 2009; Trompette *et al.*, 2014; Galvão *et al.*, 2018). By contrast, SPF mice under high-fiber diet (increasing microbial SCFA-producers), or receiving SCFA administration, are more resistant to, infection with *K. pneumoniae* or Influenza virus, and develop less allergic asthma, or sepsis- or LPS-induced lung injury (Zhang *et al.*, 2010; Trompette *et al.*, 2014, 2018; Thorburn *et al.*, 2015; Galvão *et al.*, 2018; Li *et al.*, 2018). In humans, high fiber diet is also associated with increased SCFA levels and low incidence of asthma, suggesting an overall protective role for gut microbiota-derived SCFA in respiratory health (De Filippo *et al.*, 2010). It is thought that the beneficial effects by SCFA are mediated by their capacity to modulate the lung myeloid compartment. One proposed mechanism is that SCFA regulate hematopoiesis in the bone marrow, generating monocyte differentiating in DC or macrophages with lower inflammatory properties (Trompette *et al.*, 2014, 2018). This is linked with fewer pro-inflammatory lung Th2 cell induction in an allergic asthma model, as demonstrated by propionate administration in mice which reduce lung pathology (Trompette *et al.*, 2014). Acetate also improves asthma through induction of Treg in the lungs (Thorburn *et al.*, 2015). During flu infection, high fiber diet (or butyrate administration) increased monocyte recruitment to the lungs, increased differentiation of macrophages into more anti-inflammatory cells, and decreased neutrophil infiltration, reducing lung tissue pathology. In parallel, butyrate modulated CD8⁺ T cell metabolism enhancing their ability to kill infected cells, thus improving infection resolution (Trompette *et al.*, 2018). In spite of reducing inflammation mediated by macrophages, SCFA also improved phagocytosis and bacterial killing during infection of the lungs, as observed in the gut (Galvão *et al.*, 2018). Collectively, SCFA produced by the gut microbiota enhance pathogen clearance, limit the inflammatory response, and protect the lungs from various inflammatory pathologies.

Beyond SCFA, gut microbiota MAMP (microbial associated molecular pattern) recognition by PRR mediate protection against respiratory diseases (Brown and Clarke, 2017). While bacteria from the gut microbiota are mainly restricted inside the gut lumen, bacterial components such as LPS or peptidoglycan may pass the epithelial barrier and bind PRR outside the gut (Patrice D. Cani *et al.*, 2007; Clarke *et al.*, 2010; Iwamura *et al.*, 2017). In the lungs, Nod-like receptors (NLR) signaling is particularly important. Indeed, gut microbiota-derived NLR ligands increase the IL-17 concentration in the lungs, which in turn increases that of Csf2, and, as previously described, Csf2 improves alveolar macrophage phagocytosis and bacterial killing properties, leading to better protection against respiratory bacterial infection and resistance to allergic responses (Clarke, 2014; Schuijt *et al.*, 2016; Brown, Sequeira and Clarke, 2017). Conversely, defects in NLR stimulation by microbial ligands result in increased susceptibility to *Streptococcus pneumoniae* following antibiotic treatment (Brown, Sequeira and Clarke, 2017). In addition, NLR activation by the microbiota also limits neutrophil recruitment and modulates monocyte hematopoiesis, arguing for a wide range of effects enacted by gut-microbiota MAMP outside the gut (Clarke *et al.*, 2010; Brown and Clarke, 2017; Iwamura *et al.*, 2017). Furthermore, other type of PRR may be implicated in the distal effects exerted by microbial-derived compounds. For example, TLR4 triggering by gut-derived LPS seems essential for efficient immune response to influenza infection and to prevent asthma (Ichinohe *et al.*, 2011; Qian *et al.*, 2018). Altogether, these studies clearly highlight the importance of microbiota-derived soluble compounds in the gut-lung axis and promote their exploitation for prophylactic and therapeutic purposes (this aspect will be discussed in subsection IV).

The gut-lung axis is also influenced indirectly by the capacity of the microbiota to modulate gut leukocytes. T cells induced by the gut microbiota, in particular Th17, can migrate from the gut to the lung under the influence of CCL20/CCR6 (Bradley *et al.*, 2017; Samuelson *et al.*, 2017). Gut T cells also migrate to the bone marrow, where they produce cytokines such as IL-17, and modulate myeloid cell generation and phenotype (Chen and Kolls, 2013). ILC3 primed in the gut can also migrate to the lung where they either contribute to the protection against pneumonia (Gray *et al.*, 2017) or promote lung pathology, depending on the circumstances (Bradley *et al.*, 2017).

In conclusion, the gut microbiota contributes directly and indirectly to lung immunity, emphasizing the strong need to better understand the mechanisms implicated in this process to better prevent and treat respiratory diseases. However, such understanding may not be sufficient, as it is clear now that the respiratory tract also harbors its own microbiota.

3. Role of the local pulmonary microbiota

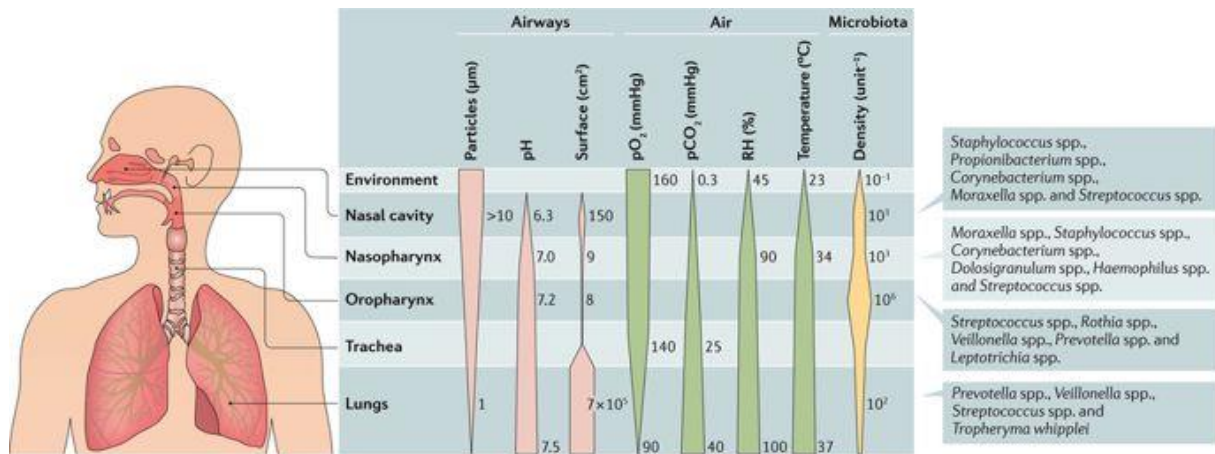
a) Composition and organization of the microbiota in the respiratory tract

The lungs were considered for a long time as a sterile organ. However, recent technological advances in 16S RNA sequencing and culture methods demonstrated that it is not the case. Detection of lung microbiota was difficult because *i*) the bacterial load in the lungs is low (10^{3-5} CFU/g, compared to 10^{11} CFU/g in the colon, for instance); *ii*) it is hardly accessible, often leading to contaminations during sampling; *iii*) intrapulmonary conditions are difficult to mimic as culture media (Charlson, Bittinger, *et al.*, 2012; Sze *et al.*, 2012; Marsland and Gollwitzer, 2014; Dickson and Huffnagle, 2015; Mathieu *et al.*, 2018). As in the gut, the respiratory tract contains different compartments with gradient of pH, oxygen and CO₂ concentrations, humidity and temperature that offer different niches for various microbial needs (Man, De Steenhuisen Piters and Bogaert, 2017) (Figure 9). In contrast to the digestive tract, the final compartment of the respiratory tract, lung alveoli, harbors low microbial diversity; they are

dominated by Bacteroidetes and Firmicutes with predominance of *Streptococcus*, *Prevotella*, and *Veillonella* genera (Erb-Downward *et al.*, 2011; van der Gast *et al.*, 2011; Charlson, Diamond, *et al.*, 2012; Man, De Steenhuijsen Piters and Bogaert, 2017). The lung microbiota share a more similar composition with the mouse microbiota compared to the nose microbiota (Figure 2), suggesting that the major source of lung microbes is immigration from the oral cavity into the upper and then lower respiratory tracts (Bassis *et al.*, 2015; Dickson and Huffnagle, 2015; Venkataraman *et al.*, 2015). Therefore, it is not surprising that the upper and lower respiratory tracts share multiple species, whose relative quantities depend on the different environmental conditions (Erb-Downward *et al.*, 2011). The respiratory microbiota composition is highly dynamic. Many of the colonizing microbes are eliminated by mucociliary clearance, cough and the immune system (Dickson and Huffnagle, 2015). In the lungs, microbes are indeed in direct contact with alveolar macrophages, which limit their growth in an environment already poor in nutrients²³. Hence, except during diseases, bacteria may only transiently be present in the lungs rather than established (Man, De Steenhuijsen Piters and Bogaert, 2017). However, it is possible that passage through the respiratory tract select bacteria entering the lungs. Indeed, IgA may prevent colonization of some species over others (Man, De Steenhuijsen Piters and Bogaert, 2017). As already mentioned, the hypothetical presence of a mucus layer in the lower respiratory track would impair gas exchange, just like the one in the colon lowers nutrient import (which is less crucial as nutrients are highly imported in preceding gut compartments). In the absence of a mucus layer in the lower respiratory system, high bacterial load would be too much of a high risk for potential infection. Is it possible then that our body provides a specialized mucus layer in the colon and cecum as an environment very favorable to microbiota colonization, in order to benefit for their global immunomodulatory functions, which may complement site-specific interactions at other mucosal tissues, through a distal effect (*e.g.*, the gut-lung axis).

In spite of its dynamic aspect, the lung microbiota composition is similar across many individuals, suggesting the existence of a “core lung microbiota” that may be composed of species that resist better than others against the toxic mechanisms (*e.g.*, IgA or AMP) encountered in the upper respiratory tract (Figure 10). Another element arguing for the presence of a specific microbiota in the lungs is the rapid alteration in microbiota composition following drastic life-habit changes of the host. As the gut microbiota, the lung microbiota composition is altered by the diet and antibiotic use, increasing susceptibility to respiratory infections or asthma (Leibovitz *et al.*, 2003; Madan *et al.*, 2012; Pettigrew *et al.*, 2012; Trompette *et al.*, 2014). Lung microbiota is also impacted by specific environmental factors such as cigarette smoking, air pollution and respiratory infections (Pettigrew *et al.*, 2012; Man, De Steenhuijsen Piters and Bogaert, 2017). Interestingly, dysbiosis of the microbiota in the lower respiratory track does not always correlate with dysbiosis occurring in the mouth microbiota, highlighting the differences in the microbiota species according to their niche (Morris *et al.*, 2013). Dysbiosis of the microbiota in the lower respiratory track is also observed in diseases such as chronic obstructive pulmonary disease (COPD), asthma or cystic fibrosis (Hilty *et al.*, 2010; Erb-Downward *et al.*, 2011; van der Gast *et al.*, 2011). In these diseases, the equilibrium between dispersion, growth and elimination, is disturbed in favor of certain species, such as Proteobacteria (Marsland and Gollwitzer, 2014). Therefore, environmental or genetic factors may induce dysbiosis that would in turn facilitate infection and/or aggravate disease outcome, but the causality relationship has not been clearly established.

²³ Nutrient sources for the microbiota are limited in the respiratory tract which seems to rely on host compounds, such as Igs, cytokines, defensins, lactoferrins, and mucins. (Mathieu *et al.*, 2018)



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Figure 9: Composition of the microbiota along the respiratory tract. Similar to the intestine, the microbiota is diverse in the lung depending on the physiological changes taking place from upper to lower respiratory tracks. Indeed, changes such as pH, relative humidity (RH), temperature, oxygen and carbon dioxide pressures, create different niches for various microbial needs. In contrast to the intestine, however, the end of the respiratory tract, lung alveoli, harbors low microbial diversity. Figure from (Man, De Steenhuijsen Piters and Bogaert, 2017).

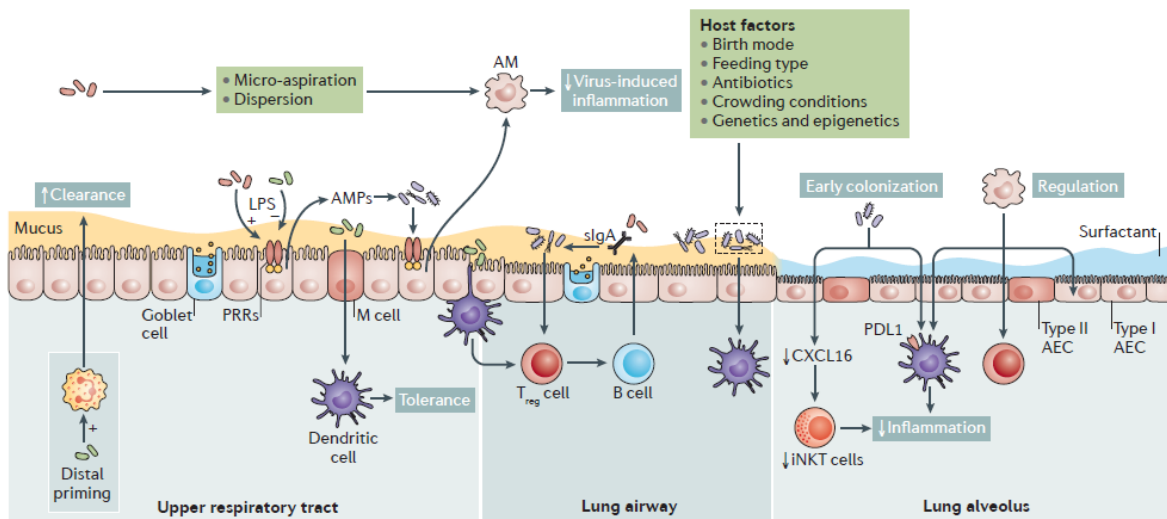


Figure 10: Host-microbiota cross-talk in the respiratory tract. Microbial invasion in the lung are prevented by mucus and AMP production by epithelial cells of the upper respiratory tract and lung airway, along with mucocilliary clearance, secreted IgA and the local immune system. Interaction between immune cells and the microbiota modulates this immune response. The respiratory microbiota is formed by micro-aspiration and dispersion of environment and resident microbes and shaped by the respiratory tract niches and genetic/environmental parameters affecting the host. The respiratory microbiota is involved in modulation of the lung immune system resulting either in the improvement of pathogen clearance or establishment of immunological tolerance. In the latter point, it participates to the development of tolerance in neonates by reducing the accumulation of inducible Natural Killer T cells (iNKT) *via* epigenetic modulations of the chemokine CXCL16; it induces a tolerogenic phenotype in DC that will promote iTreg development, reducing the incidence of inflammatory disorder like asthma susceptibility. In addition, the gut microbiota can modify lung immune response through distal priming of immune cells, among other mechanisms. AEC: alveolar epithelial cells. Figure from (Man, De Steenhuijsen Piters and Bogaert, 2017).

Because of its low abundance, many scientists doubted that the lung microbiota could exert functions on host health. However, emerging evidence suggest that stable colonization and high load are not required for microbiota to exert its functions. For example, the skin microbiota, which is of much lower density than the gut microbiota and highly transient, has a prominent role in protection against skin infections, as compared to the gut-skin axis (Naik *et al.*, 2012). Therefore, different dynamics may be responsible for the microbiota composition at different body sites, which may be crucial to sustain local health in addition to immunomodulatory properties of the gut microbiota.

b) Functions of the pulmonary microbiota on respiratory health

Early neonate colonization of lung by microbiota is involved in the respiratory tract development. Indeed, reduction of bacterial colonization is associated with altered alveoli architecture, which can be restored by nasal administration of *Lactobacillus* species (Yun *et al.*, 2014; Man, De Steenhuijsen Piters and Bogaert, 2017). Microbiota acquisition at this period is also key to shape the lung immune response. Indeed, before microbiota acquisition, SPF neonates are as sensitive to allergic asthma as germ-free ones whereas adult SPF mice exhibit protection against asthma as compared to germ-free mice or neonates (Olszak *et al.*, 2012; Gollwitzer *et al.*, 2014; Remot *et al.*, 2017). This susceptibility is associated with highly activated CD11b⁺ DC, exaggerated type-2 immunity activity, and accumulation of NKT cells. As observed in the gut, microbiota acquisition in the lung transiently modulates DC phenotype, inducing their expression of programmed death ligand 1 (PD-L1), which foments the differentiation of iTreg to establish long-term tolerance (Gollwitzer *et al.*, 2014). Importantly, nasal administration of one pulmonary *Enterococcus faecalis* strain in neonates reduced dramatically type-2 immunity and the incidence of allergic asthma (Remot *et al.*, 2017). These findings suggest that, in addition to SCFA produced by the gut microbiota, the interaction between lung microbiota with local APC promote immunological tolerance that is essential to respiratory health (Figure 10).

As of today, no study has shown that the specific absence or dysbiosis of the lung microbiota alter immune responses during infections. However, in different studies, nasal administration of pulmonary bacteria induced strong immune modulations that were protective in mouse models of infection and asthma, suggesting that this is the case (as detailed in subsection IV.4). For example, following the alteration of both gut and lung microbiota by antibiotic treatment of mice, nasal administration of high Nod2-stimulating *Lactobacillus crispatus*, *Staphylococcus aureus* or *Staphylococcus epidermidis* (species present in the lung microbiota), was sufficient to drive the production of Csf2, a cytokine promoting *Streptococcus pneumoniae* or *Klebsiella pneumoniae* clearance by macrophages-derived ROS (Brown, Sequeira and Clarke, 2017). Noticeably, oral administration of high NOD2-stimulating bacteria representative of gut microbiota species had the same protective effect, but the nasal administration of these bacteria failed to accomplish so (Brown, Sequeira and Clarke, 2017). Therefore, lung mucosal myeloid cells may have specific interactions with pulmonary bacteria, better adapted to survive in the lung environment and/or to interact with lung immune cells in comparison to strains from the gut microbiota. This study also suggests that while some mechanisms used by the gut and lung microbiota to modulate immune functions may be different and complementary, others may be conserved and redundant such as the induction of Csf2-dependent protective effects in the lungs. Of note, in this study, nasal administration of low NOD2-stimulating bacterial strains from the lung microbiota did not improve *S. pneumoniae* or *K. pneumoniae* clearance, suggesting that, as in the gut, individual bacterial strains from the lung microbiota possess particular immunomodulatory properties.

Altogether, this study highlights the strong need to further characterize strains of the respiratory microbiota and to identify those having key role in respiratory health.

Apart from immunomodulatory functions, the microbiota of the respiratory tract may also play a role in resistance to colonization by pathogens. Evidence suggests that competition between species exists in the nose; for instance, resident *S. epidermidis* secretes proteases that inhibit invading *S. aureus* biofilm formation, while resident *Corynebacterium accolens* releases antibacterial fatty acids that prevent growth of invading *S. pneumoniae* (Iwase *et al.*, 2010; Bomar *et al.*, 2016). Cooperation of the immune system and commensals present in the respiratory tract may be crucial to prevent pulmonary infections, as many pulmonary species (*e.g.*, *S. pneumoniae*) are commensals of the upper respiratory tract but, if not controlled, may reach the lower respiratory track where they become pathogenic (Wypych, Wickramasinghe and Marsland, 2019). Further studies are needed to establish whether the interactions described above also occur in the lower respiratory tract.

As previously discussed, the gut-lung axis is important for respiratory health and disease; for example, IBD causes alterations of respiratory functions (Douglas *et al.*, 1989; Songür *et al.*, 2003). Nevertheless, the opposite may be true as well as some respiratory disorders are associated with gastro-intestinal symptoms (Sze *et al.*, 2014; Samuelson *et al.*, 2016; Wypych, Wickramasinghe and Marsland, 2019). A study in the mouse model showed that influenza infection altered the gut microbiota through migration of lung-derived Th1 cells to the intestine. This microbiota alteration further results in Th17-producing IL-17 leading to intestinal damage (Wang *et al.*, 2014). Altogether, this suggests that the gut microbiota is not the only one impacting health at distant site. Further studies with other models should be performed to formally assess whether the gut-lung axis is bidirectional, and to determine if lung microbiota/pathogens affect intestinal (and other mucosal site) immunity. Indeed, we may consider the global microbiota as an entire organism with components at different sites equivalent to “microbial organs”, communicating between them and with the host, to ultimately promote the super-host health.

In conclusion, microbiota highly interacts with the immune system and pathogenic bacteria, and it is involved, consequently, in health and disease. It is, therefore, a target of choice to improve health not only of the intestine, but also at multiple other sites, including the respiratory tract.

IV. Manipulation of the microbiota to improve the host health

Long before understanding of the mechanisms by which the microbiota interacts with the immune system in health and disease, fermented products such as Kefir or Kombucha have been part of traditional regimens in different human cultures, and are now globalized, to improve digestion and prevent diseases. In the 1900's, Metchnikoff rationalized this practice, theorizing that senility was caused by the reaction of the immune system to harmful putrefactive bacteria present in the colon, which he supposed could be replaced by lactic bacteria over yogurt consumption (Mackowiak, 2013). With the discovery that many diseases are linked to microbiota dysbiosis, Metchnikoff original ideas about the modification of microbiota to improve health gained interest. Since then, different strategies have been developed that comprise fecal microbiota transplantation, probiotic, prebiotic or postbiotic. Their preventive and therapeutic potential have been assessed in animal models and for some, validated in humans. In addition to participating in the development of therapeutic applications, the studies presented in this section also contributed to improve our fundamental knowledge on host-microbiota interaction mechanisms.

1. Fecal microbiota transplantation

Many studies have assessed a causal link of microbiota dysbiosis in different diseases. This was mainly assessed by colonizing germ-free mice with fecal microbiota content from patients versus healthy donors; the majority of the results clearly demonstrated that microbiota from patients increased susceptibility to disease (Goodrich *et al.*, 2014; Blanton *et al.*, 2016; Britton *et al.*, 2019; Wong and Yu, 2019). Based on these results, it can be hypothesized that the replacement of a patient microbiota showing dysbiosis by the microbiota of a healthy individual (that usually reflects eubiosis) will reduce the incidence/outcome of a given disease. Fecalotherapy or fecal microbiota transplantation (FMT), consisting of oral administration of microbiota strains obtained from fecal material, has the potential to replace the receiver microbiota by the donor one. In some models, colonization of diseased mice with microbiota from healthy mice protected them from disease development, supporting FMT as a therapy (Seekatz *et al.*, 2015; Tian *et al.*, 2016; Zhou *et al.*, 2017; Le Bastard *et al.*, 2018). FMT was first realized in humans in the 1950's for the successful treatment of patients against *Clostridium difficile* enterocolitis (EISEMAN *et al.*, 1958). The food and drug administration (FDA)²⁴ has approved microbiota-based therapy for this disease. It is in fact the best treatment for severe and recurrent cases of this infection, where antibiotics are poorly efficient (Van Nood *et al.*, 2013; Quraishi *et al.*, 2017; Cammarota, Gallo and Bibbò, 2019). By contrast, the use of FMT against other diseases (*e.g.*, IBD) had limited effect (Libertucci and Young, 2019; Wong and Levy, 2019). This issue highlights our need for better understanding the interactions between the different species forming the microbiota, as well as their interaction with pathogens and with the host. In the case of *C. difficile* infection, the efficacy might be higher because microbiota strains interact directly with the pathogen, avoiding the complexity of host interactions. While promising, FMT raise the risk to transfer pathobionts to the receiver, causing disease in this new environment or to induce a negative interaction with natural bacteria from the receiver leading to bad outcomes (Wong and Levy, 2019). Therefore, many efforts have been invested on searching for alternative strategies targeting the microbiota.

²⁴ The **FDA** is a US federal agency responsible for protecting and promoting public health. All drugs must be approved by this agency after extensive examination of clinical trials (that are also authorized by the FDA) before commercialization.

2. Traditional probiotic approaches

a) First generation probiotics

In parallel to FMT development, many studies were performed to identify beneficial micro-organisms that could be used to improve health by modifying the host-microbiota interaction. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) named these live microorganisms, whose administration in adequate amounts confer a health benefit on the host, “probiotics” (FAO/WHO 2001). The search for probiotic candidates started with the study of *Lactobacillus* and *Bifidobacteria species* from fermented products. Many strains of this origin were found to reduce inflammation in IBD models and prevent infections through immunomodulatory properties, decreased gut permeability or direct inhibition of pathogen growth, using *in vitro* and animal models (Medellin-Peña and Griffiths, 2009; Hsieh *et al.*, 2012; Torres-Maravilla *et al.*, 2016). These bacteria have a food-grade status, namely Qualified Presumption of Safety status (QPS) in Europe and Generally Recognized as Safe (GRAS)²⁵ in the United States, which allowed their rapid commercialization for human consumption as probiotic products. Commercialized probiotics are mainly composed of the yeast *Saccharomyces cerevisiae* (boulardii); *Lactobacillus species*, such as *L. rhamnosus*, *L. plantarum*, *L. casei*, *L. salivarius*, or *L. acidophilus*; *Bifidobacterium species*, including *B. bifidum*, *B. bifidus*, or *B. lactis*; *Streptococcus thermophiles* and *Streptococcus acidophilus*; *Lactococcus lactis*, *Enterococcus SF68*; and *Escherichia coli* Nissle 1917. These probiotic strains or mixtures of them are commonly added to foods such as milk products, allowing their survival and delivery to the intestinal mucosa (Pandey, Naik and Vakil, 2015; Tsai *et al.*, 2019). Comparative studies in humans suggest that probiotics may be effective in preventing antibiotic-associated diarrhea, enterocolitis, lactose intolerance and upper respiratory tract infections in children (McFarland, 2006; Pandey, Naik and Vakil, 2015; Plaza-Díaz *et al.*, 2018). Yet, despite the generated and commercialized enthusiasm, there is a lack of clear clinical evidence to support the health benefits of these microorganisms in many disease contexts. Guidelines from the FAO and WHO emerged to regulate probiotic development, requesting the assessment of microbial strain identity and experimental proof for the strain safety, along with assessment of health effect in humans (M. J. Kim *et al.*, 2018).

Recent understanding of the mechanism of action of gut commensals and comparative studies of probiotic candidates in mice, indicate that some properties, including probiotic effects, can be either shared between bacteria from the same species, or specific to individual strains. Indeed, while secretion of lactic acid is shared by all lactobacilli, other products (*e.g.*, bacteriocins) can be strain specific as well as their expression of MAMP (Hart *et al.*, 2004; Foligne *et al.*, 2007; Medellin-Peña and Griffiths, 2009; Sahoo *et al.*, 2015). As example, *L. reuteri*, *L. casei* or *L. rhamnosus* JB-1 strains but not *L. murinus* or *L. plantarum* strains can bind to the DC-SIGN receptor²⁶, leading to delayed microbe internalization, poor expression of co-stimulatory molecules and production of IL-10 in DC, and thereby favouring an increase in Treg-producing IL-10 over effector T cells (Smits *et al.*, 2005; Konieczna *et al.*, 2015). Similarly, binding of *L. salivarius* Ls33 peptidoglycan muropeptide M-tri-Lys (but not *L. acidophilus* NCFM peptidoglycan devoided of this muropeptide) to NOD2 receptor induces IL-10 and retinoic acid production in DC, which then enable Treg differentiation to protect mice from experimental colitis (Foligne *et al.*, 2007; Fernandez *et al.*, 2011). Furthermore, not all *Lactobacillus*

²⁵ The **QPS** status is granted by the European Food Safety Authority as the **GRAS** status is given by the FDA to microorganisms used in food products that are well identified and lack pathogenic properties as demonstrated by experts. This assumption of safety exempts them of usual food additive tolerance requirements.

²⁶ Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) is PRR of the C-type lectin family presents on the surface of macrophages and DC that recognizes mannose type carbohydrates found on viruses, bacteria and fungi.

strains induce immunological tolerance through Treg activity. For example, the *L. casei* CHCC3139 strain induces highly pro-inflammatory cytokines IL-12, IL-6, and TNF- α when co-cultured *in vitro* with DC, suggesting that different strains of the same species can have opposite effect (Christensen, Frøkiaer and Pestka, 2002). In addition, while some strains have a synergistic effect, others have antagonistic effect, complicating the design of probiotic mixtures and questioning their interaction with the natural microbiota after administration (Christensen, Frøkiaer and Pestka, 2002; Chapman, Gibson and Rowland, 2011). In addition to PRR activation, secretion of soluble molecules is also involved in some lactobacilli-based probiotic effect. For instance, *L. reuteri* 17938 induction of Treg (*via* DC modulation) is recapitulated by the use of supernatant derived from this strain (Haileselassie *et al.*, 2016).

Altogether, the emerging picture is that the broad use of probiotics may not be efficient to prevent and treat all diseases, in spite of the reported efficacy achieved by specific individual probiotic for some diseases. Better knowledge of the mechanisms involved may allow the design of rationalized approaches to screen different strains using *in vitro* and animal models to select the best candidates before testing in clinical trials for a dedicated disease. Ongoing studies determining administration protocols allowing best efficacy of probiotic candidates (that may differ depending on the strains or disease) and higher standardization in pre-clinical models are also likely to improve probiotic efficiency in clinical trials (Dumas, Bernard, *et al.*, 2018). In parallel, a better characterization of traditional probiotic strains and how to improve their beneficial effect, will likely advance other microbiota-based therapeutic strategies, including the use of prebiotics/synbiotics.

b) Prebiotics and synbiotics

Prebiotics are components of the diet that change the composition or activity of the gastrointestinal microbiota to engender health in the host. Most prebiotics are oligosaccharide carbohydrates, such as fructans or galacto-oligosaccharides, which promote the growth of *Lactobacillus species* over other species (Davani-Davari *et al.*, 2019). The development of prebiotic is evolving rapidly with the use of bioinformatics tools that predict microbiota species able to metabolize a given prebiotic. However, this approach can be complex because some prebiotic may promote indirectly the growth of unexpected microorganism that feed on by-products of a given prebiotic fermentation, a process referred to as cross-feeding. Most prebiotic effects converge on increased production of SCFA (see subsection II) (Rycroft *et al.*, 2001; Artiss *et al.*, 2006; Venkataraman *et al.*, 2016). Prebiotics may also reduce pathogen binding to epithelial cells, as demonstrated for the monosaccharide mannose (Oyofe *et al.*, 1989). The effect of prebiotic is mainly beneficial against intestinal diseases, such as colorectal cancer, but it can also be relevant for skin diseases (*e.g.*, atopic dermatitis), cardiovascular diseases or mental disorders (Davani-Davari *et al.*, 2019). They may be particularly suitable to prevent acute infections in children (Lohner *et al.*, 2014). However, as for probiotics, while clinical studies globally promote the potential of prebiotics, the results concerning their efficacy remain controversial. Their advantage over probiotic is that they may be suitable for larger human populations because they target large groups of bacteria (rather than a unique one). In addition, some of them are present in natural sources (*e.g.*, the prebiotic inulin is present in chicory), inferring they can easily be added to diet to improve health.

Synbiotics refer to the combination of probiotic and prebiotic. Their function can either be complementary (action involving two different mechanisms independently promoting health) or synergistic (with the prebiotic supporting growth or activity of the probiotic). Most of the time, prebiotic allow survival of the probiotic strain through the upper intestinal tract (Pandey, Naik and Vakil, 2015). Some of them are currently tested in humans. For instance, the combination of *L. sporogens* and fructo-oligosaccharides reduced antibiotic induced diarrhea in children (La Rosa *et al.*,

2003). They represent an attractive strategy to improve traditional probiotic efficacy, but still requires further investigation.

While most studies suggest that probiotic, prebiotic and symbiotic could improve human health in many inflammatory disorders, clear evidence for their efficacy is lacking. Furthermore, even if administration of probiotic or prebiotic is safe in most individuals, cautions should be taken for their administration to immuno-compromised populations including young children. Consequently, in parallel with the optimization of traditional probiotics and the development of new candidates, additional strategies targeting microbiota are sorely needed (Tsai *et al.*, 2019). They comprise the use of strains that may have more specific and efficient protective role against dedicated diseases, and the direct delivery of active compounds from the microbiota as described in the next section.

3. Next-generation probiotics and postbiotics

a) Rational design for the development of new probiotics

Comparison of microbiota from healthy and ill individuals identified unique microbiota strains that are strikingly decreased during disease, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bifidobacterium species* or *B. fragilis*. It has been postulated that these bacterial strains, adapted to the intestinal environment, could modulate the microbiota composition and have higher beneficial effects compared to common *Lactobacillus species*, thus representing very promising microbiota candidates for the development of next-generation probiotics (Chang *et al.*, 2019). The main challenge for the probiotic use of these gut-microbiota strains was to isolate and cultivate them *in vitro*, which is a difficult task, in particular for strict anaerobic strains. Recent advances in this field have allowed a better characterization of these strains and their beneficial effect to the host (Duncan *et al.*, 2002; Derrien *et al.*, 2004). For example, administration of *A. muciniphila* ATCC BAA-835 in obese and type-2 diabetes patients, carrying low levels of this species, improved some metabolic parameters (such as increase of insulin sensitivity or decrease in plasma cholesterol). In addition, administration of the Amuc_1100 protein from this strain also mediated a protective effect in mouse models of obesity and type 2 diabetes (Everard *et al.*, 2013; Plovier *et al.*, 2017; Depommier *et al.*, 2019). Similarly, one of the most abundant bacterium in healthy adults, *F. prausnitzii*, is reduced in different diseases including IBD, colorectal cancer or obesity (Hold *et al.*, 2003; Sokol *et al.*, 2008; Rajilić-Stojanović *et al.*, 2011; Martín, Bermúdez-Humarán and Langella, 2018). Accordingly, *F. prausnitzii* A2-165 and HTF-F strains reduce inflammation in different colitis models, and restore intestinal permeability through Treg induction (Sokol *et al.*, 2008; Carlsson *et al.*, 2013; Martín *et al.*, 2014, 2017; Rossi *et al.*, 2016). The *F. prausnitzii* effect seems to be dependent on multiple effectors, including secretion of butyrate and of the microbial anti-inflammatory molecule (MAM)²⁷ (Miquel *et al.*, 2015; Quévrain *et al.*, 2016; Martín, Bermúdez-Humarán and Langella, 2018). As previously described, different mouse models suggest that *B. fragilis* (or its PSA) could also have a strong potential to reduce IBD and colorectal cancer by inducing of Treg differentiation, but its effects have not been confirmed in humans yet (Mazmanian, Round and Kasper, 2008; Lee *et al.*, 2018).

In summary, such bacterial strains are highly promising due to their strong immunomodulatory properties. However, their safety assessment requires full testing (Martín, Bermúdez-Humarán and

²⁷ The **MAM** protein secreted by *F. prausnitzii* strains seems able to reduce effector T cells induction in colitis models through modulation the nuclear factor NF-κB pathway a key determinant of inflammatory responses in immune cells, but the mechanism is not fully elucidated.

Langella, 2018). In particular, some strains of *B. fragilis* produce enterotoxins that are linked with inflammatory diarrhea (Sears *et al.*, 2008). While *A. muciniphila* ATCC BAA-835 administration was safe in the pilot study, it is associated with negative effects in models of multiple sclerosis or Parkinson's disease (Tsai *et al.*, 2019). Screening of different strains from these interesting species may allow the selection of candidates with most efficacy and safety profiles. In parallel to the use of these natural strains, two recent approaches that may be considered as safe and effective alternative microbiota-based therapies are in development that are postbiotics and engineered microbiota strains.

b) Postbiotics

Postbiotics include bacterial components with the minimal structure that possess biological activity and are devoid of live bacteria side effects. This approach only begins to be explored with the administration of microbiota-derived metabolites. Because such metabolites are naturally present in our body at high concentrations, their use in humans should be safe (Corrêa-Oliveira *et al.*, 2016; Canfora *et al.*, 2017; Wong and Levy, 2019). As previously addressed in subsection II, SCFA have been associated with pleiotropic effects improving health and their administration in mice showed promising results in colitis models (Smith *et al.*, 2013). SCFA administration in obese humans appears to modulate energy metabolism (Canfora *et al.*, 2017) and may allow a rapid control of a disproportioned inflammatory response against the microbiota within the context of disease (Wong and Levy, 2019). This strategy is also suitable to large human populations, as it does not rely on the patient microbiota composition that may compete with a probiotic candidate (Wypych, Wickramasinghe and Marsland, 2019). As yet, the effects enacted by postbiotics are difficult to predict since they may differ depending on the organ and immune cell type exposed. Therefore, the choice of the route of administration and of the dosage are critical before this new strategy become an efficient therapeutic.

c) Engineered strains for the delivery of microbiota compounds

While microbiota-derived metabolites could be administered directly to the host, this is not the case for microbial antigens. One possible strategy to deliver antigens relies on safe bacteria used as carriers. First generation GRAS probiotics (e.g., lactic acid bacteria) are ideal candidates for this purpose. Several studies in animal models demonstrated that *Lactococcus lactis*, *Lactobacillus plantarum* or *Escherichia coli* Nissle 1917, are able to efficiently deliver therapeutic molecules in the intestinal lumen or vaginal mucosa (Bermúdez-Humarán and Langella, 2018). This include pathogen-derived antigens for vaccination purposes (later developed in Section 2), molecules inhibiting pathogen colonization, and host-derived molecules with therapeutic interest, such as anti-inflammatory cytokines that are promising to protect against infections or to treat IBD and diabetes (Bermúdez-Humarán *et al.*, 2005, 2015; Lagenaur *et al.*, 2011; Duan, Liu and March, 2015; Hwang *et al.*, 2017). Some of these recombinant strains have shown efficacy in non-human primates and are currently being tested in humans (Baat *et al.*, 2006; Lagenaur *et al.*, 2011; Limaye *et al.*, 2013; Isabella *et al.*, 2018). Thanks to current knowledge about the specific beneficial properties of different first-generation probiotics, it is now possible to strategize synergetic approaches to combine natural probiotic properties with the effects predicted by the molecule being delivered. For instance, there is an improved protection against *S. pneumoniae* infection by co-administration of antigens with the immunomodulatory strain *L. rhamnosus* CRL 1505 during nasal immunization, suggesting that antigen delivery by this strain would be a promising strategy (Laiño *et al.*, 2018). In parallel, while past studies have focused on the delivery of host-derived molecules, new strategies now include the delivery of microbiota antigens with high immunomodulatory properties, such as the Amuc_1100 protein from *A. muciniphila*, microbial anti-inflammatory molecule MAM from *F. prausnitzii*, or PSA from *B. fragilis* (Quévrain *et al.*, 2016; Plovier

et al., 2017; Lee *et al.*, 2018). In fact, one study has already shown that the delivery of MAM *via L. lactis* strain to epithelial cells was protective in a mouse model of colitis (Quévrain *et al.*, 2016).

4. Perspectives

Microbiota-based therapy hold many advantages compared to other treatments. First, the microbiota is associated with the development of many diseases, and therefore it represents an etiological agent that should be targeted. Second, to modulate deleterious host-microbe interactions, the microbiota is a more accessible compartment to modulate in comparison to the host immune system. Last, in the infectious context, targeting the microbiota to boost the host immune response should avoid selection of multi-drug resistant super bugs in comparison to the further use of antibiotics.

Even though there are multiple challenges to make microbiota-based therapy a reality, significant progress has been made on this front. Considering the complexity of the mechanisms underlying the relationship of the microbiota with its host, it is not surprising that our first blinded attempts to design microbe-based therapy show few efficiency. The design of rationalized approaches to identify best promising strains based on growing knowledge of interactions between microbiota members and the immune system promoting health or disease is likely to be successful in the near future. For instance, while lactobacilli-based probiotics may be highly efficient to prevent vaginal-related diseases, or diseases in young children, where they are naturally abundant and protective, other species studied as next-generation probiotics should be investigated for other contexts as well as complementary approaches (Mur Pérez *et al.*, no date; Schwarzer *et al.*, 2016; Gosmann *et al.*, 2017; Libertucci and Young, 2019). Likewise, the development of computational tools may allow us to better decipher the complex relationships among the microbiota communities, and to better predict which microbiota strains are responsible for a given phenotype and with whom it interacts, improving again microbiota-based therapies (Faith *et al.*, 2014).

Eventually, the effect of some strains may be dependent on the host age, nutritional status, genetic polymorphism or antibiotic use (Segers and Lebeer, 2014). Development of personalized medicine will represent a watershed moment in the way we treat patients and prevent diseases and this may be possible by targeting the microbiota. Data collected from the direct comparison of healthy individuals and patients have already delivered some microbiota-derived biomarkers of health and disease. With the reduction of DNA sequencing costs, it is possible that in the near future there will be combined host and microbiota genome sequencing to predict susceptibility to diseases. The development of different animal models mimicking specific host populations (e.g. use of neonates, models of malnutrition or antibiotic use) should enable the targeting of host populations which will benefit the most for a given microbiota strain candidate (Agüero *et al.*, 2006; Schwarzer *et al.*, 2016; Remot *et al.*, 2017; Khan *et al.*, 2019). Altogether, while still in its infancy, microbiota-based therapies are likely to represent a consequent section of future preventive and therapeutic strategies to improve health.

5. Microbiota-targeted strategies for respiratory diseases

Reflecting the protective impact of the microbiota outside the gut, the development of microbiota-targeted strategies has a strong potential to improve respiratory health. In mouse models, administration of different lactic acid bacteria was found to dramatically modify the immune response to influenza infections or bacterial-derived pneumonia resulting in improved survival, decreased weight loss, diminished viral titer or bacterial load and a lower incidence of tissue damages (Dumas, Bernard, *et al.*, 2018). In addition, administration of *Lactobacillus species* or *Bifidobacterium longum* improved the early set-up of a pro-inflammatory response characterized by increased activation of alveolar macrophages (producing more ROS), accelerated recruitment of neutrophils and natural killer lymphocytes, and elevated levels of pro-inflammatory cytokines such as TNF- α and IL-6, associated with rapid clearance of pathogens and infected cells. This inflammatory response appeared, however, highly controlled at later time points. Indeed, they were characterized by a diminution of inflammatory cells and cytokines, increase of Treg and IL-10 resulting in an overall decrease of lung injuries as compared to non-treated mice (Racedo *et al.*, 2006; Kechaou *et al.*, 2013; Khailova *et al.*, 2013; Park *et al.*, 2013; Zelaya *et al.*, 2015; Vieira *et al.*, 2016; Belkacem *et al.*, 2017).

Likewise, probiotics are also potent immunomodulators of lung immune response. Indeed, while germ-free mice were highly susceptible to *Klebsiella pneumoniae* infection due to unwanted levels of IL-10 production, FMT with microbiota from SPF mice or mono-association with *Bifidobacterium longum* 5(1A) restored the control of the pathogen to the level of SPF mice (Fagundes *et al.*, 2012; Vieira *et al.*, 2016). Furthermore, this probiotic treatment also boosted the immune response of conventional mice, improving pathogen killing by alveolar macrophages and decreasing lung injuries, indicating that this strong immunomodulatory strain is an interesting probiotic candidate. One advantage of probiotic over more targeted treatments could be additional protection against secondary infections, as evidenced in mice, for influenza infections and Respiratory Syncytial Virus (RSV)-*Streptococcus pneumoniae* super-infection (Clua *et al.*, 2017; Jung *et al.*, 2017; Kanmani *et al.*, 2017).

Importantly, while few studies demonstrated that the protective effect of probiotics in respiratory diseases was due to their immunomodulatory role, one study showed that *L. rhamnosus* CRL1505 protective effect was due at least in part to increase levels in IFN- γ -producing T cells (reducing *S. pneumoniae* bacterial burden), IL-10-producing T cells (reducing epithelial damages), and alveolar macrophages producing IFN- β (reducing epithelial barrier permeability and limiting *S. pneumoniae* colonization). In this model, the use of antibiotic improved bacterial clearance but did not reduce immunopathology; in contrast, its combination with *L. rhamnosus* CRL1505 administration (or with inflammation inhibitor dexamethasone) improved both parameters, highlighting a new potential probiotic strategy (Damjanovic *et al.*, 2013; Clua *et al.*, 2017). Beyond the infectious context, some probiotics can decrease the pathological Th2 response, demonstrating a strong potential for the treatment of asthma (Pellaton *et al.*, 2012; Secher *et al.*, 2018). While most probiotic protective effects in the lung appear to be driven by similar immunomodulation events, these effects are strain-specific (Youn *et al.*, 2012; Tomosada *et al.*, 2013).

Several clinical studies have shown a beneficial effect of the consumption of probiotic (mostly *Lactobacillus species*) characterized by a reduction of infection incidence and the use of antibiotics (Alexandre *et al.*, 2014; Lehtoranta, Pitkäranta and Korpela, 2014). However, the difference with placebo group was often low and a Cochrane evaluation²⁸ suggested that this may be due to poor

²⁸ The Cochrane Reviews are part of the Cochrane Library databases. They are systematic reviews and meta-analyses which summarize and interpret the results of medical research and in particular of clinical trials to inform healthcare decision-making.

quality of trials (Hao, Dong and Wu, 2015). Thus, standardization of protocols and methods is needed to improve probiotic efficacy in the clinics, including a description of how bacteria modulate the immune system in a given disease context. The use of animal models will help for the selection of the most promising strains to test in future clinical trials.

Administration of postbiotics may also be useful in the context of respiratory diseases. Several studies using inactivated bacteria reported a partial protection against various infections, suggesting that both membrane compounds such as peptidoglycan but also metabolites may contribute to the full effect of live bacteria (Youn *et al.*, 2012; Vieira *et al.*, 2016; Brown, Sequeira and Clarke, 2017; Clua *et al.*, 2017; Kanmani *et al.*, 2017). In addition, administration of propionate was protective in a mouse model of allergic asthma, and administration of acetate reduced susceptibility to *K. pneumoniae* infection, explaining in part the overall beneficial effect imparted by *B. longum* in this model (Trompette *et al.*, 2014; Galvão *et al.*, 2018). Likewise, administration of prebiotics improving SCFA production is promising. Indeed, administration of inulin, favoring butyrate production, protects mice from influenza-induced pathology (Trompette *et al.*, 2018).

While our growing knowledge of the first-generation probiotic mechanisms of action may allow to improve their efficiency, the identification of more efficient candidates is needed. Strains highly involved in the gut-lung axis, such as SCFA producers, may be good candidates. Beyond microbiota strains (and their compounds) from gut origin, the discovery of the respiratory microbiota has opened new avenues for their use as probiotic candidates. This approach may be promising as studies employing microbiota strains from mucosal sites other than the gut (*e.g.*, skin) demonstrate a strong potential to enact local beneficial effects. For example, administration of the skin commensal *Staphylococcus epidermidis* modulates the local DC compartment to induce IL-17⁺ CD8⁺ T cells that limit *Candida albicans* invasion of the skin (Naik *et al.*, 2015). In addition, studies using first generation probiotics indicates improved protection by nasal compared to oral administration (Pellaton *et al.*, 2012; Youn *et al.*, 2012).

Moreover, in recent studies, presented hereafter, mice respiratory tract colonization using nasal administration of bacterial species that are naturally observed in this location (but that have not been isolated from the respiratory microbiota) decreased susceptibility to respiratory infections. For example, intranasal delivery of *Staphylococcus aureus* protects mice from influenza-mediated death. In this study, *S. aureus* binding to TLR2 signaling induced the recruitment of monocytes polarized toward anti-inflammatory macrophages secreting IL-10 and TGF- β , which then decrease the recruitment of pro-inflammatory cells and attenuate consequently lung immune injury (Wang *et al.*, 2013). Likewise, intranasal administration of high NOD2- stimulators such as *L. crispatus*, *S. aureus* or *S. epidermidis*, improve the mouse resistance to *S. pneumoniae* and *K. pneumoniae* infection (Brown, Sequeira and Clarke, 2017). Last, intranasal administration of *Corynebacterium pseudodiphtheriticum*, which is present in the respiratory tract, was able to reduce features of both RSV primary infection and of secondary *S. pneumoniae* infection in infant mice, decreasing both pathogen burden and lung damage (Clua *et al.*, 2017; Kanmani *et al.*, 2017). Outside the infection context, intranasal administration of *Acinetobacteri lwoffii* F78, or *Lactococcus lactis* G121, which are present in cowsheds of farms, reduced the development of allergic reactions in mice associated with an increase in Th1 cytokines and IL-10 that reduce type 2 immunity. This result suggests that natural acquisition of such strains in the respiratory tract may explain protection against asthma mediated by the farm environment that may represent another source of promising probiotic strains (Debarry *et al.*, 2007).

Recently, a pioneering study from the laboratory of Muriel Thomas and Philippe Langella, reported the isolation of 20 lower respiratory commensal bacteria species from mice neonates (Remot *et al.*, 2017). They demonstrated that these strains have different immunomodulatory properties *in vitro* and in an

allergic asthma model. As such, nasal administration of one *Enterococcus faecalis* strain strikingly decreased type-2 immunity, reducing most asthma features. This study demonstrated for the first time that the use of lung bacterial strains has the potential to improve respiratory health. In theory, these strains may have improved fitness for the lung environment and exert unique immunomodulatory pathways, and therefore represent highly attractive candidates.

The use of probiotic is promising in the context of respiratory diseases. Determining which interactions between the microbiota and the immune system are required to promote health should foment the design of more efficient next-generation probiotics. In particular, as for skin diseases, recent studies suggest that commensals from lung origin should be considered as promising candidates. The development of such alternative strategies could improve treatment of many respiratory diseases for which we lack antibiotics, or in combination with a shorten antibiotic treatment, to protect tissue integrity from deleterious inflammation, and delay the emergence of multi-drug resistant pathogens. As explained in the following section, such approaches are highly needed, and may be particularly suitable to improve treatment of one deadly respiratory disease: tuberculosis.

Section 2: Tuberculosis

I. Tuberculosis, the ever-present threatening disease

Tuberculosis (TB) is a pulmonary infection caused by the pathogenic bacterium *Mycobacterium tuberculosis*. While most people in Western countries believed the disease does not exist anymore, it is still the main cause of death on earth from a single infectious agent, ranking above acquired immune deficiency syndrome (HIV/AIDS) and malaria (Global and Tuberculo, 2018). As summarized in this chapter, the disease persistence in humans is partly due to specific properties of the bacillus, which is difficult to detect, control and kill, and partly due to the dedicated immune response against this pathogen, which is imperfect and difficult to improve.

1. *Mycobacterium tuberculosis*, a singular bacterium

The tubercle bacillus belongs to the *Mycobacterium tuberculosis* complex (MTC or MTBC) composed of mycobacteria causing tuberculosis (e.g., *M. tuberculosis* and *M. bovis*) in human or animals. It forms part of the *Mycobacteriaceae* family that is also composed of mycobacteria causing leprosy disease (*M. leprae*), and nontuberculous mycobacteria that are mainly environmental bacteria. Recent findings support the hypothesis that the common ancestor of this complex is an environmental mycobacterium, called *M. prototuberculosis*. Genetic material exchange through horizontal gene transfer with other bacteria, and clonal expansion, have given rise to this heterogeneous family (Gutierrez *et al.*, 2005).

M. tuberculosis is a microorganism difficult to study, which complicates the development of tuberculosis treatments. First, *M. tuberculosis* is a slow growing bacterium (under optimal growth conditions, its generation time is 18-24h). This implies long-term experimentation. Second, it is a dangerous pathogen, belonging to the risk group 3²⁹, which implies the need for particular and expensive biocontainment infrastructures along with complicated procedures. Many studies are using alternative model of mycobacteria, such as *M. smegmatis*, a fast-growing, non-pathogenic mycobacterium or *M. bovis* BCG (Bacille Calmette Guerin)³⁰, a slow-growing highly attenuated strain, used as a live vaccine in humans. However, they do not reflect all *M. tuberculosis* characteristics and, in particular, its complex interaction with the host. Therefore, most studies aiming at understanding the immune response to tuberculosis use virulent *M. tuberculosis* strains despite their constraining handling. Third, pathogenic mycobacteria harbor a strict host specificity. For example, *M. tuberculosis* is a facultative intracellular pathogen that only infects human leukocytes. Understanding its interaction with humans as a natural host dictates complex ethical, socio-economical and technical issues, among others (Pai *et al.*, 2016b). Although a number of alternative animal models exist to study *M. tuberculosis* interactions with the host, they do not perfectly mimic the human pathology.

²⁹ **Risk Group 3 organisms** cause serious disease in humans and represent a serious hazard for workers. They are likely to spread to the community, but effective treatments and vaccines exist.

³⁰ **BCG** is the only current anti-tuberculous vaccine, an attenuated form of *M. bovis* obtained by Calmette and Guerin. As *M. smegmatis*, it is a risk group 2 bacteria, that may cause only non-severe and treatable disease.

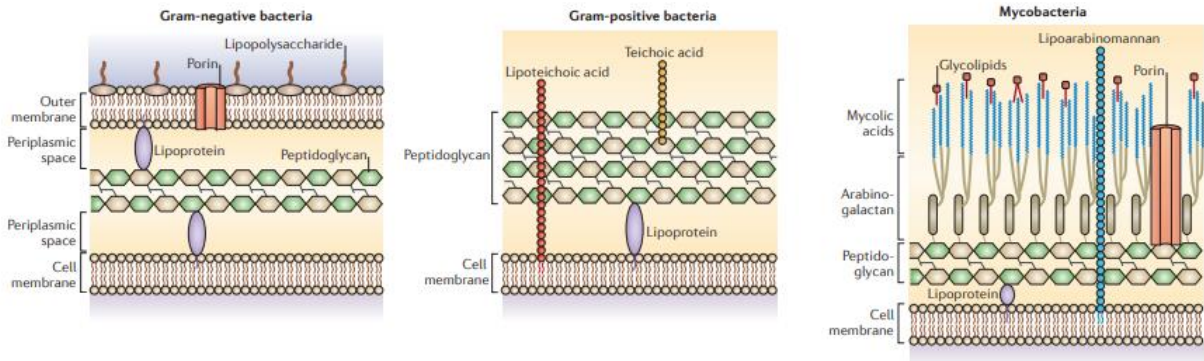


Figure 11: Structure of bacterial envelopes. Like Gram-positive bacteria, mycobacteria have no outer membrane. Their cell wall core is composed of layers of Peptidoglycan, Arabinogalactan and Mycolic acids. The Mycolic acid layer (also called mycomembrane) is surrounded by the capsule (not represented) containing highly mannosylated polysaccharides, such as Phosphatidyl-myo-inositol mannosides (PIM), Lipomannans (LM) or Lipoarabinomannans (LAM). The mycomembrane and capsule of mycobacteria have all a shared structure, but their components varied among mycobacteria species and are highly involved in their interaction with the immune system. Figure adapted from (Brown *et al.*, 2015).

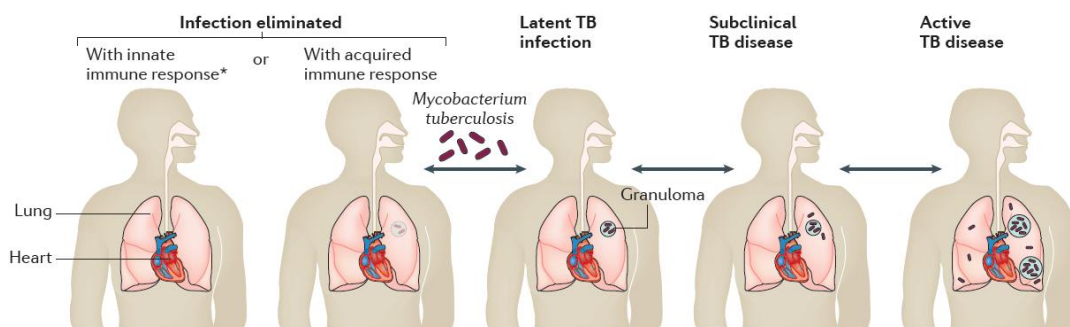


Figure 12: The different stages of tuberculosis infection. Individuals infected with *M. tuberculosis* can undergo different stages of disease depending on their immune system status going from infection clearance to active disease. TB: tuberculosis. Figure adapted from (Pai *et al.*, 2016b).

For instance, key findings in the mouse model (the most widely used in tuberculosis research) must be verified in translational research in non-human primates before a human attempt. For all these reasons, *M. tuberculosis* remains an ever-constraining and elusive micro-organism to be studied in the context of its natural host.

Nevertheless, the tubercle bacillus offers some advantages that can be exploited for therapeutic purposes. Unlike most bacteria, mycobacteria metabolism is mainly devoted to the synthesis and degradation of lipids. Some of them, the mycolic acids, are very important in the formation of the complex cell wall. This structure, specific to mycobacteria also contains very particular polysaccharides (Guinn and Rubin, 2017) (Figure 11). Because of its specificity, the mycomembrane allows the specific detection of mycobacteria (such as the Ziehl-Neelsen staining) and is the target of several of the current regime of anti-tuberculosis antibiotics. However, because of its impermeability, it also render other antibiotic targets difficult to reach (Jackson, 2014). *M. tuberculosis* lipids are essential components for the bacterial growth but also for its pathogenesis and interaction with the host.

The fact that *M. tuberculosis* is both a lethal pathogen and a micro-organism having co-evolve with humans for many thousands of years, makes these interactions highly complex. This for sure complicates the development of treatments but highlight the benefit of studying this pathogen to better understand the host-microbe interactions in health and disease even with the risk and level of difficulty involved.

2. Overall view of the characteristics, development and transmission of tuberculosis

As aforementioned, tuberculosis is caused by bacteria of the *M. tuberculosis* complex (in particular *M. tuberculosis*) (Pai *et al.*, 2016b). *M. tuberculosis* is primarily a pulmonary pathogen, but it can disseminate outside of the lung, creating a whole spectrum of highly morbid diseases, called extra-pulmonary tuberculosis (*e.g.*, meningitis) (Pai *et al.*, 2016a). Depending on the host status, the infection can lead to different outcomes depicted in Figure 12.

There are people in contact with patients who never develop active disease and are thought to naturally eliminate the pathogen either during the early step of the innate immune response or after recruitment of adaptive immune cells. If the infection is not cleared at the earliest stages, different immune cells including T cells will migrate to the site of infection and become part of the aggregates forming around infected cells, culminating in hallmark tuberculosis-related structures called granulomas (the immune response to tuberculosis is described in Section 2.II). Among individuals who do not eliminate the bacillus, the majority will efficiently contain the bacteria inside these granulomas leading to latent and asymptomatic disease. About 5-10% of infected people will fail at some time to control the pathogen growth, developing subclinical disease if few bacteria escape immune control, or active tuberculosis disease if many bacteria are able to replicate and trigger an over-activation of the immune system (as detailed in subsection II). Bacterial replication inducing over-activation of the immune system in active tuberculosis will result in mild to severe symptoms associated with pneumonia including coughing leading to pathogen transmission (Churchyard *et al.*, 2017), and in the more severe cases lung tissue lesions leading to the patient death (Taylor *et al.*, 2005; Hunter, 2011; Guinn and Rubin, 2017). Patients with active tuberculosis can remain infectious for up to one year in some settings, while patients with subclinical disease are sporadically infectious and develop mild or none symptoms (Pai *et al.*, 2016b). Importantly, patients can advance or reverse disease stages depending on reinfection or immune status of the host.

In fact, it is estimated that ¼ of the global population is infected today with tuberculosis, highlighting the fact that the low proportion of infected individuals developing active disease do not represent a low proportion of the global population. Because infection does not manifest any immediate visible or symptomatic signs, it is difficult to diagnose tuberculosis, a step needed to stop this transmission. In addition, even after pathogen eradication (naturally or following treatment), patients are susceptible to reinfection. Because progression to active disease depends on some settings, such as poor sanitary conditions, malnutrition or co-infection, weakening the immune system, the proportion of people developing active tuberculosis and dying from the disease is heterogeneous. As such, tuberculosis was pandemic in Europe and America in the past, and while lower proportions of patients develop active tuberculosis in these areas today and are in the majority of cases well treated for this disease, this is not the case in other areas of the world like Asia and Africa.

3. A historical perspective of tuberculosis

a) Tuberculosis pandemic in the pre-antibiotic era

Recent data suggest that human infection by *M. prototuberculosis* may have occurred dating back to the Paleolithic period in Africa (Donoghue *et al.*, 2004; Gutierrez *et al.*, 2005). From this environmental ancestor, it seemed that the *M. tuberculosis* complex later diversified and evolved along with the human species as its natural host, following historical human migration patterns ((Hershberg *et al.*, 2008) and reviewed in (Gagneux, 2012)). Along with the industrial revolution, tuberculosis peaked and reached epidemic levels at the start of the eighteenth and throughout nineteenth centuries in Europe and North America; its dissemination increased dramatically with migration, crowding, poor sanitation and famines associated with this period. More than 70% of the human population in these geographical areas were infected with *M. tuberculosis*, and up to 80% of these people died from this disease (Pezzella, 2019). Tuberculosis was given a variety of names such as phthisis, white plague or consumption (Pezzella, 2019). Since Antiquity, the nature/etiology of the tuberculosis (spontaneously generated, hereditary or transmissible) was unclear until the nineteenth century, when Jean-Antoine Villemin clearly demonstrated that it was an infectious and transmissible disease. Few years later, Robert Koch identified *M. tuberculosis*, also known as the “Koch’s bacillus”, in the patient sputum by using a new staining method, the Ziehl-Neelsen staining. He demonstrated that this bacterium was the disease causative agent using what are known now as the Koch Postulates³¹, a key step for the development of treatment. He also developed a glycerin extract from the bacteria, tuberculin, which was later used by Mendel and Mantoux to develop a skin detection test (PPD for Purified Protein Derivative). General advances in medicine, such as radiography, also improved the disease diagnosis allowing isolation of patients in sanatorium and reducing transmission of the disease.

b) Development of first preventive and therapeutic strategies

In 1908, Albert Calmette and Camille Guérin developed the BCG (Bacille Calmette Guerin) vaccine, an attenuated form of *M. bovis*. This is still today the only available vaccine. It has been extensively used to vaccinate infants due to its low cost, safety and stability (Ernst, 2018). While it shows efficiency to prevent TB-meningitis in children, its efficacy to prevent TB in adults varies widely and was not sufficient to stop expansion of tuberculosis (Rodrigues, Diwan and Wheeler, 1993; Ernst, 2018).

³¹ Koch Postulates are criteria developed by Robert Koch for judging whether a micro-organism is the cause of a given disease. These are: (i) The bacteria must be present in every case of the disease; (ii) the bacteria must be isolated from the host with the disease and grown in pure culture; (iii) the specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host; (iv) the bacteria must be recoverable from the experimentally infected host.

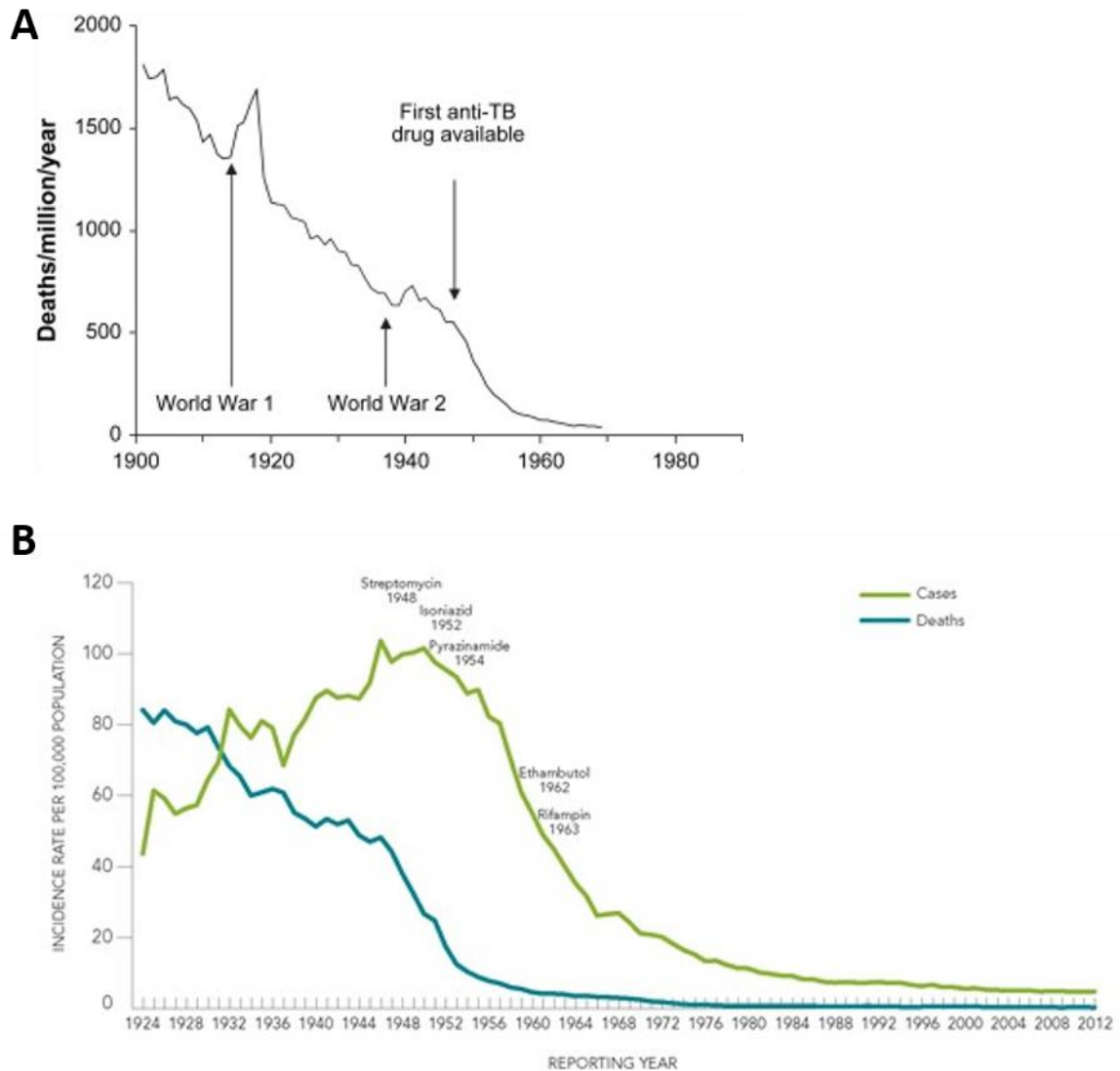


Figure 13: Mortality rate and incidence of tuberculosis in England and Wales (A) or Canada (B). Tuberculosis was a major cause of death in Europe and America in the eighteenth-nineteenth century. Mortality declined greatly with improvement of sanitary conditions and isolation of infected individuals. Of note, tuberculosis caused increased death during World Wars (WW) in Europe, suggesting that nutritional deficiencies, crowding and reduced sanitary conditions decrease outcome in infected individuals. Discovery of antibiotics after 1940 eventually allow reduction in mortality and incidence. Figure adapted from (Lönnroth *et al.*, 2009; *Public health agency of canada, TB case reporting*, 2012).

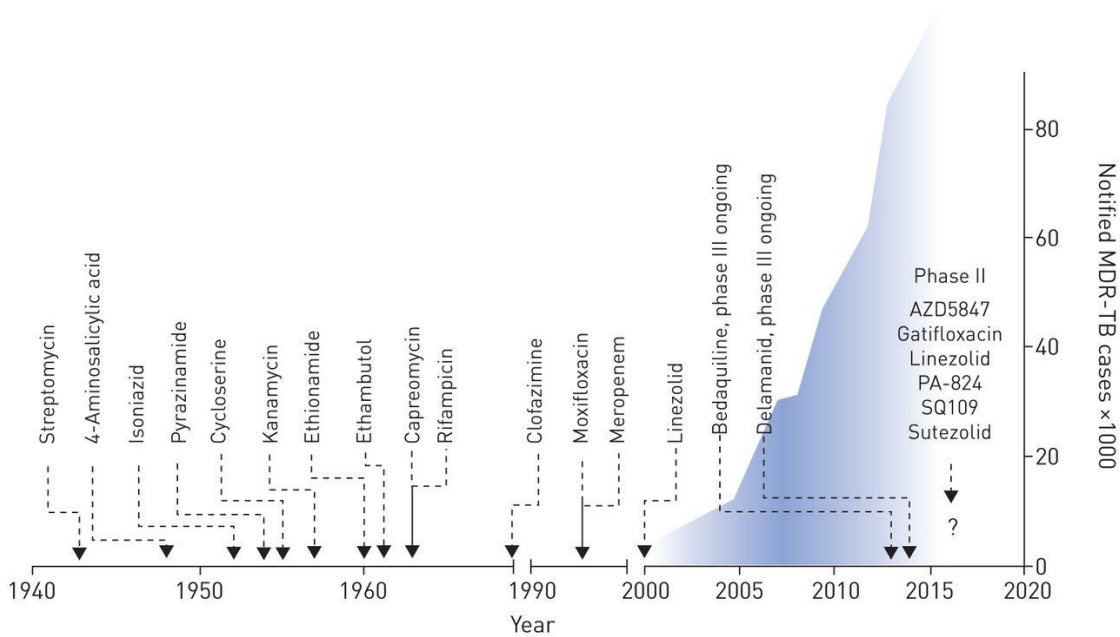


Figure 14: Anti-tuberculosis antibiotic pipeline. Since 1940, various antibiotics have been developed to the present time. New drugs, however, are still needed because of the progress of drug resistance and, in particular, multi-drug resistance (MDR-TB) (represented by the blue area). Figure adapted from (Olaru *et al.*, 2015).

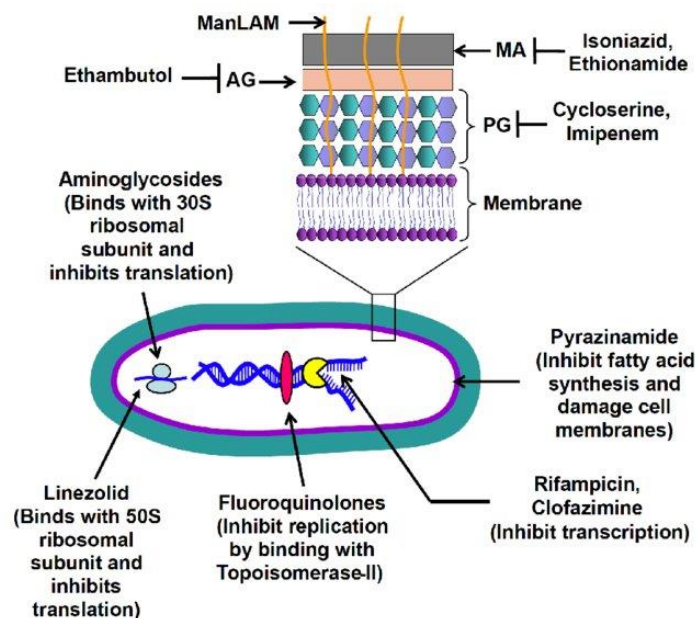


Figure 15: Anti-tuberculosis drug targets. Many anti-tubercular drugs target cell wall or membrane synthesis: Isoniazid and Ethionamide inhibit mycolic acid synthesis (MA), Cycloserine and Imipenem inhibit peptidoglycan synthesis (PG), Ethambutol inhibits arabinogalactan (AG) synthesis, and Pyrazinamide impacts cell membrane function. Other drugs interfere with vital processes such as bacterial replication (Fluoroquinolones), transcription (Rifampicin, Clofazimine) and translation (Aminoglycosides, Linezolid). Figure adapted from (Sukhithasri *et al.*, 2014).

In addition, its inoculation to HIV-infected infants may lead to the development of disseminated BCG disease³² (Hesseling *et al.*, 2007). Therefore, there is a critical need for vaccine development that would efficiently prevent the tuberculosis (Flynn, Chan and Lin, 2011; Pai *et al.*, 2016b). After 1820's, the advance in thoracic surgery also helped the treatment of the disease through lung collapse or resection (Pezzella, 2019). Since 1944, however, antibiotics were discovered and used to treat tuberculosis instead of surgery, marking a significant progression in tuberculosis treatment (Figure 13). In addition to antibiotherapy, it is thought that improvement of sanitary conditions mainly contributed to the decrease in tuberculosis incidence and mortality in Europe and America (Figure 13). Even if there is a low incidence of tuberculosis in these regions today, it is still a major health issue in other parts of the world where the hygiene, nutrition and socio-economic conditions are still a major issue (as detailed in subsection I.4).

The use of the first anti-tuberculosis antibiotic, Streptomycin, was quickly limited by the appearance of resistant *M. tuberculosis* strains (Pezzella, 2019). Since then, antibiotics targeting different essential pathways of the bacteria were discovered, and their use in combination strongly decreased the appearance of resistant strains (Figure 14 and 15). Classical therapy regimen has not evolved much at the present moment. It consists in treatment with four first-line antibiotics (isoniazid, rifampicin, pyrazinamide and ethambutol) for two months, followed by treatment with isoniazid and rifampicin alone for two additional months (Furin, Cox and Pai, 2019). Yet, patients with high bacterial loads and tissue damages require longer treatment. In many parts of the world, such complicated regimen is almost impossible to sustain in the long-term treatment, leading to incomplete treatments that favor the selection of multi-drug resistant (MDR) strains³³ (Russell, 2011). The discovery of new drugs and repurpose of existing drugs have hopefully increased alternative treatments. The second-line treatment (use to treat MDR-TB) comprise fluoroquinolones, acid para-amino-salicylic, aminoglycoside, ethionamid and cycloserine (Pai *et al.*, 2016a). This treatment is even longer (up to two years) and has adverse effect. They are also less efficient. While treatment of sensitive tuberculosis reaches around 95% success, MDR-TB are only cured at 50% when efficiently diagnosed (WHO | *Global tuberculosis report*, 2018). Different strategies are being studied to shorten the treatment duration. They include the use of higher dose of rifampicin or the use of other antibiotics. The development of the drug pipeline is not a subject detailed here but is reviewed in (Pai *et al.*, 2016a; Furin, Cox and Pai, 2019). These strategies seem promising but may not be sufficient. Indeed, the appearance of extensively drug resistant strains (XDR, that are also resistant to fluoroquinolone and second-line aminoglycoside antibiotics) and totally-drug resistant strains increases the difficulty to treat all patients (Pai *et al.*, 2016b). If duration, cost and side effect of these treatments are not reduced by strategies in development, patient compliance may rely low and resistance appearance quick.

³² Disseminated BCG disease is a rare life-threatening complication of BCG application that resemble tuberculosis and that significantly occurs in immuno-compromised individuals.

³³ Different class of resistant tuberculosis strains exist. A strain is simply called resistant (R-TB) if resistant to at least one antibiotic used for therapy. Multi-drug resistant (MDR-TB) are resistant to at least Rifampicin and isoniazid. Extremely-drug resistant (XDR-TB) are also resistant to fluoroquinolone and second-line aminoglycoside antibiotics while totally-drug resistant (T-DR) are resistant to all available antibiotics.

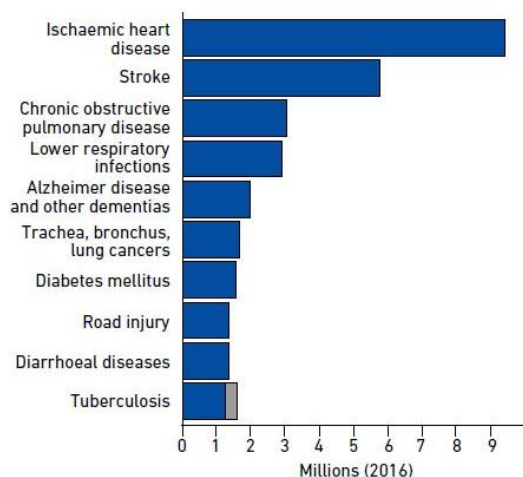


Figure 16: Top causes of death worldwide in 2016. Tuberculosis is the 9th cause of global death, and the 1st one from a single infectious agent. 25% of tuberculosis-related deaths are caused by co-infection with the HIV virus (represented in grey). Figure adapted from (*WHO | Global tuberculosis report, 2018*)

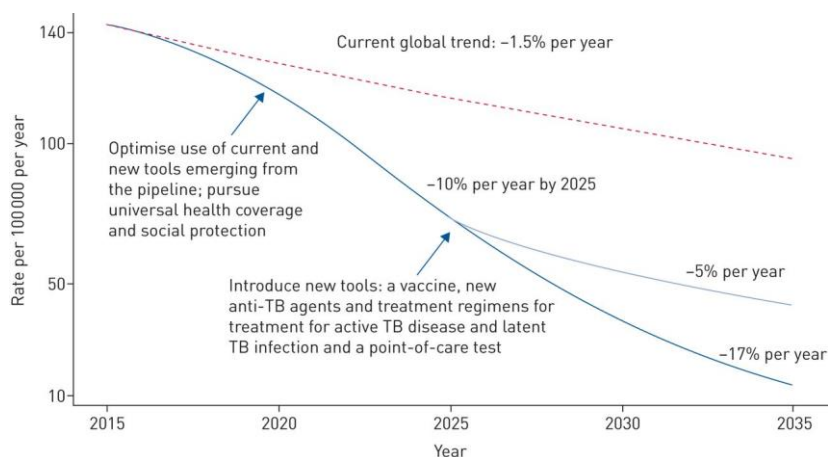


Figure 17: Projected global trajectory of tuberculosis (TB) incidence rate required to reach targets of the End TB Strategy. Current incidence of tuberculosis is around 1.5% per year. Achievement of the End TB Strategy of 1 case per 100 000 population per year requires not only the optimization of the current diagnosis, prevention and treatment tools, but also the development of new ones. Figure adapted from (*WHO | Global tuberculosis report, 2018; Gilpin et al., 2018*).

c) Diagnostic tools to detect tuberculosis infection

While new diagnostic tools have been developed since Robert Koch visualization of *M. tuberculosis* by microscopy, serious challenges still remain today to identify latent or subclinical tuberculosis. Tuberculosis infection can be diagnosed based on identification of *M. tuberculosis* compounds, reactivity of the patient's immune system to such compounds, or visualization of granulomatous structures *via* radiography. Active tuberculosis can be identified through observation of sputum smear in light or fluorescence microscopy, culture in liquid medium (allowing to test for drug resistance but with a long delay before results), detection of lipoarabinomannans³⁴ in urine samples (at the moment not sensitive enough to detect active tuberculosis in HIV-negative individuals), or molecular tests such as the Xpert MTB/RIF and the Line Probe Assays (that rapidly detects genetic material from *M. tuberculosis* along with mutations that cause resistance to define antibiotics) (Zijenah, 2018; Furin, Cox and Pai, 2019). Tests based on immune system reactivity to *M. tuberculosis* antigens, such as the tuberculin skin test (detecting high immune recruitment following PPD subcutaneous administration) or Interferon- γ release assays (measuring *ex-vivo* blood T cell activation following exposure to antigens), allow the detection of active and latent tuberculosis. Yet, they do not allow to distinguish these two forms of tuberculosis, yield false positives in patients having eradicated already the infection or vaccinated with BCG, and have low sensitivity in immunocompromised patients (Pai *et al.*, 2016b). With a better understanding of the pathology, disease biomarkers are under development that may help to predict risk of infection, disease, or cure. Eventually, chest radiography and PET-scan are used to detect active tuberculosis, in particular among HIV-positive patients, and are improved by the development of digital radiography and computer-aided diagnostic software (Pai *et al.*, 2016b). One remaining problem is the determination of the disease stage, availability and cost of technologies (Furin, Cox and Pai, 2019).

Even if improved sanitary/hygiene conditions, BCG vaccination, antibiotics and diagnostic tools, have together dramatically decreased the incidence and mortality of tuberculosis in Europe and America, we have still failed to end the pandemic tuberculosis.

4. Why is there still tuberculosis today?

a) From current epidemiology toward the end of tuberculosis

Tuberculosis remains today a major public health issue, with more than 6 million of new cases every year and about 1.6 million deaths in 2017. Additionally, tuberculosis has become recently the leading cause of infectious death above malaria and HIV infection. It is in fact the only infectious disease among the top 10 causes of death (Figure 16) (WHO | *Global tuberculosis report*, 2018). As tuberculosis is a major global threat, the WHO has proposed strategies to coordinate efforts all around the world to fight the disease, the latest being the "End TB strategy". It is built on the agreement of all member governments to accelerate their individual actions against tuberculosis and to include specific actions to prevent and cure drug-resistant tuberculosis. The aim of this program is to achieve by 2030 an 80% decrease in tuberculosis incidence compared to 2015 (corresponding to one new case per 100 000 people per year), and a 90% decrease in deaths. Today tuberculosis incidence is reduced by 2% every year and its mortality by 5% (decreasing in particular among HIV-positive people) (Figure 17) (WHO | *Global tuberculosis report*, 2018).

³⁴ Lipoarabinomannans are polysaccharides present in *M. tuberculosis* mycomembrane.

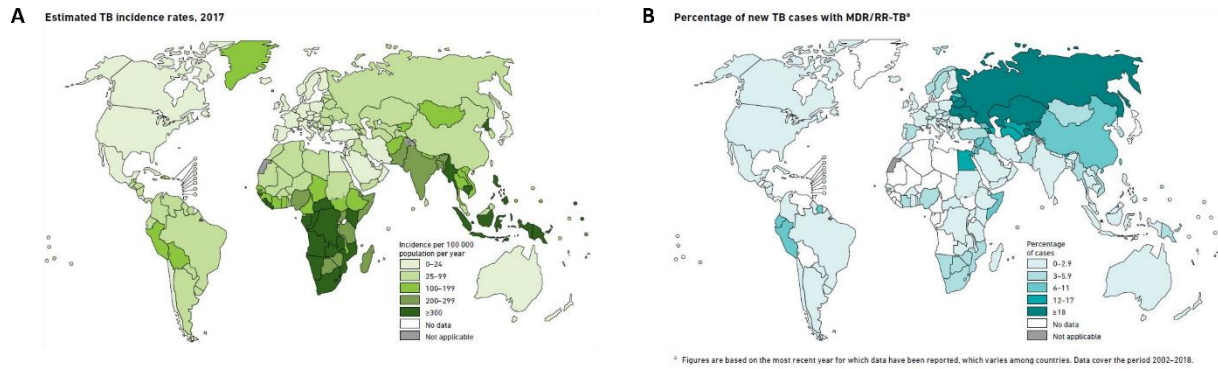


Figure 18: Estimated incidence of total or resistant tuberculosis (TB) cases. Tuberculosis still has a high incidence. However, the disease is not randomly distributed and is mainly present in low- to mid-income countries (A). Among the positive cases for tuberculosis, the frequency of drug resistance is increasing, but its prevalence do not coincide with global high incidence. Figure adapted from (*WHO | Global tuberculosis report, 2018*)

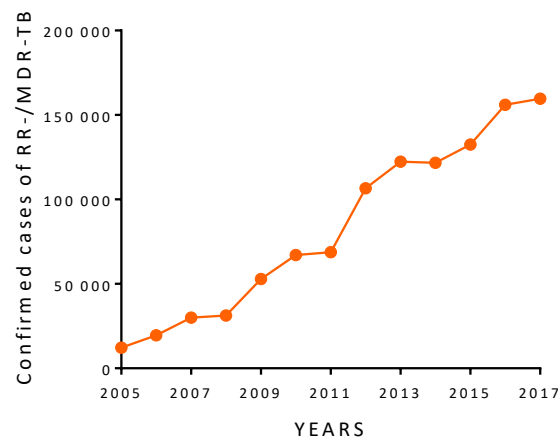


Figure 19: Increase of global drug resistant tuberculosis (TB). Even if the global tuberculosis incidence is decreasing, the number of cases of Rifampicin (RR) or multidrug resistance to tuberculosis (MDR-TB) exhibits a constant increase. Since these patients are more difficult to treat, it may result in global increase of deaths caused by tuberculosis in absence of a better treatment for these cases. Figure adapted from (*Global Health Observatory - WHO, 2018*).

b) Obstacles in tuberculosis eradication

Several pitfalls make tuberculosis eradication particularly difficult. First, most tuberculosis cases are currently appearing in low- and middle-income countries (two thirds of tuberculosis patients are living in only eight countries: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh or South Africa) (Figure 16 and 18). In these countries, co-morbidity parameters such as HIV infection, diabetes, malnutrition, crowding, sanitation and reduced healthcare are all increased (see section 4.d for details on co-morbidity parameters). This implies a strong need for funds to better detect and report tuberculosis cases, but also to provide appropriate health care. Also, the disease characteristics and epidemiology vary greatly among countries. For example, high incidence of tuberculosis in South Africa is associated to high HIV prevalence, while in India this is mainly caused by a high transmission rate, in particular in hospitals (Dye *et al.*, 2013). Together with the development of new treatments, the disparity in tuberculosis prevalence reinforces the need for therapeutic strategies that are adapted for each country. Second, more and more patients are infected with drug-resistant *M. tuberculosis* strains; in 2017, half a million patients were infected with *M. tuberculosis* strains resistant to Rifampicin, and about 80% of them were infected with multidrug-resistant *M. tuberculosis* strains (MDR-TB) (Shah *et al.*, 2017) (Figure 18 and 19). Global treatment success is today elevated with cure achieved in 82% of cases but does not progress, maybe due to a lower success rate among MDR-TB treatment (55%) (WHO | *Global tuberculosis report*, 2018). Finally, about 1.7 billion people (23% of the global population) are estimated to have latent TB and are at risk of developing active TB later in life. They represent an important and undetected reservoir of carriers of the disease. However, this estimation has recently been questioned with a new study suggesting that most cases of TB reactivation are due to re-infection rather than reactivation of a latent infection (WHO | *Global tuberculosis report*, 2018).

c) Requirements for tuberculosis eradication

Achievement in early detection of infection, prevention of new infections and neutralization of existing (latent) infections, are needed to achieve the End TB strategy goals (Dye *et al.*, 2013). Current actions in this direction include BCG vaccination of children and preventive treatment of latent TB for HIV-positive patients and children in contact with active pulmonary TB patients, but they will not be sufficient. One limitation to the success of the End TB Strategy is the lack of funding, needed partly to improve health services (with increase of health coverage and reduction of the cost faced by tuberculosis patients) in order to reduce tuberculosis incidence. The second one is the lack of more effective diagnostic (in particular to test drug susceptibility), preventive and therapeutic tools, as it is estimated that current tools are insufficient to eradicate tuberculosis (WHO | *Global tuberculosis report*, 2018). Predictive models suggest that action to block transmission and to cure existing (and in particular latent) infection should be a simultaneous effort to achieve synergistic effects (Dye *et al.*, 2013). Development of only new anti-tuberculous drugs may not be sufficient to achieve these goals. Targeting the host with the development of new vaccines or host-directed therapies may thus allow the synergic effect needed (current developments are detailed in subsection III). This implies a requirement to better understand the immune response during tuberculosis infection and how risk factors modify it.

d) Known co-morbidity parameters for tuberculosis

M. tuberculosis elimination and containment require a complex immune response. Therefore, an immuno-compromised host may develop active disease upon infection or upon activation of asymptomatic latent tuberculosis. This is particularly true for HIV-1-infected patients, who display reduced CD4⁺ T cell counts, especially of *M. tuberculosis*-specific CD4⁺ T cells (essential for the control of *M. tuberculosis* growth in the granuloma). In the co-infection with *M. tuberculosis*, HIV-1⁺ individuals are more prone to develop active tuberculosis (Esmail *et al.*, 2018), and often exhibit extra-pulmonary tuberculosis (Naing *et al.*, 2013). In the case of type II diabetes mellitus, patients displayed an altered pro-inflammatory metabolism in CD4⁺ T cells and macrophages (Yorke *et al.*, 2017). During malnutrition, which leads to secondary immunodeficiency, patients show micronutrients deficiencies such as vitamins or metal ions that are essential for cellular functions and proper activation of the immune system to fight the bacterial pathogens (Kant, Gupta and Ahluwalia, 2015). Tuberculosis is also aggravated by cigarette smoke, air pollution, and high alcohol consumption (Lönnroth *et al.*, 2009; Duarte *et al.*, 2018; Silva *et al.*, 2018). Indeed, food supplementation and smoking reduction are associated with improved tuberculosis recovery. Altogether, these co-morbidity parameters clearly indicate that a better understanding of the immune response during tuberculosis infection is essential to achieve the goals proposed by the End TB Strategy (Duarte *et al.*, 2018).

II. The immune response to tuberculosis

The immune response to *M. tuberculosis* is quite complex and difficult to decipher. It is highly circumvented and subverted by the pathogen in multiple ways that are unique for this disease. As described in this section, the mechanisms leading to pathogen transmission are detrimental for the host. At the same time, pathogen survival and replication are dependent on host survival. Therefore, it is sometimes difficult to decipher if some immune mechanisms benefit the host or the bacteria. In addition, the host has to make compromises between engaging a hostile war against this pathogen to destroy it at all cost (via resistance mechanism), and the truce our bodies make to tolerate it and preserve tissue integrity (Russell, 2011; Bussi and Gutierrez, 2019). Altogether, the immune response to tuberculosis is fascinating and still today not fully characterized. Our current understanding of this immune response, based on intense investigations in animal models and human samples is summarized in this section.

1. Overview of the immune response initiation in tuberculosis

M. tuberculosis infection begins by inhalation of expectorated droplets containing bacteria released by an infected patient with lung lesions. Transmission is highly efficient as it is estimated that arrival of one bacterium could be sufficient to drive the infection. In addition, high bacterial load in the inoculum or repeated exposure correlate with the exacerbation of the disease (Russell, Barry and Flynn, 2010). After inhalation, small (but not larger) droplets may achieve entry into the lower lungs. Once in the airways, *M. tuberculosis* first encounters epithelial cells and alveolar macrophages that phagocytize it, a phenomenon resulting in the infection of these cells (Cohen *et al.*, 2018).

It is possible that alveolar macrophages are able to clear the infection at this early stage. Indeed, many humans in contact with the bacteria do not become infected or rapidly eliminate the infection without showing immunological reactivity to *M. tuberculosis* antigens (Dannenbergh, 1991; Flynn, Chan and Lin, 2011; de Martino *et al.*, 2019). Yet, these observations need to be confirmed experimentally. By contrast, most experimental studies suggest that after internalization of the bacteria, intracellular killing of *M. tuberculosis* is impaired in infected macrophages. In fact, several mechanisms aimed at killing the bacteria may even benefit the pathogen.

After phagocytosis, most pathogens are degraded when the phagosome compartment is fused with lysosomes, which are organelles enriched in acidic, oxidative compounds and in hydrolases. However, numerous studies have shown that *M. tuberculosis* is able to inhibit the acidification of the phagosome by blocking its fusion with lysosomes (Figure 20) (Sturgill-Koszycki *et al.*, 1994; Clemens and Horwitz, 1995; Via *et al.*, 1997; Vergne, Chua and Deretic, 2003; Lugo-Villarino and Neyrolles, 2014). Nevertheless, a recent study using live imaging suggests that, in some cases, maturation of the phagosome may occur, trapping the bacteria in a proteolytic phagolysosome (Schnettger *et al.*, 2017). Even if it seems that phagosome maturation occurs, it is not sufficient to kill *M. tuberculosis*, which is able to survive in this acid environment (Armstrong and Hart, 1975). The bacterium also induces phagosome membrane damages, which induce the release of bacterial compounds (and maybe living bacteria) inside the cytoplasm. This mechanism seems dependent on 6 kDa early secretory antigenic target (ESAT-6) secretion by *M. tuberculosis* ESX-1 secretion system, and phthiocerol dimycocerosates (DIM that are part of the bacteria outer membrane) expression by the bacteria (Mishra *et al.*, 2010; Augenstreich *et al.*, 2017).

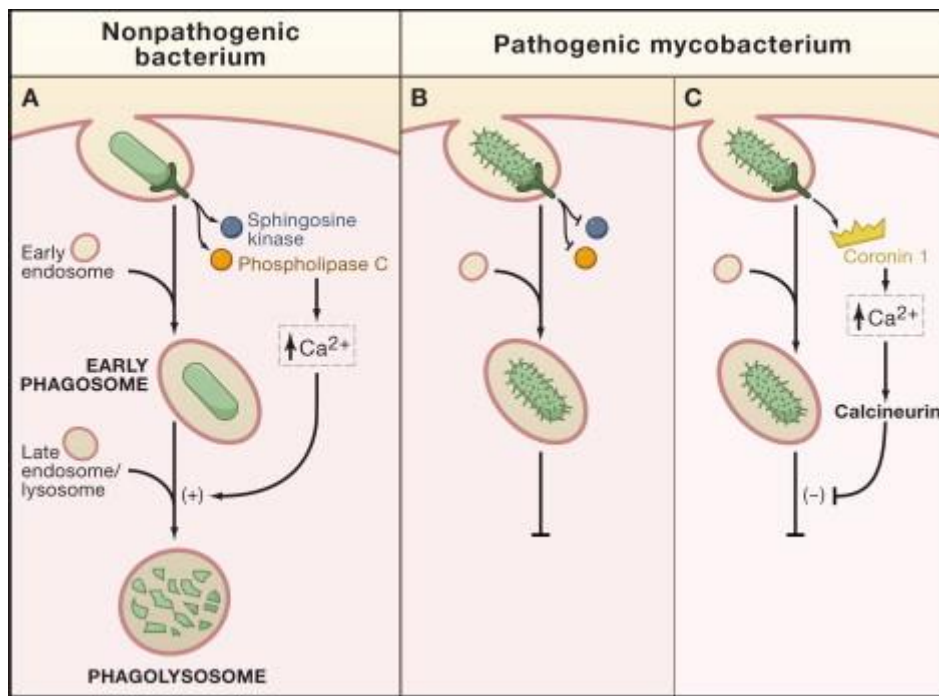


Figure 20: Mechanisms of phagosome maturation. A: Receptors that engage the bacteria or molecules on its surface activate sphingosine kinase and/or phospholipase C (PLC), which induce an increase in the amount of cytosolic free calcium ions (Ca²⁺). Nascent phagosomes fuse with early endosomes to generate early phagosomes, which in turn fuse with late endosomes/lysosomes to become phagolysosomes. Lysosomal enzymes in the phagolysosome destroy the bacterium. B: In the presence of pathogenic mycobacteria, such as *M. tuberculosis*, bacterial components, possibly lipoarabinomannan, preclude the elevation of Ca²⁺ and thereby prevent fusion with late endosomes/lysosomes, arresting maturation at the early phagosome stage. C: Another model proposes that receptors induce an elevation of Ca²⁺ by a process that requires the actin-binding protein coronin 1. The rise in Ca²⁺ activates calcineurin, which blocks fusion of early phagosomes with late endosomes/lysosomes by an unknown mechanism. Both models would allow the bacterium to persist in macrophages. Figure adapted from (Trimble and Grinstein, 2007).

Bacteria released in the cytosol are usually targeted by xenophagy (a form of autophagy)³⁵ in macrophages, but *M. tuberculosis* is again able to escape the following lysosomal degradation (Ouimet *et al.*, 2016) and reviewed in (Kimmey and Stallings, 2016).

During macrophage infection, various *M. tuberculosis* compounds are sensed by macrophages through PRR, such as TLR2, TLR9, DC-SIGN (Tanne *et al.*, 2009), mannose receptor (Schlesinger, 1993), Mincle (Ishikawa *et al.*, 2009), NOD2 (Divangahi *et al.*, 2008) or NLR family, such as the NLR pyrin domain containing 3 (NLRP3) (Mishra *et al.*, 2010), among others. Binding of the bacteria to those receptors and subsequent signal transduction is key for the activation of the infected macrophage. Recognition of bacterial antigens in the cytosol (after phagosome membrane damages) by PRR induces the activation of inflammasome and IL-1 β production. IL-1 β production by infected epithelial cells and macrophages is involved in alveolar macrophage translocation to the lung parenchyma (Cohen *et al.*, 2018) and in the recruitment of other immune cells to the site of infection (Davis and Ramakrishnan, 2009), such as neutrophils. *M. tuberculosis* transfer to the lung epithelium may also occur through migration of the bacteria inside infected epithelial cells (which seems also important for its dissemination to other organs) (Nair *et al.*, 2016). Production of the metalloprotease Matrix Metalloproteinase 9 (MMP-9) by epithelial cells is also implicated in the recruitment of immune cells to the site of infection and in the formation of an environment favoring bacterial growth (Volkman *et al.*, 2010).

The bacteria is able to limit apoptotic cell death (again through ESX-1 secreting system elements) of epithelial cells (Dobos *et al.*, 2000) and macrophages (Divangahi *et al.*, 2010). This is important since apoptosis of infected cells may result in phagocytosis of compartmentalized bacteria by other macrophages through efferocytosis³⁶, a mechanism suggested to efficiently lead to *M. tuberculosis* killing (Martin *et al.*, 2012). In absence of apoptosis, *M. tuberculosis* will multiply inside macrophages. Indeed, the bacteria not only inhibits phagosome lysosome fusion, but also resists to other killing mechanisms developed by macrophages, such as intoxication by transition metals (Botella *et al.*, 2011) or reactive oxygen and nitrogen species (Lugo-Villarino and Neyrolles, 2014), allowing its intracellular replication. If the bacterial load is too high, burst size cytolysis (a necrotic cell death) of infected cells may occur, releasing free viable bacteria (Repasy *et al.*, 2013). This leads to infection of newly recruited monocytes or neutrophils (Eum *et al.*, 2010; Cohen *et al.*, 2018; L. Huang *et al.*, 2018). Recruited cells are usually grouped in aggregates around the released bacteria and infected cells, in order to contain the pathogen. But at this stage, newly recruited cells are also permissive to bacterial infection and represent more a reservoir rather than a threat for the pathogen (Ernst, 2012). Their recruitment is even accelerated by the secretion of exosome enriched in *M. tuberculosis* lipids by infected macrophages, suggesting that new target cells recruitment is directly induced by the pathogen (Davis and Ramakrishnan, 2009; P. P. Singh *et al.*, 2012). Recruited cells also display an increased motility. Therefore, after infection, these cells may migrate to other sites of the lungs, resulting in the dissemination of the bacteria and to the formation of new aggregates (Davis and Ramakrishnan, 2009).

³⁵ **Autophagy** is a cellular process aiming at removing unnecessary or damage compounds. It occurs through engulfment of these compounds in an autophagosome vacuole that will fuse with lysosome for degradation. A form of autophagy, called xenophagy, uses similar pathways to eliminate intracellular pathogens.

³⁶ **Efferocytosis** consists on phagocytosis and subsequent degradation of apoptotic bodies from a dying cell. It prevents tissue exposure to toxic compounds such as enzymes contained in the apoptotic bodies.

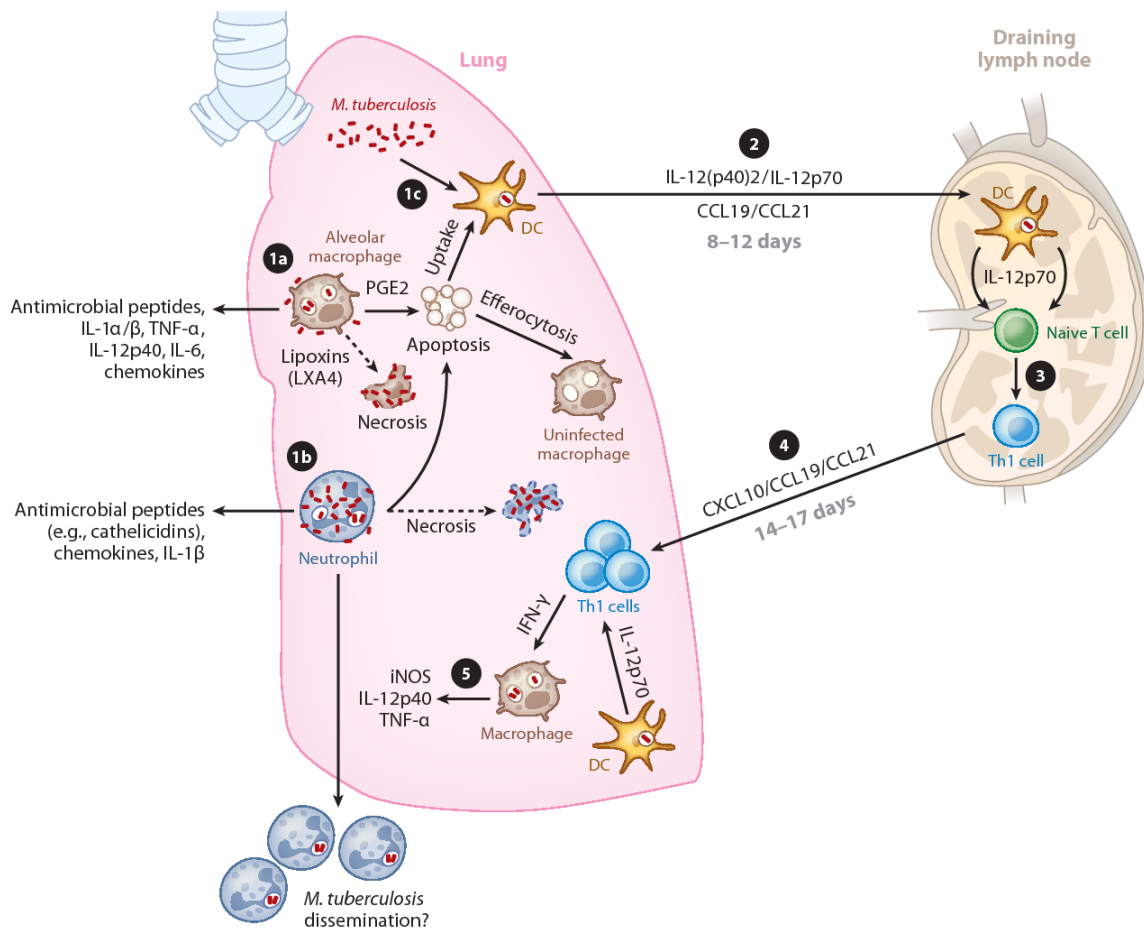


Figure 21: The cellular immune response to *M. tuberculosis*. Following aerosol infection with *M. tuberculosis*, resident lung alveolar macrophages (1a), neutrophils (1b) and lung DCs (1c) can become infected, leading to the production and secretion of antimicrobial peptides, cytokines, and chemokines. The balance of lipid mediators, such as prostaglandin E2 (proapoptotic) or lipoxin (LX) A4 (pronecrotic), within infected macrophages plays a major role in determining downstream pathways leading to the induction of either apoptosis or necrosis. Infected apoptotic cells can be taken up by resident lung DC or efferocytosed by uninfected lung macrophages (1c). *M. tuberculosis*-infected DC migrate to the local lung-draining lymph nodes by 7–14 days post infection. DC migrate to the lymph nodes under the influence of IL-12(p40)2 and IL-12p70, and that of the chemokines CCL19 and CCL21 (2), to drive naive T cell differentiation toward a Th1 phenotype (3). Protective antigen-specific Th1 cells migrate to the lungs in a chemokine-dependent manner 14–17 days after the point of initial infection/exposure (4) and produce IFN- γ , leading to macrophage activation, cytokine production, the induction of microbicidal factors including iNOS (5), and bacterial control. Figure adapted from (O’Garra *et al.*, 2013)

This stage of the infection, characterized by the replication of the bacteria, continues until the T cell response is activated and reinforces the microbicide activities in innate immune cells to overcome the pathogen's influence, as described in the next subsections (Havlir and Barnes, 1999; Moguees *et al.*, 2001).

2. T cell induction in the draining lymph nodes

DC are also present early at the infection site and can become infected by *M. tuberculosis*. However, unlike macrophages and neutrophils, they do not represent a reservoir for the bacteria in the lungs (Cohen *et al.*, 2018). Upon interaction with the pathogen, these cells migrate to the lung draining lymph node to present antigen and prime a dedicated T cell response. Intriguingly, while this process happens within the first 24 to 72 hour post-infection with most pathogen, DC migration to the lymph nodes is quite delayed in *M. tuberculosis* infection, ranging between 9-14 days post-infection (Cooper, 2009; Roberts and Robinson, 2014). In addition, DC are thought to contribute to dissemination of the bacteria when infected (Chackerian *et al.*, 2002; Wolf *et al.*, 2007) (Figure 21). Lymphatics and lymph nodes represent the main sites of extrapulmonary tuberculosis (Behr and Waters, 2014), but the bacteria can disseminate to any organ. T cell activation occurs in the draining lymph node where clonal expansion and activation of *M. tuberculosis* specific T cell is observed (Reiley *et al.*, 2008; Wolf *et al.*, 2008). One main feature of *M. tuberculosis* infection is the delayed adaptive immune response. Indeed, in human, adaptive immune response appears 21-42 days post-infection (Wallgreen, 1948; Poulsen, 1950) (in mice it takes up to 21 days (Lai *et al.*, 2018)), which is quite long compared to many other infections. As previously mentioned, this is due to a delayed migration of DC to the lymph node (Wolf *et al.*, 2008; Lai *et al.*, 2014), while migration of T cells to the lung after their activation seems to take place at a normal speed (Reiley *et al.*, 2008).

A rapid initiation of T cell response is essential to the host and may constitute one factor that differentiates resistant C57BL/6 mice from susceptible C3H, DBA/2 or CBA/J mice strains to tuberculosis (Turner *et al.*, 2001; Chackerian *et al.*, 2002). It is possible that this delay is necessary for this slow growing pathogen to replicate in permissive non-activated myeloid cells. This may explain why delayed migration of DC seem directly caused by the bacteria. Indeed, their migration does not seem impaired when antigens are picked up from infected neutrophils compared to free bacteria (Blomgran and Ernst, 2011). By contrast, blockade of apoptosis in *M. tuberculosis*-infected cells reduces antigen presentation resulting in delayed adaptive immunity priming (Hinchey *et al.*, 2007; Divangahi *et al.*, 2010). Of note, migration and maturation of DC seemed dependent on the presence of IL-12p40, rendering them responsive to chemokines such as CCL19 (Khader *et al.*, 2006; Wolf *et al.*, 2007). DC migration may be inhibited during homeostasis due to suppression of CCR7 by IL-10, a mechanism latter counteracted by IL-12p40 (Khader *et al.*, 2006).

M. tuberculosis-infected DC express low levels of MHC-II and are poor antigen presenters *in vivo* (Pai *et al.*, 2003; Sendide *et al.*, 2005). However, these cells are able to transfer *M. tuberculosis* antigens to more powerful uninfected bystander DC in the draining lymph node (Wolf *et al.*, 2007; Srivastava and Ernst, 2014). Interestingly, this may be an adaptation of our immune system to overcome the decreased antigen presentation capacity of infected DC (Hudrisier and Neyrolles, 2014). Yet, presentation by bystander cells do not compensate for the reduced bacteria presentation by infected cells (Srivastava, Grace and Ernst, 2016), leading to an overall delay in T cell priming.

Recent studies suggest that the two populations of lung cDC may not behave similarly during the infection. Both lung CD11b⁺ and CD103⁺ DC are infected by *M. tuberculosis* and will migrate to the lung

draining lymph nodes, but CD103⁺ DC carry higher bacterial loads (Lai *et al.*, 2018). In the lymph node, only CD11b⁺ DC are able to mediate CD4⁺ T cells activation toward a Th1 phenotype. Conversely, CD103⁺ DC inhibit this priming by producing the anti-inflammatory cytokine, IL-10 (Lai *et al.*, 2018). This is maybe due to their higher index of infection, which may render them susceptible to bacterial inhibition of their maturation and modification of cytokine secretion (Geijtenbeek *et al.*, 2003; Madan-Lala *et al.*, 2014). Because CD103⁺ DC are also known to induce Treg (Suffia *et al.*, 2005; Janine L Coombes *et al.*, 2007), they also indirectly reduce effector T cell activation. It is clear that IL-10 (Redford *et al.*, 2010) and Treg (Shafiani *et al.*, 2010) delay priming of CD4⁺ and CD8⁺ T cells during tuberculosis. Of note, other mechanism besides the activity of CD103⁺ DC may exist to induce Treg in the lungs or lung draining lymph nodes, such as alveolar macrophages (Soroosh *et al.*, 2013).

3. T cell mediated containment of the infection

M. tuberculosis-infected DC mainly induce the generation of both Th17 and Th1. Generation of Th1 producing IFN- γ by DC seems dependent on IL-12. Among Th1, different CD4⁺ T cells are present, comprising early activated T cells producing IL-2, classical Th1 cells producing IFN- γ and multifunctional T cells producing both IL-2, IFN- γ and TNF (Winkler *et al.*, 2005). The latter category seems crucial for the development of a protective immune response. Type-1 immunity cytokines IFN- γ , TNF, IL-1 and IL-6 are produced in the lungs after Th1 activation and are involved in control of the infection (Da Silva *et al.*, 2015). IFN- γ and TNF production by Th1 cells activates macrophage microbicidal activities (Figure 21). Macrophage activation increases interactions of *M. tuberculosis* containing phagosome with the endosomal network, targeting mycobacteria to spacious, proteolytic compartments that efficiently reduce its replication and access to the cytosol (Schnettger *et al.*, 2017). An activated phagosome is characterized by the presence of nitric oxides (in mouse), hypoxia and carbohydrate reduction, which perturb the *M. tuberculosis* envelop (Chan *et al.*, 1992; Macmicking *et al.*, 1997). Bacterial killing is also induced by vitamin D activation of macrophages, increasing autophagy (Gutierrez *et al.*, 2004; Yuk *et al.*, 2009) as well as anti-microbial peptide cathelicidin³⁷ production (in humans) (Liu *et al.*, 2007; Martineau *et al.*, 2007). Some bacteria are eliminated at this step, but not all. Indeed, the bacteria is able to survive these toxic stress by shifting its metabolism and enter dormancy (Wayne and Hayes, 1996; Schnappinger *et al.*, 2003; Rohde, Abramovitch and Russell, 2007; Leistikow *et al.*, 2010).

Macrophage activation by T cells is required to control bacterial growth. Indeed, IFN- γ -deficient mice, and human carrying genetic mutations in IFN- γ or IL-12 receptors, or infected by HIV are highly susceptible to tuberculosis (Cooper *et al.*, 1993; Ottenhoff, Kumararatne and Casanova, 1998; Esmail *et al.*, 2018). Similarly, the deleterious effects are observed in TNF-deficient mice (Flynn *et al.*, 1995) or patients under anti-TNF therapies (Keane *et al.*, 2001). Likewise, mice deficient in T-bet show increased IL-10 and decreased IFN- γ levels, associated with impaired macrophage activation and bacterial control (Sullivan *et al.*, 2005). IL-12 is not only essential to induce and maintain IFN- γ producing Th1 (Feng *et al.*, 2005), but also for induction of TNF- α and nitric-oxide production by macrophages (Jana *et al.*, 2003). Of note, other mice models show that protection by IFN- γ can be compensated by other mechanisms such as production by T cells of Csf2 (Rothchild *et al.*, 2017; Sallin *et al.*, 2018). These results highlight the key role of IL-12 mediated production of IFN- γ and TNF- α by

³⁷ Cathelicidin is an anti-microbial peptide produced in phagocyte lysosomes involved in lipoprotein membrane disruption, leading to bacteria death.

Th1 cells to control the infection, while other mechanisms may be also involved and need to be investigated.

Maintenance of macrophage activation is achieved partly because of T cell persistence. While T cells should have a short lifespan under chronic antigen stimulation due to replicative exhaustion, this is not the case during this infection. Even if their proliferation is reduced after the acute phase of infection, these lymphocytes are maintained (Winslow *et al.*, 2003). In the mouse model, most antigen-specific Th1 expressed programmed death-1 (PD-1), a marker of exhaustion, or killer cell lectin-like receptor G1 (KLRG1), a marker of terminal differentiation. PD-1⁺ cells were highly proliferative and, instead of becoming exhausted, they differentiated into KLRG1⁺ cells. These cells exhibited a short lifespan and secreted the pro-inflammatory cytokines IFN- γ and TNF- α . Therefore PD-1⁺ cells could constitute a reservoir of progenitors that continually produces terminal effector cells (Reiley *et al.*, 2010).

Th17 produce IL-17 leading mainly to neutrophil recruitment, but their role in tuberculosis progression is less clear. In IL-23 p19-deficient mice, Th17 cells are absent and IL-17 and IL-22 are poorly produced. In this model, the unaltered Th1 compartment seems sufficient to control the pathogen growth at early time points but not on the long term (Khader *et al.*, 2005, 2011). In addition, Th17 become important to control the pathogen growth in absence of IFN- γ , prolonging mice survival, but also inducing potential tissue damage through high neutrophil infiltrates (Wozniak *et al.*, 2010). This phenomenon was exacerbated in a model of repeated BCG vaccination before *M. tuberculosis* infection, inducing high amount of IL-17 responsible for tissue damage (Cruz *et al.*, 2010). Therefore, IFN- γ is also implicated in the control of IL-17 production to reduce neutrophil infiltrates, which is associated with immunopathology (Cruz *et al.*, 2006; Nandi and Behar, 2011). This may occur through induction of IDO in lung epithelial and endothelial cells, which then inhibit IL-23-mediated induction of Th17 (Desvignes and Ernst, 2009). Another detrimental role for IL-17 consists in the inhibition of apoptotic death of infected macrophages, which restricts the pathogen growth and infection of other target cells (Cruz *et al.*, 2015). However, Th17 may not be totally detrimental. Indeed, they are involved in the early recruitment of Th1 during vaccination, by induction of the CXCL9, CXCL10 and CXCL11 chemokines (Khader *et al.*, 2007), and inhibition of IL-10 induced by BCG, which limits Th1 activation (Gopal *et al.*, 2012). Also, Th17 are able to mediate bacterial killing, but to a lesser extent than Th1 (Gallegos *et al.*, 2011). IL-17 is also essential for mature granuloma development and thus containment of the bacteria. In this case, the source of IL-17A responsible for this effect seems to be $\gamma\delta$ T cells³⁸ rather than Th17 (Okamoto Yoshida *et al.*, 2010). Further studies are needed to determine their role during the infection, and if this compartment could be modulated to benefit the host.

³⁸ $\gamma\delta$ T cells and MAIT cells, like iNKT cells are subsets of unconventional T cells highly present in mucosal sites. **$\gamma\delta$ T cells** have a TCR composed of gamma and delta chains by opposition to classical T cells harboring alpha and beta TCR chains. They do not need antigen presentation by MHC molecules and seems able to recognize various antigens with different receptors including lipid antigens, presented on CD1 by myeloid cells or phosphoantigens. Upon activation, they produce cytokines (TNF- α , IFN- γ or IL-17), chemokines and mediate lysis of infected cells. In mucosal sites, they seem to be the main source of IL-17.

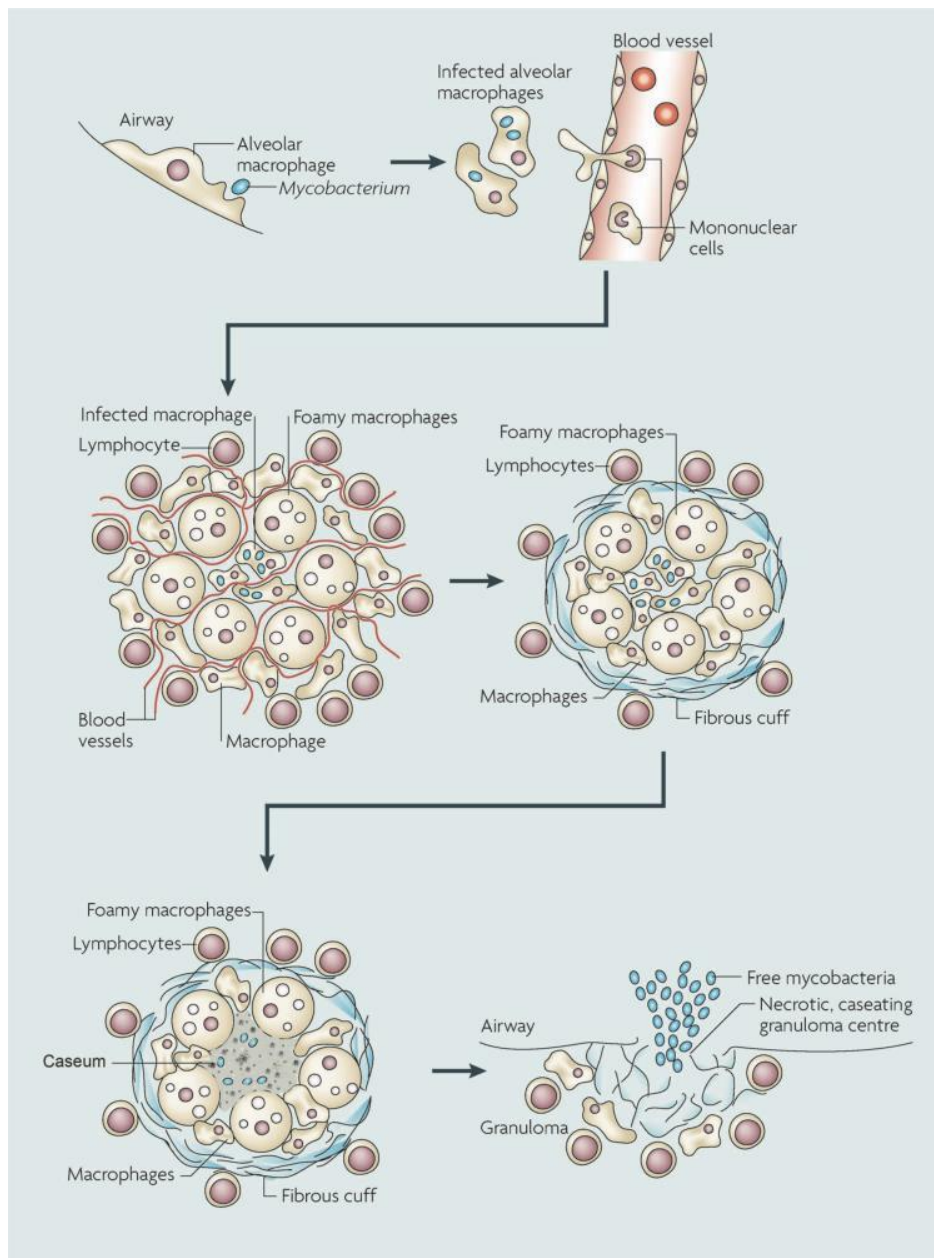


Figure 22: Illustration of the progression of the Human Tuberculosis Granuloma. Once in the lung the bacilli are phagocytosed by alveolar macrophages that will translocate into the lung interstitium. This leads to recruitment of mononuclear cells from neighboring blood vessels that will form the cellular matrix of the early granuloma. In its early stage, the granuloma has a core (inner ring) of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by an outer ring composed by T lymphocytes and anti-inflammatory macrophages. This tissue response contains the infection and spells the end of the period of rapid replication for *M. tuberculosis*. As the granuloma matures, it is less vascularized and develops an extensive fibrous capsule that encases the macrophage core and excludes the majority of lymphocytes from the granuloma structure. At this stage, there is a noticeable increase in the number of foamy macrophages within the fibrous capsule. Necrosis of these cells may be responsible for the accumulation of caseous debris in the center of the granuloma, which portends progression to active disease. In a progressive infection the caseous, necrotic center of the granuloma may liquefy and cavitate spilling thousands of infectious *M. tuberculosis* into the airways. This damage to the lungs trigger development of a productive cough facilitating generation of the infectious aerosol and completion of the bacterium's life cycle. Figure from (Russell *et al.*, 2009).

Apart from CD4⁺ T cells, CD8⁺ T cells and unconventional T cells such as $\gamma\delta$ T cells, iNKT cells or MAIT³⁹ cells are also able to stimulate infected macrophages through cytokine production (Sada-Ovalle *et al.*, 2008; Gold *et al.*, 2010; Harriff *et al.*, 2014; Nunes-Alves *et al.*, 2014; Lerner, Borel and Gutierrez, 2015). Cytotoxic granule released by CD8⁺ T cells, $\gamma\delta$ T cells, or iNKT also triggers apoptosis of infected cells (Gansert *et al.*, 2003; Chen *et al.*, 2013). Of note, granulysin (another anti-microbial peptide) contained in these granules also allow the delivery of proteases inside bacteria leading to their direct elimination (Gansert *et al.*, 2003; Chen *et al.*, 2013). In macaques, CD8⁺ T cells and $\gamma\delta$ T cells seemed also important to protect against reinfection, and are part of the BCG efficacy (Shen *et al.*, 2002; Chen *et al.*, 2009). However, it seems that CD8⁺ T cells and unconventional T cells are more dispensable than CD4⁺ T cells in the mouse model (Nunes-Alves *et al.*, 2014). Indeed, CD8⁺ T cell-deficient mice infected with low dose of *M. tuberculosis* have higher bacterial load than WT mice, but these mice achieved a better control of the infection over time and survive much longer than mice deficient for CD4⁺ T cells, IFN- γ or inducible nitric oxide synthase (iNOS)⁴⁰ (Mogues *et al.*, 2001). In fact, uncontrolled replication of *M. tuberculosis* led to a necrotic lung pathology, resulting in the rapid death of CD4⁺ T cell-deficient mice (Mogues *et al.*, 2001). MHC-II-deficient mice are also very susceptible to this disease due to the delayed activation of infected phagocytes, which is not efficiently compensated by CD8⁺ T cell or NK cell activity and exhibit high dissemination of the bacteria in the organism.

Therefore, macrophage activation by CD4⁺ T cells is essential for the host survival. However, while pathogen replication is certainly reduced by the arrival of T cells to the site of the infection, its elimination from the host is not guaranteed. Rather, macrophage activation mainly leads towards an equilibrium (or stationary phase) where the pathogen enters into dormancy state inside of well-structured granulomas. Without a sustained T cell response over a long period of time, the dormant bacteria represent an ever-present danger to wake up and lead to lung disease.

4. Granuloma dynamics

Arrival of *M. tuberculosis*-specific T cells, as well as B cells that surround phagocytes at the site of the infection, lead to the formation of an organized early granuloma that is highly vascularized (Figure 22) (Russell, Barry and Flynn, 2010). In this structure, phagocyte activation leads to the arrest of pathogen proliferation and entry into a latent state. However, *M. tuberculosis* is still importantly remodeling macrophages within the granuloma. During the infection, they give rise to different specialized cell types, such as epithelioid macrophages (activated macrophages mainly observed in humans), foamy macrophages (containing multiple lipid droplets that may sustain the bacteria) (Peyron *et al.*, 2008), and multi-nucleated giant cells (derived from the fusion of several macrophages such as epithelioid macrophages) (Puissegur *et al.*, 2007). T cell activation drives macrophages toward a microbicidal (M1) phenotype, characterized for example by nitric oxide production in mice. To counteract these actions, *M. tuberculosis* changes the macrophage metabolism toward a tolerogenic (or M2) phenotype, characterized by ARG1 expression, with fewer killing elements (*e.g.*, reactive species), reduced capacity to die by apoptosis, and increased matrix metalloproteinase MMP-12 production (Kahnert *et al.*, 2006; Pessanha *et al.*, 2012; Lugo-Villarino and Neyrolles, 2014). In fact, a precise controlled immune

³⁹ MAIT are activated by binding of their low variant TCR to bacterial vitamin B metabolites presented by the non-conventional MHC I MR1. Similar to $\gamma\delta$ T cells, they are implicated in recruitment and activation of other immune cells through production of chemokines and cytokines such as TNF- α , IFN- γ or IL-17.

⁴⁰ The inducible nitric oxide synthase (iNOS or NOS2) is the enzyme responsible for nitric oxide production that is part of the microbicidal molecules produced by macrophages activated by IFN- γ to kill pathogens.

response is needed so that mature granulomas persist without a potential escape of the bacteria. Ideally for the host, this implies the presence of microbicidal macrophages at the inner ring of the granuloma in contact with the bacillus, and tolerogenic macrophages in the outer ring to avoid an excessive inflammation causing tissue damage (Flynn, Chan and Lin, 2011).

Progression toward disease is associated with reduced granuloma vascularization and development of a fibrous capsule around the inner ring-macrophage core that will exclude lymphocytes from the granuloma structure (Russell *et al.*, 2009). This strongly impairs macrophage activation by CD4⁺ T cells. Foamy macrophages are highly present in the granuloma at this stage of the disease (Hunter, 2011). Growth of the bacteria in these permissive cells, releasing multiple antigens, may conduct to delayed-type hypersensitivity reaction (Dannenberg, 1991). This over-reaction of the immune system, reflected by excessive TNF- α (through ROS increase) or IFN- γ signaling, leads to necrotic death of the infected cells (Dannenberg, 1991; Roca and Ramakrishnan, 2013; Wong and Jacobs, 2013). Necrosis seems also to be directly triggered by the modulation of lipid mediators in cells infected by virulent *M. tuberculosis* (Chen *et al.*, 2008). Indeed, virulent *M. tuberculosis* strains increase the levels of lipoxin LXA4, perturbing the balance between prostaglandin E2 (PGE2) and LXA4⁴¹ that determine the cell type of death. This reaction may reflect an attempt of the immune system to destroy this bacterial niche, but it may directly be driven by the pathogen. Necrosis seems to appear more often in granuloma with immature macrophages in susceptible host, and is associated with disease progression, or post-primary tuberculosis (Dannenberg, 1991).

At this stage, the granuloma is characterized by the formation of the caseum at its center, giving the term “caseous granuloma”. Caseum is a material composed of lipid rich cell debris that are released by necrotic cells and extracellular trap (Wong and Jacobs, 2013), which is also thought to sustain bacterial survival (Russell *et al.*, 2009) (Figure 22). In humans, the caseous granuloma contains hypoxic regions that impair the bacterial growth (Via *et al.*, 2008). However, the bacteria is resistant to slow decrease in oxygen, entering an anaerobic persistent phase (Wayne and Hayes, 1996) from which it can rapidly recover (Leistikow *et al.*, 2010).

The caseous center of the granuloma is also highly pro-inflammatory, and characterized by the presence of antimicrobial peptides, reactive oxygen species and pro-inflammatory eicosanoids, while the surrounding outer ring is more anti-inflammatory that presumably provides tissue protection and repair (Marakalala *et al.*, 2016). Depending on immune regulation, it appears that each granuloma will later evolve individually and independently, displaying a different pattern of progression, cell composition and outcome (Martin *et al.*, 2017; Cohen *et al.*, 2018). Progression toward active disease seems dependent on the number of granulomas experiencing bacterial growth and immune over-reaction.

The lesion development may be dependent on B cells that appear during granuloma progression and localized into lymphoid cuff regions surrounding the granuloma (Gonzalez-Juarrero *et al.*, 2001). Mice deficient in B cells are not affected in the IFN- γ production and control of the bacteria (C. M. Johnson *et al.*, 1997), except when there is a high dose infection level (Vordermeier *et al.*, 1996). However, they display increased Th17 activation, leading to neutrophilia and worsening of lung pathology (Taylor *et*

⁴¹ Eicosanoids derived from arachidonic acid such as **PGE2** and **LXA4** are cell metabolites with important signaling properties. Different eicosanoids can have opposite functions. For example, PGE2 and LXA4 are important determinant of cell death, LXA4 favoring necrotic death while PGE2 promotes apoptosis. LXA4 also decreased inflammatory cytokine production and Th1 induction. Depending on environmental factors, arachidonic acid is more converted in some eicosanoids compare to others leading to different signal and outcome.

al., 2005; Kozakiewicz *et al.*, 2013). The role of B cells in tuberculosis will not be further discussed here, but they appear to be essential for optimal immunity against *M. tuberculosis*, by modulating cytokine production, neutrophil infiltration, macrophage phenotype and opsonization⁴² of the pathogen through antibody production (Maglione and Chan, 2009; Flynn, Chan and Lin, 2011; Torrado *et al.*, 2013; Bénard *et al.*, 2018).

Necrosis progression leads the granuloma to cavitate (Hunter, 2011). This phenomenon appears amplified in region of the lungs with mechanical stress (Ihms, Urbanowski and Bishai, 2018). In these regions, pressures applied on the caseous granuloma composed of a soft center surrounded by a fibrotic rigid ring lead to ruptures (Ihms, Urbanowski and Bishai, 2018). Action of matrix remodeling enzymes also seemed involved in this process; the collagenase MMP-1, induced by IL-17, is increased in this type of granuloma while the metalloproteinase TIMP3 is decreased, resulting in the degradation of the lung parenchyma (Kübler *et al.*, 2015; Squeglia, Ruggiero and Berisio, 2018) (Figure 23). Neutrophils are highly present in the granuloma at this stage of the disease (Nolan *et al.*, 2013), recruited by Th17 and correlate with high inflammation and poor disease outcome (Gopal *et al.*, 2013). This may be linked to the MMP-8 and -9 that are released upon infected neutrophil death in Neutrophil Extracellular Trap (NET) (Ong *et al.*, 2015). One parameter involved in the disease progression is IFN- γ . Indeed, IFN- γ signaling is depressed in active tuberculosis and correlates with the disease severity in human (Sahiratmadja *et al.*, 2007). Neutrophilia may also be explained by impaired IFN- γ signaling, which normally limit neutrophil recruitment (Cruz *et al.*, 2006; Nandi and Behar, 2011).

At this stage, lesions allow a contact between the granuloma center and the airways (Wong and Jacobs, 2016). This may lead to an environment prone to bacterial growth reactivation (rich in oxygen), as abundant free bacteria are found in cavitated lesions. Granuloma rupture also allows liquid caseum containing bacteria to be released in the bronchial tree and transmit it to new host *via* cough droplets. This suggests that contrarily to most accepted hypothesis, the development of caseous granuloma may benefit the pathogen. Therefore, cavitation of the granuloma is a state of active tuberculosis.

⁴² Opsonization is an immune process involving the binding of antibodies surrounding a pathogen that will favor its elimination by immune cells. The constant fragment (Fc) of antibodies is indeed recognized by Fc receptors present at the surface of phagocytes facilitating its phagocytosis, a process called antibody-dependent cellular phagocytosis. Fc binding on monocytes, neutrophils, eosinophils or Natural Killer cells also activates the release of lysis products by these cells, leading to bacterial elimination without phagocytosis, a process called antibody-dependent cell-mediated cytotoxicity.

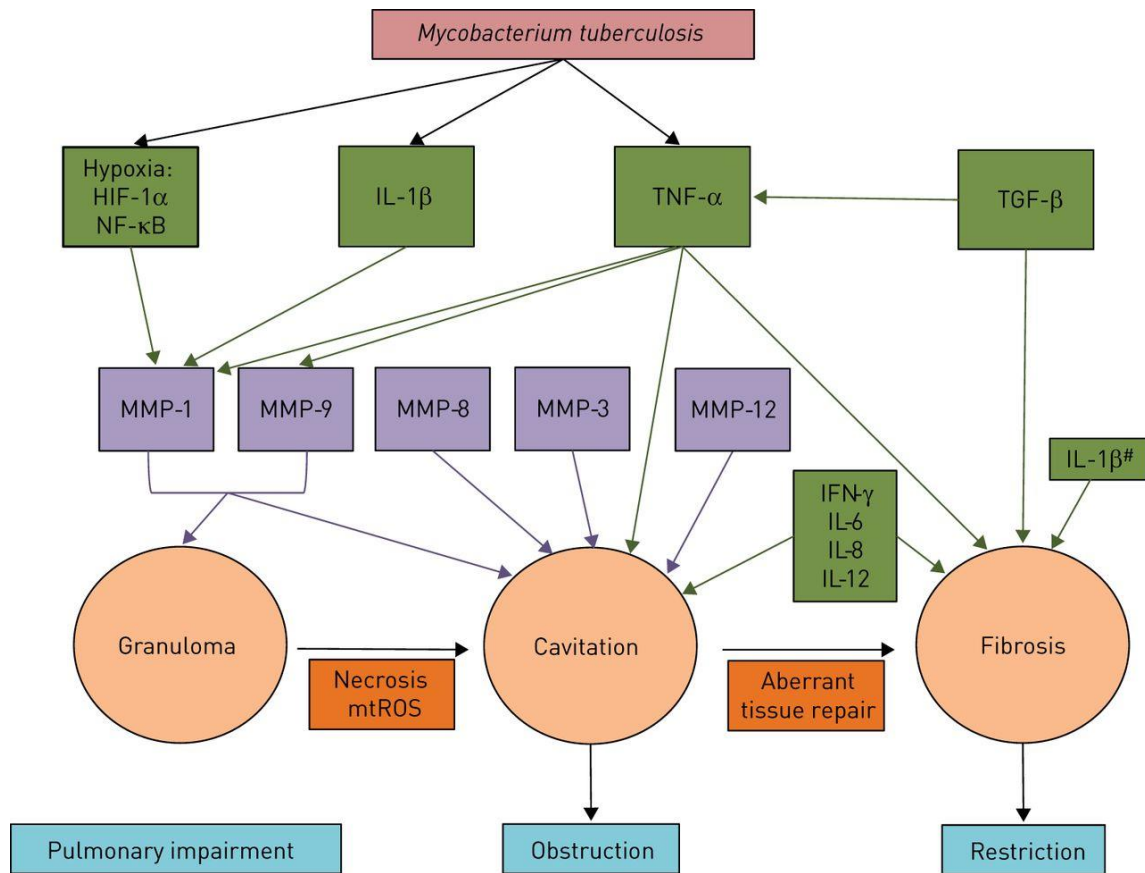


Figure 23: Immune mediators of tissue remodelling and lung function impairment in tuberculosis. Transcription factors, cytokines and chemokines that drive expression of tissue-degrading enzymes or directly mediate cavitation and/or fibrosis are shown in green. Matrix metalloproteinases (MMP) that promote granuloma and cavitation are depicted in purple. HIF, hypoxia inducible factor; NF, nuclear factor; mtROS, mitochondrial reactive oxygen species. #: IL-1 β regulates fibrogenesis in idiopathic pulmonary fibrosis and may play a role in tuberculosis. Pathological processes contributing to the progression of lesions may influence the development of airflow obstruction and restrictive ventilatory patterns of pulmonary impairment. Figure adapted from (Ravimohan et al., 2018)

In patients and mouse model of tuberculosis reactivation, the disease is characterized by lipid pneumonia and thrombosis with bronchial obstruction (Hunter, 2011). Patients in this state are highly contagious, experience strong tuberculosis symptoms and will die eventually, if untreated. Granuloma may harden (through fibrosis) or be healed by calcification (Wong and Jacobs, 2016), a state characterized by a decreased inflammation and elimination of the bacteria (Flynn, Chan and Lin, 2011). TNF- α production increases fibroblast recruitment and activity following granuloma formation, leading to collagen synthesis, matrix degradation, and thus development of fibrosis (Shkurupiy *et al.*, 2014). In addition to TNF- α , aberrant TGF- β and IL-1 appear to be involved in the development of fibrosis (Tsao *et al.*, 2000; DiFazio *et al.*, 2016; Ravimohan *et al.*, 2018). Fibrosis may help pathogen containment but could also impair the host homeostatic lung functions. (Figure 23).

Factors leading to active tuberculosis are still today not well understood. Indeed, while the early stages of tuberculosis immune response may be similar in many host species, few of them mimic the latter stages. For example, most mouse strains do not develop necrotic granuloma structures that resemble those in humans. Even if similar cell types are recruited, the organization of the granuloma is not the same and their center is less anaerobic compared to humans (Tsai *et al.*, 2006). Difficulties to obtain tissue from patients, and the unknown impact of antibiotic treatment on granuloma dynamics, have also restrained validation of findings in animal models (Hunter, 2011). While no animals are known to develop cavities as in humans, some of them, including C3HFeJ mice, guinea pigs, rabbits or non-human primates, develop exudative lesions with foamy macrophages and are therefore very helpful to understand disease development (Hunter, 2011; Williams and Orme, 2016). As humans, non-human primates develop heterogenous disease following infection, confirming that latent tuberculosis is not a fixed and well defined stage, but rather it is manifested in different forms such as a well-contained disease, or subclinical and asymptomatic disease at high risk to progress to active disease (Capuano *et al.*, 2003).

It was thought that progression toward active tuberculosis occurs mainly in immunocompromised patients or in patients having genetic polymorphisms rendering them more susceptible to the disease, reviewed in (Levin and Newport, 2000). Nevertheless, the fact that all granulomas in a single individual do not evolve similarly, suggest that this may be more complicated (Cadena, Fortune and Flynn, 2017; Martin *et al.*, 2017). In fact, it is still unclear if active disease occurs after reactivation or reinfection, in particular in patients presumably cured by antibiotic treatment. It is possible that some bacteria are not killed by the treatment, leading to relapse or reactivation of the primary infection that may occur early after infection (Malherbe *et al.*, 2016). Conversely, reinfection may be the main cause of post-primary tuberculosis, especially when the disease manifests one year or later after primary infection (Marx *et al.*, 2014; Behr, Edelstein and Ramakrishnan, 2018). Even if the treatment results in clearance of the pathogen, the patients may keep pulmonary dysfunctions (such as cavitation, fibrosis, nodular infiltrates that impact ventilation) that are aggravated in case of reinfection and render patients more susceptible to death from respiratory causes, such as Chronic Obstructive Pulmonary Disease (COPD) (Hnizdo, Singh and Churchyard, 2000; Wohlfert *et al.*, 2011). A better understanding of the immune response, and above all the reasons why the arrival of T cells do not allow disease resolution, may help to improve treatments as discussed in the following section.

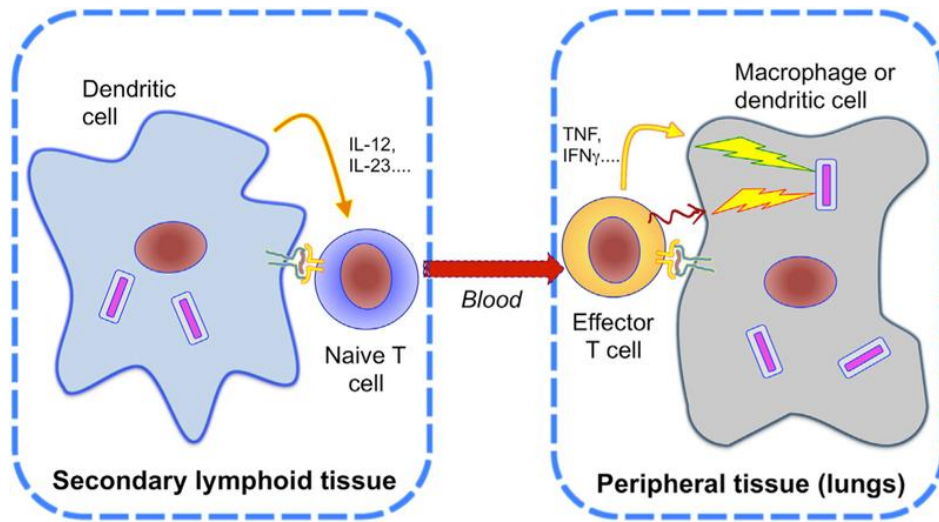


Figure 24: Effector T cell activation by infected target cells is required for pathogen control. Naive T cells are primed by dendritic cells (DC) presenting their cognate antigens in lung draining lymph nodes. Priming initiates the clonal expansion of T cells and is influenced by cytokines produced by DC, leading to their differentiation into effector or memory T cell subsets, and egress from the lymph node via the blood. To accomplish effector functions, T cells must traffic to the site of infection and be activated by recognizing their cognate epitope presented by infected cells, to secrete cytokines (curved solid arrow in figure) and express surface ligands for activating receptors (squiggly arrow in figure) that activate intracellular microbicidal mechanisms. If T cells cannot be activated at the site of infection, they will not contribute to control of the infection. Figure from (Ernst, 2018).

5. T cell response limitations

The fact that the pathogen is not eliminated after the arrival of T cells suggests that the host immune response is not fully efficient (Ernst, 2012). The mechanisms responsible for this phenomenon have been investigated and appear to be multiple.

First, several CD4⁺ T cells may not be efficient because of antigen modulation. Many immunodominant antigens are shared between different *M. tuberculosis* lineages, suggesting that development of CD4⁺ T cells against these antigens is not fully detrimental to the bacteria. Indeed, *M. tuberculosis* highly expresses the immunodominant antigen Ag85A during the early stage of infection, and reduces its expression after the recruitment of CD4⁺ T cells. This results in the expansion of multiple CD4⁺ T cells specific for this antigen in the lymph nodes that will not find their target cells upon arrival to the site of infection, and thus cannot participate in the control of the pathogen (Bold *et al.*, 2011). Indeed, direct recognition of infected cells by CD4⁺ T cells is needed to control of *M. tuberculosis* by infected cells (Figure 24) (Srivastava and Ernst, 2013). This is not the case for all antigens; for example, ESAT-6 expression is maintained throughout all stages of infection. However, as previously mentioned, infected APC are able to transfer antigens to non-infected APC. While this phenomenon improved antigen-specific CD4⁺ T cells generation in the lymph node, it also results in reduced antigen expression by the infected cells leading to decreased recognition of these infected cells by antigen-specific CD4⁺ T cells in the lungs, even if the antigen is expressed by the pathogen (Srivastava, Grace and Ernst, 2016).

In addition, the architecture of the granuloma, which keeps away T cells from infected phagocytes, may limit the T cell response efficiency (Kauffman *et al.*, 2018). The proximity of T cell and infected macrophages is dependent on TNF- α and lymphotoxins (Roach *et al.*, 2001). Impaired localization may also be caused by IDO expression in *M. tuberculosis*-infected macrophages, as its silencing improved the localization of CD4⁺ T cell in the center of the granuloma, reduced the bacterial burden, and improved macaques survival (Gautam *et al.*, 2018). In addition, co-culture of infected macrophages (silenced for IDO1) with CD4⁺ T cells resulted in an increased bacterial killing (Gautam *et al.*, 2018).

Interestingly, impaired T cell activity, characterized by reduced polyfunctional IFN- γ ⁺ IL-2⁺ TNF- α ⁺ in patients with active compared to asymptomatic tuberculosis, becomes restored during antibiotherapy, suggesting that this process is masterfully controlled by the pathogen (Day *et al.*, 2011).

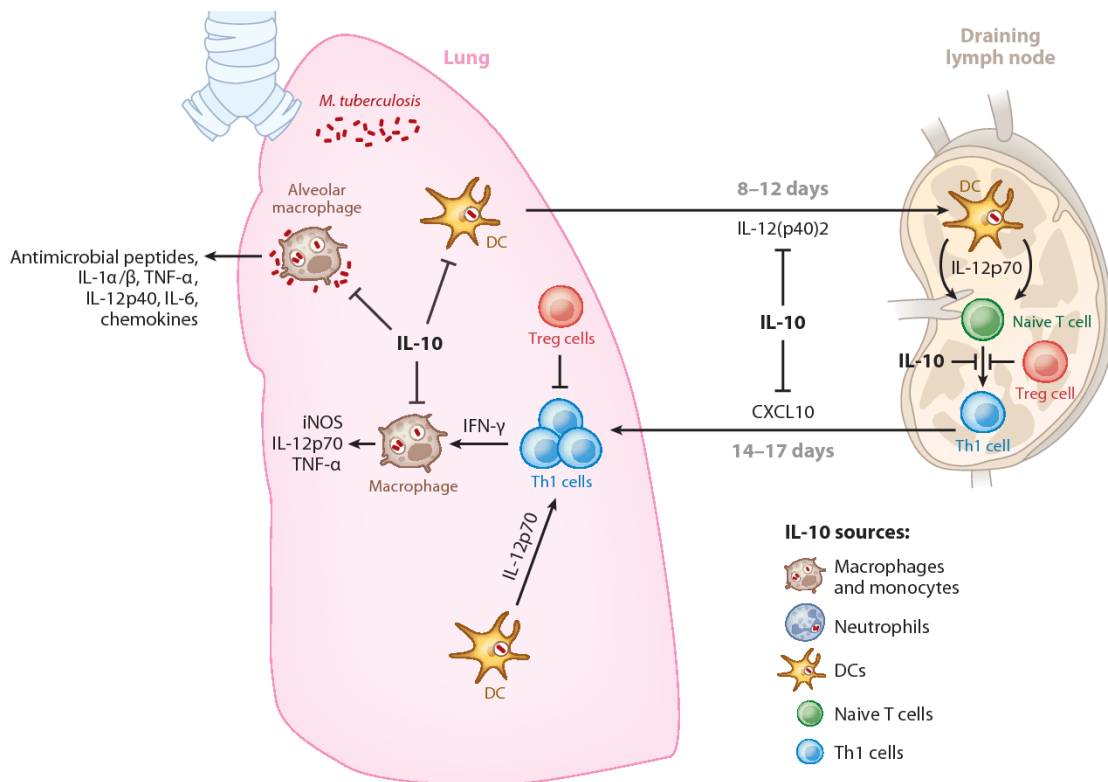


Figure 28: Regulation of the immune response during *M. tuberculosis* infection. Following infection with *M. tuberculosis*, specific regulatory pathways that normally serve to limit host-induced immune pathology may inadvertently promote pathogen persistence. Two such regulators include IL-10 and regulatory T cells (Treg). The induction of IL-10 during infection can lead to the inhibition of macrophage effector functions, with reduced bacterial killing and impaired secretion of cytokines/chemokines. IL-10 can also block chemotactic factors that control DC trafficking to the draining lymph nodes. In the lymph nodes, both IL-10 and Treg can block the differentiation of naive T cells to IFN- γ -producing Th1 cells, predominantly through direct effects on the DC. Furthermore, IL-10 can block T cell chemotactic factors such as CXCL10, which mediates Th1 cell trafficking back to the lungs, in addition to blocking macrophage activation and downstream antimicrobial pathways in response to IFN- γ . In the lungs, Treg can inhibit Th1 cell activation of infected cells. Figure from (O’Garra et al., 2013).

6. Immune regulation

Anti-inflammatory and suppressive mediators are highly involved in tuberculosis. They are needed to avoid late over-inflammation leading to necrosis and tissue damage. Nevertheless, *M. tuberculosis* appears to activate these factors too early during the infection, which lead to an overall impairment of bacterial killing. Therefore, understanding their role and finding ways to modulate their activation, is crucial to better understand and design the diagnosis and treatment of tuberculosis.

One example of a molecule that is highly implicated in immunosuppression is the PD-1⁴³ receptor expressed by T cells. PD-1-deficient mice show high bacterial proliferation and succumb to the disease, early after expansion of highly pro-inflammatory CD4⁺ T cells, leading to immunopathology (Lázár-Molnár *et al.*, 2010; Barber *et al.*, 2011). In fact, increased IFN- γ production by CD4⁺ T cells results in early death of infected mice without better control of the bacterial load, demonstrating the importance of the suppressive activity of PD-1 expression on T cells in this type of chronic infection (Sakai *et al.*, 2014). Interestingly, the expression of PD-1 on CD4⁺ T cells, which represents a pool of activated KLRG1⁺ T cell progenitors, may be a key mechanism to restrict the generation of such activated T cell and prevent immunopathology at some stages of the infection (Urdaahl, Shafiani and Ernst, 2011).

As expected, the conventional suppressive Treg, which are highly present in infected mice (Scott-Browne *et al.*, 2007), non-human primates (Green *et al.*, 2010) and patients (Guyot-Revol *et al.*, 2006), are also involved in the control of the immune response. In humans, their abundance seems to correlate with *M. tuberculosis* load, low Th1 response and disease progression suggesting that the pathogen favors their induction (A. Singh *et al.*, 2012; Pang *et al.*, 2013; Arram, Hassan and Saleh, 2014). As a matter of fact, there are antigen-specific Treg that are expanded in the pulmonary draining lymph node along with effector CD4⁺ T cells. This may be because of increased PD-L1 ligand expression on infected DC (Trinath *et al.*, 2012). These Treg inhibit effector T cell priming through CTLA-4 expression (Figure 25), participating in the delay in their recruitment into the lungs (Shafiani *et al.*, 2010). Subsequently, Treg migrate to the lungs at same speed and same location as effector T cells do, including inside granulomatous regions, being ideally situated to inhibit effector T cell functions (Scott-Browne *et al.*, 2007). Treg express high level of ICOS and PD-1 molecules and are associated with increased anti-inflammatory TGF- β levels in human patients suggesting that they acquired an activated anti-inflammatory phenotype (Pang *et al.*, 2013). Surprisingly, in mice, depletion of Treg using anti-CD25 antibodies had modest effect in reducing bacterial loads and did not increase the immunopathology (Quinn *et al.*, 2006; Ozeki *et al.*, 2010). However, these results must be taken with caution given that, while not all lung Treg express CD25, some activated effector T cells do (Parkash, Agrawal and Madhan Kumar, 2015). Also, depletion of Treg by anti-Thy1.1 administration in either mixed bone marrow chimeric mice (in which all cells capable of expressing Foxp3 expressed Thy1.1) (Scott-Browne *et al.*, 2007), or in a model of Rag1^{-/-} receiving adoptive transfer of CD4⁺ CD25⁻ T cells (Kursar *et al.*, 2007), led to a better control of *M. tuberculosis* growth. These authors also showed that impairment of the control of *M. tuberculosis* growth was due specifically to the ability of Treg to suppress CD4⁺ T cell activity in an IL-10-independent manner (Figure 25) (Scott-Browne *et al.*, 2007; Kursar *et al.*, 2007). Surprisingly, their deletion did not result in increased level of TNF- α , IFN- γ or NO

⁴³ **PD-1** is a receptor regulating inflammation upon binding of PD-L1 expressed by myeloid cells or cancer cells by favoring apoptosis of effector T cells while reducing apoptosis of regulatory T cells. Over-expression of PD-1 is associated to T cell exhaustion, a state of T cell linked with poor effector functions.

(Kursar *et al.*, 2007). In a model of InterCellular Adhesion Molecule (ICAM-1)⁴⁴ deficient mice that exhibit lower level of T cells including iTreg, *M. tuberculosis* infection resulted in high morbidity associated with high immune cell infiltration, which became reduced after adoptive transfer of CD25⁺ Treg (Windish *et al.*, 2009). Therefore, while Treg may have an early deleterious role in delaying the T cell response, their presence may be essential latter to prevent immunopathology. In the non-human primate model, IL-2 administration mediating expansion of both effector T cell and Treg resulted in reduced immunopathology associated with decreased bacterial load (Chen *et al.*, 2012). These results complement data from the mouse model and suggest a novel therapeutic approach to benefit from Treg protective role while limiting the impairment of the early effector T cell response. Besides Treg, *M. tuberculosis* induction of other CD4⁺ T cell subsets that regulate the Th1 response, such as Th2, Th22 or Th9, may also contribute to disease progression. Yet, at the moment, data regarding the role of the cells in this disease are scarce and controversial, highlighting the need for further investigation , as reviewed in (Da Silva *et al.*, 2015).

Cytokines and their receptors are also part of the anti-inflammatory response limiting the T cell response in tuberculosis. Indeed, mice deficient in regulatory cytokines, such as IL-10 (Jung *et al.*, 2003; Beamer *et al.*, 2008; Cyktor *et al.*, 2013; Moreira-Teixeira *et al.*, 2017) or IL-27 (Pearl *et al.*, 2004; Hölscher *et al.*, 2005), are better able to control the pathogen growth or to limit reactivation (Turner *et al.*, 2002). The role of IL-10 in particular has been extensively studied. The source of IL-10 may be diverse and even produced by Th1 or Th17 that could co-produce IL-10 and IFN- γ following chronic antigen stimulation (Da Silva *et al.*, 2015). In the mouse model, overproduction of IL-10 by T cell *in vivo* was detrimental for bacterial control while IFN- γ was produced in large amounts (Murray *et al.*, 1997). This suggests a direct role of IL-10 on macrophage activation rather than through effector T cell inhibition (Figure 25). In mice depleted of IL-10, the bacteria was not eliminated, and in fact, regrew at late time point, associated with excessive Type-1 immune inflammation, leukocyte infiltration, leading eventually to reduced survival (Higgins *et al.*, 2009). In susceptible CBA/J mice depleted from IL-10, a mature, fibrotic granuloma is formed, associated with generation of multifunctional T cells, increased IFN- γ levels and reduced bacterial loads (Cyktor *et al.*, 2013). Therefore, as for Treg, its presence to control immune activation after (but not before) initiation of the T cell response may be required for the host survival. Inflammatory status of the host may highly determine its protective *versus* detrimental role. Along with these findings in mice, it has been shown that in patients under clinical cure, IL-10 seems induced in combination to IFN- γ and TNF- α and might allow eradication of the pathogen with minimum tissue damage (da Silva *et al.*, 2013). In addition to cytokines, other regulatory molecules like the eicosanoid LXA4, (Bafica *et al.*, 2005) or DNAX-activating protein of 12 kDa (DAP12)⁴⁵ are able to inhibit Th1 induction (Divangahi *et al.*, 2007).

Similar to Treg, anti-inflammatory mediators such as IL-10 or DAP12 seem essential to prevent death related to increased chronic inflammation (Hölscher *et al.*, 2005; Divangahi *et al.*, 2007). Therefore, even if the anti-inflammatory response seems inappropriate, pathogen survival is maybe a sacrifice granted by the host in order to protect tissue integrity. This phenomenon is not unique to tuberculosis. In fact, tolerance to microbes rather than resistance seems to be an option often considered by the host when facing an infection (Ayres and Schneider, 2012). As in its interaction with the microbiota, containment rather than elimination may be the less detrimental option for the host in a well-defined

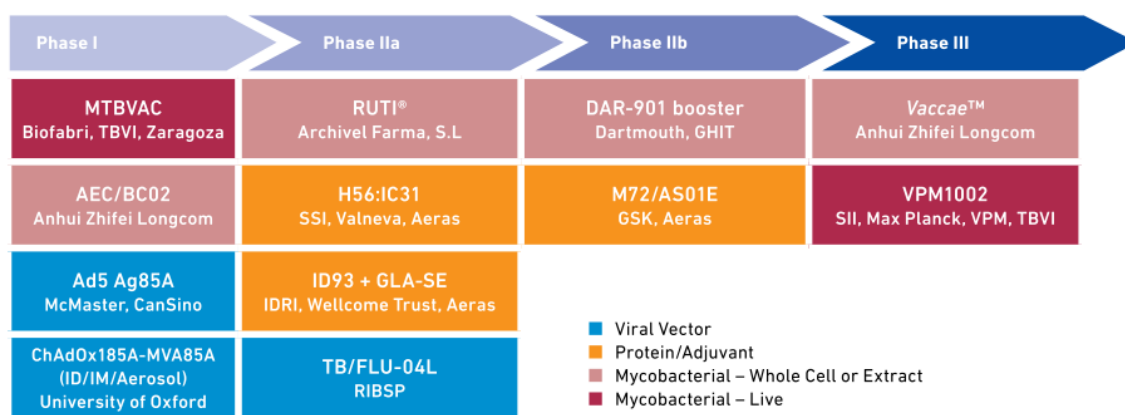
⁴⁴ **ICAM-1** is an adhesion molecule expressed on endothelial cells and immune cells, allowing leukocyte transmigration from blood vessels to tissue.

⁴⁵ **DAP12** is a transmembrane signaling adaptor mainly expressed on myeloid cells that can either activate or inhibit the immune response depending on the receptor it is associated with.

situation. However, while being the better of two evils at a define point of the infection, tolerance is on the long term a risky option because it depends on perfect regulation of the immune system. This fault may have been exploited by pathogens such as *M. tuberculosis* during their long evolution with humans.

Since the immune response to tuberculosis is imperfect, the different studies detailed in the following section aimed to improve it through vaccination and more recently *via* host-directed therapies. Complexity of the natural immune response to tuberculosis, and in particular of the mechanisms involved in the balance between resistance and tolerance have been a major challenge conducting to failure of many strategies. However, our growing understanding of such mechanisms, partly revealed by these approaches modifying the immune response, will likely allow the delivery of successful strategies in the future.

The global development pipeline for new TB vaccines, August 2018^a



^a Information was self-reported by vaccine sponsors, and the Stop TB Partnership Working Group on New TB Vaccines supported the review of their feedback.

Figure 26: Pipeline for new tuberculosis vaccines development. Currently, multiple vaccine candidates at different clinical phases form part of a promising pipeline. They represent different strategies, being viral vector, subunit vaccines (protein/adjuvant), mycobacterial cell wall or living mycobacteria. In particular, there are two candidates in the phase III that may soon be used to prevent tuberculosis. Figure from (*WHO | Global tuberculosis report, 2018*).

III. Development of preventive and therapeutic strategies targeting the host

1. Design of new vaccines in tuberculosis

a) Classic strategies used for the development of the new pipeline

Long-lasting immune protection following BCG inoculation and immunity generated in latent *M. tuberculosis* infection suggest that protective immunological memory can be induced by vaccination. However, developing more efficient vaccines has not been as easy as expected, and promising candidates are only emerging (Figure 26).

New vaccine candidates can be categorized in three classes: (i) preventive pre-exposure vaccines, or priming vaccines, designed to be administered to neonates, prior to first exposure to *M. tuberculosis*, (ii) preventive post-exposure vaccines, or boosting vaccines, targeting adolescents and adults with LTBI and prior BCG immunization, and (iii) therapeutic vaccines, which are to be co-administered with antibiotics, notably to persons at higher risk of developing recurrent disease (Kaufmann, Weiner and von Reyn, 2017). They can also be classified depending on their constituent as follow: subunit vaccines, viable whole-cell vaccines, and inactivated whole-cell vaccines.

Subunit vaccines are composed of one or more antigens that are often combined to improve vaccine efficacy. Protection of these vaccines is often low. Proteins efficacy is usually improved using adjuvants or through DNA delivery directly to host cells by a recombinant viral vector. One challenge for the design of a subunit vaccine is the choice of antigen. Investigated antigens, such as Ag85, ESAT-6 or 10 kDa culture filtrate antigen (CFP-10)⁴⁶, were considered as good candidates for pre-exposure vaccines due to their high immunogenicity and early expression during infection. However, as the immune response to tuberculosis is still not fully understood, delivery of a single antigen may fail to induce protection. MVA85A, was a promising candidate consisting in an attenuated poxvirus viral vector delivering Ag85A. It induced a high frequency of T cell responses, and was found to be safe and immunogenic in humans (McShane *et al.*, 2004). However, it failed to prevent *M. tuberculosis* disease or new infections in two phase II clinical trials (one in HIV infected adults (Ndiaye *et al.*, 2015) and in another one in infants (Tameris *et al.*, 2013)). The design of hybrid vaccines such as H1 (ESAT-6/Ag85B) and H4 (TB10.4⁴⁷/Ag85B) was based on combination of antigens to decrease the risk of such issue (Voss *et al.*, 2018). However, these candidates do not help prevention of disease reactivation in latent patients as the antigen used seem to be poorly expressed during latency. Therefore, dormancy related antigens, such as Heat-shock protein X (HspX)⁴⁸, or antigens in the DosR regulon⁴⁹, appear to be more suitable for a post-exposure strategy. Ideally, a combination of both types of antigens could be used for the development of a unique vaccine, called multi-stage vaccine. Fusion proteins were used to

⁴⁶ CFP-10 is a *M. tuberculosis* antigen secreted in complex with ESAT-6 by the ESX-1 secretion system, with the capacity to attach to host cell membranes, an important step for the prevention of phagolysosomal fusion.

⁴⁷ **TB10.4** is another protein secreted by members of the MTBC, part of the ESAT-6 family. It is highly recognized by T cells, in particular by T cells from BCG vaccinated individuals suggesting its potential for the design of new subunit vaccines but its functions are poorly described.

⁴⁸ **HspX**, or Hsp 16.3 or Acr, is a protein highly produced by *M. tuberculosis* during stress conditions such as hypoxia, nutrient scarcity or in presence of NO during which it can represent 25% of total protein expression. It has a key role for bacterial survival in macrophages, reducing bacterial growth and acting as a chaperone allowing stability of proteins and cellular structures in the latent phase.

⁴⁹ **DosR regulon** is a genetic program also induced in stress conditions such as reduced oxygen levels. It is involved in the metabolic shift allowing bacterial survival in hypoxia and in the rapid bacterial regrowth when oxygen level increased.

design vaccine belonging to this class, including H56, M72 and ID93 that are today in clinical phase II (Figure 26) (Kaufmann and McMichael, 2005; Kaufmann, Weiner and von Reyn, 2017; Voss *et al.*, 2018). Importantly, recent results from the phase IIb trial evaluating the M72/AS01E vaccine (composed of the fusion of protein M72 derived from MTB32A and MTB39A antigens and of the AS01E adjuvant) suggest that vaccination with this candidate mediates an immune response that reduces the risk of latent infected individuals to develop pulmonary disease (Tait *et al.*, 2019). Even if highly promising, this strategy is still risky today, as it induces a response to a limited number of antigens. The fact that the T cells induced by such vaccination will confer protection largely depends on our understanding of the immune response that is much more complicated than previously thought. In particular, the conservation of *M. tuberculosis* immunodominant antigens in the different lineages infecting different populations around the world suggest that their expression may not be to the disadvantage of the pathogen (Gallegos, Pamer and Glickman, 2008).

Therefore, even if less controlled, the two other strategies, using more antigens, must be considered as promising. They comprise the use of viable or whole-cell vaccine. The complexity of their antigen components allows for interaction with innate immune cells, and induction of conventional and unconventional T cell responses, as well as humoral responses (Voss *et al.*, 2018). However, the *M. tuberculosis* mutant MTBVAC, and the recombinant BCG vaccine VPM1002, have shown promising results in phase I and II, respectively, and seemed well tolerated (Figure 26). However, the use of living vaccines is complicated as there is still a risk that modified strains may revert or induce pathology in immuno-compromised individuals. The use of whole cell mycobacterial vaccines also remains central in tuberculosis vaccine development with two candidates tested as boosting vaccine: DAR-901, derived from SRL172, which is an inactive whole-cell vaccine derived from a non-tuberculous mycobacterium, and *Vaccae* vaccine, consisting of a heat-inactivated whole-cell from *M. vaccae*. They have respectively entered phase IIb and phase III (Figure 26) (Voss *et al.*, 2018).

Developing a therapeutic vaccine would be particularly critical for the treatment of drug resistant tuberculosis infections. The RUTI vaccine candidate, composed of fragmented *M. tuberculosis* cell wall delivered in liposome, inducing protection and safety in latent tuberculosis patients, is now evaluated as adjunctive treatment for MDR-TB patients (Kaufmann, Weiner and von Reyn, 2017). The *Vaccae* vaccine has also been evaluated as adjunctive therapy for MDR-TB patients. In first studies it was well tolerated and improved both bacterial clearance and closure of tuberculosis cavity suggesting that therapeutic vaccines are highly promising (Weng *et al.*, 2016).

Several pitfalls may limit the efficacy of vaccines. First, the diversity of human populations and environmental factors, which may comprise risk factors for tuberculosis, render the establishment of correlations between protection in animal models and clinical trials difficult. This may indicate that several vaccines will be needed to achieve tuberculosis elimination (Kling *et al.*, 2014). This and other factors certainly limit the efficacy of BCG. Indeed, our understanding on how BCG protects against disseminated tuberculosis, and why it fails to protect against pulmonary tuberculosis, is still incomplete (Pai *et al.*, 2016b). Adoptive transfer of specific CD4⁺ T cell seemed unable to induce protective response before seven days post-infection (Gallegos, Pamer and Glickman, 2008). Even if it consists in an accelerated T cell response, it still gives too much time to the pathogen to replicate. However, such T cells may be able to prevent the bacterial dissemination (that occurs during this timeline), which may explain the protective effect of BCG against disseminated tuberculosis diseases. Reasons for this delay are still not fully understood. It may be explained by the ability of *M. tuberculosis* to block phagosome maturation, delaying the expression of antigens at the surface of infected cells (Singh *et al.*, 2006; Russell, 2011; Srivastava and Ernst, 2013). Also, even when antigen presentation is restored, the fact that the bacteria reduced the expression of some immunodominant antigens, such

as Ag85A, would abolish the protection mediated by effector T cell (Bold *et al.*, 2011). This may explain failure of the MVAg85A vaccine candidate and complicate the design of subunit vaccines. As detailed previously, the proximity of T cell to infected cells in the lung parenchyma and within the granuloma structure is also a key parameter for protective immune response. Intramuscular vaccination may not be the best delivery way/system to achieve such a goal (Lai *et al.*, 2015). Indeed, BCG subcutaneous delivery only induces the recruitment of T cells in the lung parenchyma, but not to the airways. This may be directly responsible for the delayed protection observed after *M. tuberculosis* infection. Indeed, intratracheal transfer of primed T cells before the infection accelerates the immune response, decreasing the bacterial load (Horvath *et al.*, 2012). Interestingly, intratracheal or intranasal delivery of BCG can also lead to the recruitment of lung resident PD-1⁺ KLRG1⁻ and PD-1⁻ KLRG1⁺ T cells, leading to a decreased bacterial load both in the lung and spleen after infection, compared to subcutaneous BCG delivery (Chen *et al.*, 2004; Perdomo *et al.*, 2016; Bull *et al.*, 2019). *M. tuberculosis* subversion of the immune system seems highly dependent on the bacterial load. Therefore, bacterial load reduction by mucosal administration may allow better immune clearance of the pathogen. Mucosal administration of subunit vaccines in mouse models also resulted in better protection (Wang *et al.*, 2004; Santosuosso *et al.*, 2005, 2007). This effect seems even improved using systemic priming followed by mucosal boosting (Wang *et al.*, 2004). Such protocols may induce both improved CD4⁺ and CD8⁺ T cell responses (Wang *et al.*, 2004). However, in NHP, mucosal boosting of BCG using subunit vaccines failed to improve protection (Darrach *et al.*, 2019). Protective lung homing of T cells may also be achieved by injection of chemokines or retinoic acid (Riccomi *et al.*, 2019). In human, MVAg85A aerosol delivery was compared to intradermal administration. Both routes were shown to be well tolerated and immunogenic. Although the aerosol route induced a better response in cells collected from bronchoalveolar fluid, suggesting a higher induction of local response (Satti *et al.*, 2014), this induction was not efficient to protect the host from developing the disease. Therefore, this strategy seems promising while efficacy studies are needed.

Many strategies have been used to develop a new vaccine to prevent tuberculosis. They have given rise to multiple candidates currently forming a rich pipeline. However, as many candidates fail to prove efficacy during the last stages of clinical trials or may not be adapted to all patients, the search for new strategies is still ongoing.

b) Non-pathogenic bacteria as tuberculosis vaccines

As described in Section 1.IV of this introduction, bacteria from the microbiota represent attractive carrier of molecules of interest to mucosal sites. In particular, the use of recombinant lactic bacteria, such as *Lactococcus lactis* or *Lactobacillus plantarum*, as vaccine vectors has been extensively tested in other diseases. They offer specific advantages compared to other strategies. For example, they are more immunogenic than naked purified antigens and can carry more foreign DNA than viruses (Bermúdez-Humarán *et al.*, 2011; Rosales-Mendoza, Angulo and Meza, 2016). Their safety status and possibilities to further modify them for increased biocontainment is also very attractive (Steidler *et al.*, 2003). Two strategies using such vehicle have been developed. The first one consists in the production of antigens from the pathogen by the recombinant bacteria. Co-expression of immunomodulatory proteins such as IL-12 is also possible, and it has been shown to improve immunization effect (Bermúdez-Humarán *et al.*, 2005; Hugentobler *et al.*, 2012). The second strategy uses the bacteria as a DNA carrier, transmitting DNA to eukaryotic cells after its internalization (through phagocytosis or infection) (Yurina, 2018). To improve this strategy, integration of invasion-encoding genes for invasions has been used. For example, integration of the fibronectin-binding protein A (FnBPA) from *Staphylococcus aureus* has been expressed in *L. lactis* and increases its invasive power (Pereira *et al.*, 2015, 2017; Mancha-Agresti *et al.*, 2017). Such strategy is beginning to be tested in the field of

tuberculosis. Delivery of Ag85A, ESAT-6, or MPB/MPT51 antigens in target cells using DNA delivery approach is successful in mouse model, inducing systemic Th1 response (Pereira *et al.*, 2015; Mancha-Agresti *et al.*, 2017) and protection against *M. tuberculosis* infection (Miki *et al.*, 2004). Similarly, expression of ESAT-6 at the surface of *L. lactis*, or Ag85B-ESAT-6 fusion antigen expression at the surface of *L. plantarum*, induced an immune response in mice (Pereira *et al.*, 2015; Kuczkowska *et al.*, 2017). However, this field is quite recent and at the moment few studies really show the protective potential of these approaches after *M. tuberculosis* infection. Therefore, further studies are needed to demonstrate efficacy of this approach retaining a great potential.

One major advantage of this approach is its suitability for mucosal immunization that may improve vaccine efficacy. Indeed, nasal administration of bacteria delivering antigens seems potent for immunization in mice (Cortes-Perez *et al.*, 2007; Kuczkowska *et al.*, 2017). It is also suitable for mucosal boosting following BCG subcutaneous priming (Pereira *et al.*, 2017). For example, intranasal boost using *Bacillus subtilis* spores coated with *M. tuberculosis* antigens after BCG priming, increases the level of specific IgG (in serum) and IgA (in the lungs), and the generation of tissue resident memory T cells in lung parenchyma, resulting in improved *M. tuberculosis* lung clearance after infection (Copland *et al.*, 2018). In addition, the use of recombinant bacteria may be used to deliver therapeutic proteins or may be used as post-exposure vaccines (Bermúdez-Humarán *et al.*, 2011; Jacouton *et al.*, 2018).

Achieving high efficacy may be dependent on the vector strain. *L. lactis* has been extensively used because it is easier to transform than, for example, *L. plantarum*. However, because *L. plantarum* is able to persist longer than *L. lactis* in the lung, it can be a candidate of choice in the case of tuberculosis (Oliveira *et al.*, 2006; Cortes-Perez *et al.*, 2007). Nevertheless, as protein expression differs between bacterial species, testing different strains may be needed to choose the best vector depending on proteins and disease (Oliveira *et al.*, 2006; Cortes-Perez *et al.*, 2007). The use of probiotic strains (such as *L. plantarum*), having specific immunomodulatory properties as a delivery vector, may have synergistic effect. This option is very promising and represents one main advantage of this strategy. Yet, more studies are needed to assess the proof-of-principle that bacterial strains can improve protection both by delivery of antigens and by their natural properties. More fundamental knowledge about the microbiota interaction with the immune system in the lung is also needed to choose the best vector candidates.

While the development of microbiota-based vaccine is still in its infancy, this strategy is promising for safe mucosal delivery of *M. tuberculosis* antigens and may be strongly improved by studies of the lung microbiota that are on the rise.

2. Appearance of host-directed therapies

While preventing tuberculosis is important, improving treatment is also essential in the global fight against tuberculosis. Current anti-tuberculosis therapy is long and constraining. Appearance of drug-resistant *M. tuberculosis* strains highlights the need not only to develop new antibiotics, but also new strategies to complement them. During the past years, increasing interest focuses on strategies aiming at improving the host natural ability to fight infections, rather than the classical and historical strategy to directly target the pathogen components. Such host-directed therapies appear particularly appropriate for tuberculosis, as patients suffer either from an insufficient or excessive immune response. Therefore, augmenting host defense mechanisms or modulating the excessive inflammation, or both, should improve clinical treatment outcomes (Flynn, Chan and Lin, 2011; Zumla *et al.*, 2016). Many components of different nature, including drugs approved for other applications,

have been tested for their ability to improve bacterial killing by macrophages, delivery of antibiotics to the bacteria, or reduction of immune over-activation leading to necrosis and tissue degradation (Figure 27). The most advanced ones are presented below to illustrate the advantages and limitations of this strategy.

One interesting target for tuberculosis host-directed therapies are macrophages, as the activation of their killing properties is delayed in tuberculosis. In line with its role in the activation of macrophages, IFN- γ administration is one promising target for host-directed therapy. Its administration in patients have already been tested in humans, including in one clinical trial and was well tolerated, even with aerosolized administration. Administration was tested in patients with MDR-TB with or without antibiotic treatment depending on the studies. Improvement of bacterial clearance and reduction of the lung immunopathology was observed in most patients following IFN- γ administration in the different studies (Condos, Rom and Schluger, 1997; Koh *et al.*, 2004; Dawson *et al.*, 2009). These results suggest that host-directed therapies improving rapid bacterial killing also reduce immunopathology. Other molecules aiming at improving bacterial killing have been studied. They include compounds improving autophagy and phagosome maturation, such as metformin, gefitinib and carbamazepine⁵⁰, which appear highly promising in the mouse model and a human-based study (Figure 27B) (Singhal *et al.*, 2014; Stanley *et al.*, 2014; Schiebler *et al.*, 2015), or statins⁵¹, which reduce the incidence of active tuberculosis (Kang *et al.*, 2014; Parihar *et al.*, 2014; Skerry *et al.*, 2014; Dutta *et al.*, 2016; Lai *et al.*, 2016). Of note, some components, such as vitamin D, are not as encouraging in human trials. Indeed, vitamin D supplementation is beneficial for patients with polymorphism in the vitamin D receptor, but not in other people for whom this vitamin is already found in saturating concentrations (Martineau *et al.*, 2011; Coussens *et al.*, 2012; Salahuddin *et al.*, 2013; Harishankar, Anbalagan and Selvaraj, 2016). These data suggest that different host-directed therapies may suit different type of patients. The development of multiple therapies may allow their adaptation to patients depending on genetic susceptibility and co-morbidity factors. Increased macrophage killing capacity could also be achieved through accelerated T cell priming and expansion. In this regard, IL-2 administration is promising and show great results in the non-human primate model in which it results both in decreased bacterial load and decreased immunopathology (Chen *et al.*, 2012). However, at the moment, administration of IL-2 in clinical studies show either protection or no effect (B. J. Johnson *et al.*, 1997; Johnson *et al.*, 2003; Shen *et al.*, 2015). Administration protocol was different in these studies, which may explain differences in results. This is observed for many host-directed therapy trials, suggesting that determination of doses and administration frequency is not trivial, complicating the identification of favorable candidates.

⁵⁰ **Metformin** is an antihyperglycemic agent used in treatment of type 2 diabetes and activates adenosine monophosphate-activated protein kinase (AMPK) that is an inducer of autophagy. **Gefitinib** is an inhibitor of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase used for the treatment of some cancer. It seems also to inhibit the EGFR/p38 MAPK pathway that restricts autophagy. **Carbamazepine** is an anticonvulsant drug and induces a myo-inositol-dependent activation pathway of autophagy.

⁵¹ **Statins** are cholesterol-lowering drugs, targeting HMG-CoA reductase, used in treatment of coronary disorders and hypercholesterolemia. They seem also to reduce cholesterol levels in phagosomal membranes which promotes phagolysosomal fusion and autophagy.

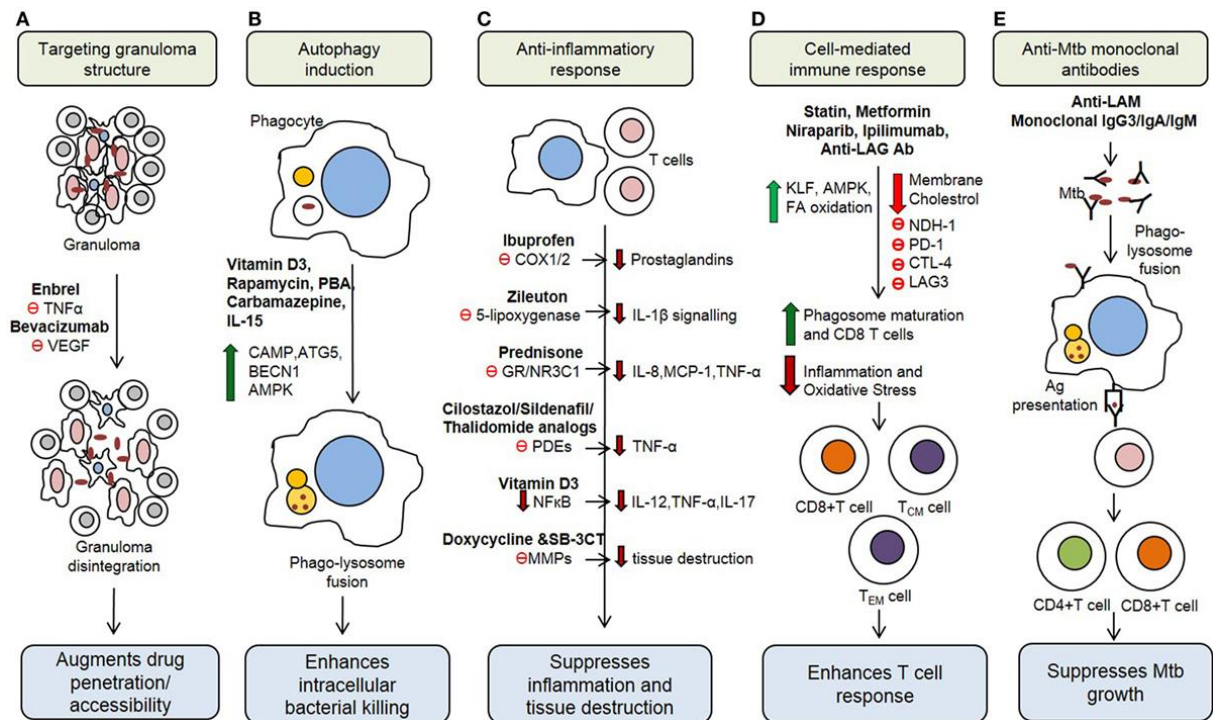


Figure 27: Host-targeted therapies developed to improve tuberculosis treatment overview. Current strategies for host-targeted therapies include: A) granuloma structure disruption (to improve bacteria accessibility to antibiotics), B) autophagy induction (to improve bacterial killing in macrophages), C) the induction of anti-inflammatory pathways to protect tissue integrity, D) T cell enhancement, and E) delivery of anti-*M. tuberculosis* antibodies that would improve opsonisation, bacterial killing and T cell activation. VEGF, vascular endothelial growth factor; PBA, phenylbutyrate; CAMP, cathelicidin antimicrobial peptide; ATG5, autophagy-related protein 5; BECN1, beclin-1; AMPK, AMP-activated protein kinase; COX1/2, cyclooxygenase-1/2; GR, glucocorticoid receptor; PDE, phosphodiesterases; MMPs, matrix metalloproteinases; KLF, Kruppel-like factor; LAM, Lipoarabinomannan. Figure from (Kolloli and Subbian, 2017).

Although TNF- α is required to induce macrophage-killing activities, excessive production is detrimental to the host as it mediates necrosis, worsening tissue pathology (Roca and Ramakrishnan, 2013) (Figure 27A,C). Interestingly, in infected mice treated with etanercept (a soluble TNF- α receptor), enbrel (a soluble TNF- α receptor antagonist), or zileuton (inhibiting 5-lipoxygenase mediated TNF- α production) (Figure 28), in combination with standard antibiotics, the level of TNF- α was reduced along with a decreased bacterial burden and lung damage (Skerry *et al.*, 2012; Bourigault *et al.*, 2013). Granuloma disruption by these anti-TNF- α agents improved bacterial killing and lung resolution after chemotherapy (Robert S Wallis *et al.*, 2004). However, asymptomatic tuberculosis patients treated with etanercept or infliximab (another TNF- α inhibitor) for non-related inflammatory diseases, developed tuberculosis (R S Wallis *et al.*, 2004). Thus, targeting TNF- α in human seems to have more complex outcome. In fact, polymorphism in 5-lipoxygenase (involved in TNF- α production) (Figure 28) is directly responsible for the sensitivity of patients to the disease, and for the poor response to therapy in TB meningitis (Tobin *et al.*, 2012). Another clinical trial using the glucocorticoid prednisolone (which inhibits TNF- α) showed an improved bacterial load clearance by antibiotics, but it also exerts adverse effects (Mayanja-Kizza *et al.*, 2005). Therefore, approaches targeting TNF- α production should be carried out with caution. Targeting other effectors involved in necrosis may be safer. For instance, PGE2 that favors apoptosis over necrosis (Figure 28), improves bacterial killing *in vitro* (Chen *et al.*, 2008). Administration of PGE2 with antibiotics also reduced type I interferon (promotes bacterial survival via IL-1 β inhibition) levels in plasma, bacterial loads and mortality in infected mice (Mayer-Barber *et al.*, 2014; McNab *et al.*, 2015). Interestingly, tuberculosis severity correlates with changes in IL-1, IFN-I and eicosanoids in patients, suggesting that PGE2 administration is promising and should be tested in patients (Mayer-Barber *et al.*, 2014). Surprisingly, the use of aspirin or ibuprofen seems also promising despite the fact that they inhibit PGE2 synthesis (Figures 27B and Figure 28). Indeed, in infected mice, ibuprofen strongly reduced bacterial loads and tissue pathology (Vilaplana *et al.*, 2013). Aspirin may also be a good candidate, but results in mice were more controversial (Sean T Byrne, Denkin and Zhang, 2007; Sean T. Byrne, Denkin and Zhang, 2007). However, combining glucocorticoid with aspirin to standard chemotherapy decreased mortality induced by meningitis in a clinical trial (Ariel *et al.*, 2003; Misra, Kalita and Nair, 2010). Therefore, the modulation of eicosanoids may also have broad effects and, while encouraging, should be considered with caution.

There are other strategies with strong potential, but most have not been tested in humans yet. They involve the reduction of tissue damage by inhibition of metalloproteases (Elkington, D'Armiento and Friedland, 2011; Walker *et al.*, 2012; Majeed, Radotra and Sharma, 2016) or inhibition of immunoregulators phosphodiesterases⁵² (PDE) (Figure 27C and Figure 28) (Koo *et al.*, 2011; Subbian *et al.*, 2011, 2016; Maiga *et al.*, 2012, 2013). Others target the granuloma structure that, while helping to contain the bacteria, reduce antibiotic activity by targeting the abnormal vascularization of this structure with anti-angiogenic agents (Figure 27A) (Datta *et al.*, 2015; Oehlers *et al.*, 2015). These alternative strategies are very promising in animal models, and could be good substitutes to cytokine administration, but their safety in humans has not been assessed yet.

⁵² Phosphodiesterases are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes. Inhibitors of PDE3 and 4 possess immunomodulatory properties, decreasing TNF- α production by increasing intracellular cAMP in macrophages.

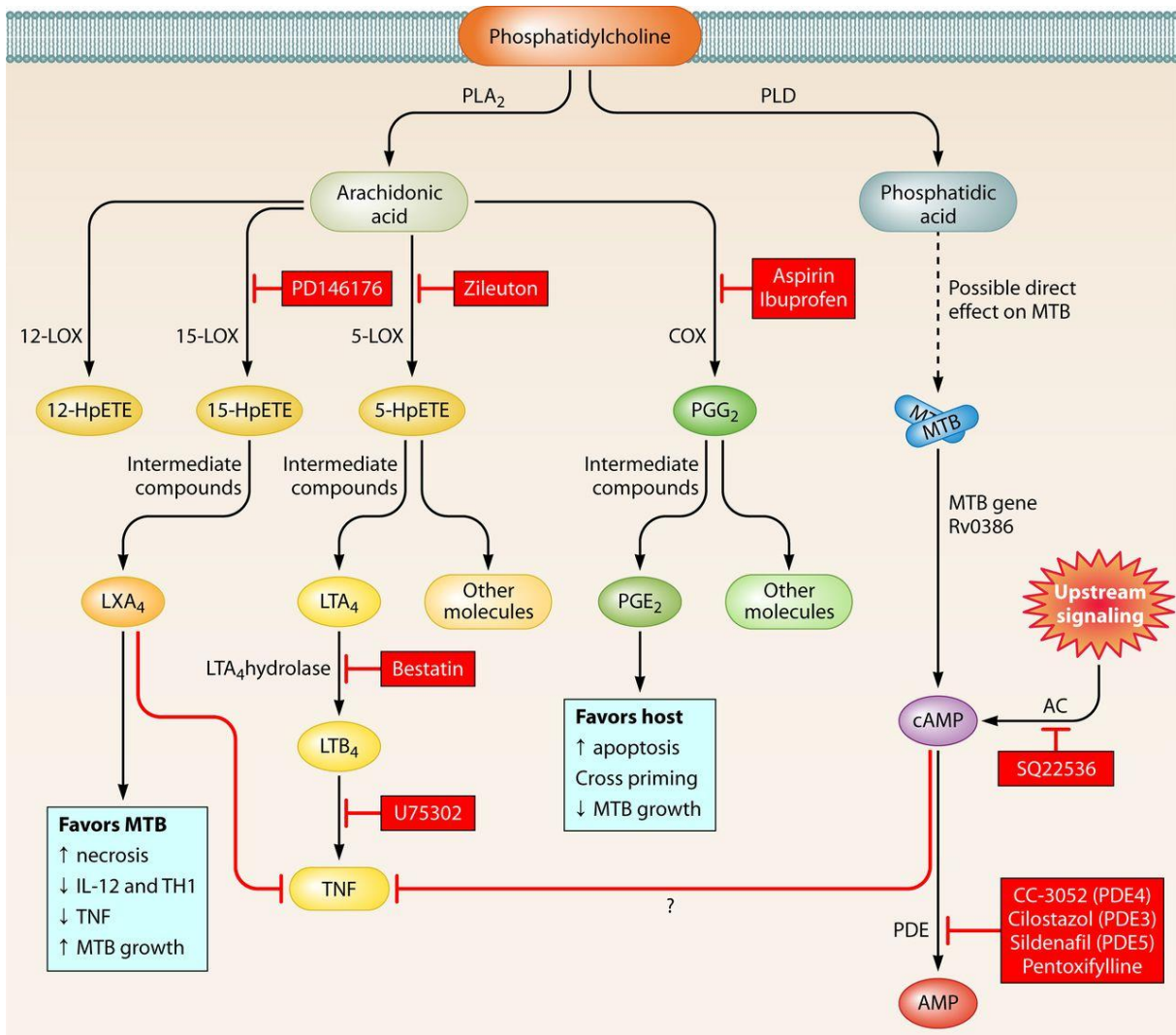


Figure 28: Host-targeted therapies targeting the eicosanoid pathway. Degradation and conversion of arachidonic acid by cyclooxygenase (COX) or lipoxygenase (LOX) produce different eicosanoids. In particular, LXA₄, PGE₂ and AMP, have different immunoregulatory properties that may either favor the host or the bacillus. Some of them also induce TNF production. Generation of these different eicosanoids can be inhibited by different drugs, altering their immunomodulatory properties for host-targeted therapies. Figure from (Hawn *et al.*, 2013).

These encouraging studies established host-directed therapies as an important element in the fight against tuberculosis. Such approaches should be integrated to existing (or future) chemotherapies to improve treatments, and are particularly promising for the treatment of infection with MDR- or XDR-tuberculosis. Of importance, host-targeting strategy should not induce antibiotic resistance in the pathogen. Ideally, it could even reduce antibiotic resistance directly by improving killing host mechanisms against the surviving bacteria, and indirectly by shortening antibiotic treatment length (as they improve their efficacy) and patient compliance (Zumla *et al.*, 2016). While most of the discussed candidates are at the preclinical stages, the identification of existing drugs that can be repurposed for tuberculosis treatment for the rapid therapy development. One other big advantage of host-targeting therapies is that they allow the reparation of tissue damage that is not triggered by the antibiotic therapy alone (Zumla *et al.*, 2016). Importantly, several identified compounds are able to improve bacterial killing and tissue repair. However, the determination of the appropriate administration and efficiency in human is lacking for most candidates. Personalized medicine may allow better success of such therapies and treatment of all patients (Ndlovu and Marakalala, 2016). However, because immunomodulatory molecules may have broad effect, their use is not trivial. For example, granuloma structure disruption may lead to activation of latent or subclinical tuberculosis, if not combined with antibiotic therapy. The use of host-directed therapies decreasing inflammation would theoretically safer as adjunctive therapy rather than on its own (Kolloli and Subbian, 2017). Also, the cost of some candidates, such as cytokines, is an obstacle to their use in low-income countries, where the tuberculosis incidence is the highest (Hawn *et al.*, 2013). Original strategies that would fulfill low cost and different immunomodulatory properties have been recently investigated. For example, the beneficial use of herbal plants, such as the one used in Chinese medicines, as adjunctive therapeutics was reported in humans with accelerated bacterial clearance and improved lesions resorption (Tomioka *et al.*, 2019). Alternatively, probiotics (as described in Section 1.4) are cheap treatments having promising effect in respiratory diseases. And yet, there are no studies investigating their effect in tuberculosis patients. In Section 3, arguments in favor of their hypothetical protective role to treat or prevent tuberculosis are presented.

Table 1: Studies conducted on lung microbiota communities in *M. tuberculosis* infection. Adapted from (Hong *et al.*, 2016; Tarashi *et al.*, 2018).

Study	Country	Type of analyze	Groups	Subject nb	Type of sample	Antibiotic use before sampling	Changes in diversity	Other main findings
Cui 2012	China	16S rDNA sequencing	Control	24	Saliva, pharyngeal samples	yes	Diversity ↑ in TB patients	↓ <i>Bacteroidetes</i> , ↑ <i>Phenylbacterium</i> , <i>Stenotrophomonas</i> , <i>Stenotrophomonas</i> , <i>Cupriavidus</i> , <i>Pseudomonas</i> , <i>Thermus</i> , <i>Sphingomonas</i> , <i>Methyllobacterium</i> , <i>Diaphorobacter</i> , <i>Comamonas</i> , and <i>Mabillitococcus</i> in TB patients
			TB patients	31	Sputum			
Cheung 2013	China	16S rDNA sequencing	Control	14	Sputum	no	Similar diversity	↑ <i>Maglibacterium</i> , <i>Moryella</i> and <i>Oribacterium</i> , ↓ <i>Lactobacillales</i> in TB patients
			TB patients	22				
Wu 2013	China	16S rDNA sequencing	Control	20	Throat swab	yes	↓ in TB patients ↓ ↓ in recurrent TB patients	↑ <i>Streptococcus</i> , <i>Gramulicella</i> and <i>Pseudomonas</i> , ↓ <i>Prevotella</i> , <i>Leptotrichia</i> , <i>Treponema</i> , <i>Catonella</i> and <i>Caproecoccus</i> in all TB patients compared to healthy controls. <i>Pseudomonas</i> is associated with recurrent TB or treatment failure, <i>Treponema</i> with new TB
			New TB	25	Sputum			
			Recurrent TB	30				
			Treatment failure TB	20				
Botero 2014	Colombia	16S rDNA sequencing	Control	6	Nasal, oropharynx sample	no	↓ in TB patients	↑ <i>Streptococcus</i> , <i>Candida</i> and <i>Aspergillus</i> ↓ <i>Cryptococcus</i> in TB patients
			TB patients	6	Nasal, oropharynx sample, sputum			
Zhou 2015	China	16S rDNA sequencing	Control	24	Saliva, pharyngeal secretion	yes		<i>Cupriavidus</i> dominates the lung microbiota of TB patients. <i>Mycobacteria</i> and <i>Porphyromonas</i> are increased in lesions
			TB patients	32	BAL			
			Control	16	Sputum			
TB patients	25							
Krishna 2015	India	16S rDNA sequencing	Control	16	Sputum	no	↓ in TB patients	↑ Firmicutes and Actinobacteria in TB samples dominated by <i>Streptococcus</i> , <i>Neisseria</i> and <i>Veillonella</i>
			TB patients	25				
Dube 2016	South Africa	Multiplex real-time PCR	Children unlikely to have TB	94	Nasopharyngeal sample, sputum	yes (72h before study)		↑ <i>Chlamydia pneumoniae</i> , <i>metapneumovirus</i> , <i>coronavirus</i> , <i>influenza virus C virus</i> , <i>rhinovirus</i> and <i>oxtomegalovirus</i> in children with definite TB
			Children with TB	34				
Querido 2011*	Brazil	Phenotypic characterization and PCR	Control	50	Oral rinses subgingival samples	yes		↑ fungi species, in particular <i>Candida albicans</i> in TB patients
			TB patients	50				
Nakhaee 2018*	Iran	Phenotypic characterization and PCR	Control (lung cancer)	5	BAL	no		↑ <i>Streptococcus</i> in TB patient. In patients, association between T-bet expression and <i>Haemophilus</i> . In controls, association between GATA-3 and T-bet expression and <i>Streptococcus</i> or <i>Neisseria</i> frequency.
			TB patients	10				

* Studies having only looked for specific species. nb: number, TB: tuberculosis, BAL: bronchoalveolar lavage, ↑/↓: increase/decrease in relative abundance

Section 3: *M. tuberculosis*, the microbiota and the host, a complex cross-talk

I. Microbiota alterations during tuberculosis

1. Perturbations of the lung microbiota in tuberculosis patients

In many diseases, dysbiosis of the microbiota is correlated with susceptibility and aggravation of pathology (as detailed in Section 1). In the last ten years, different groups tried to assess if this was the case in tuberculosis. Until now, nine studies have described respiratory microbes in the context of tuberculosis (summarized in [Table 1](#)). Seven of them performed broad characterization of the respiratory microbiota, while the other two compared the presence of specific species. Overall, the findings reported by these studies are divergent in terms of the microbiota composition differences between TB patients and healthy controls, some describing an increase in microbiota diversity while others describe a decrease. Such divergence is not specific to tuberculosis studies and is rather an important problem in studies of the respiratory microbiota (Adami and Cervantes, 2015). Differences in microbiota extraction methods, which can affect detection and relative abundance of species detected, or in sequencing platforms, which have different coverage and depth of sequencing, partly contribute to the divergence between studies (Hong *et al.*, 2016). Another major issue for the studies of respiratory microbiota in humans, and in particular in the studies presented here, is the use of different sampling methods between studies and even in the same study between tuberculosis patients and healthy controls (Cui *et al.*, 2012; Zhou *et al.*, 2015).

The ideal sample to determine the composition of the respiratory microbiota is actually lung tissue, but biopsies are an invasive procedure and are therefore only obtained when useful for patient treatment, and never obtained from healthy subjects for ethical reasons. For this reason, many studies use bronchoalveolar lavage fluids (BAL), considered as the second most representative sample to assess composition of the lung microbiota. While still invasive, this method allows the sampling of microbiota from the lower respiratory tract, without contamination from that found in the upper respiratory tract (Cabrera-Rubio *et al.*, 2012). Most studies use induced sputum (for patients) or oropharyngeal samples (for healthy controls), which are non-invasive methods, allowing a greater access to samples and performance of longitudinal studies. The disadvantage of these samples is that their microbiota composition is a poor representative of that found in the lower respiratory tract (Adami and Cervantes, 2015; Tarashi *et al.*, 2018).

As our knowledge of the microbiota composition progresses, it is now clear that different regions of the respiratory tract harbor different communities, as described in Section 1.III. In spite of the differences in microbiota composition observed in the same individual when using different sampling methods, the sputum and oropharynx samples yield comparable results, suggesting that these two samples can be used to compare composition of the upper respiratory tract between healthy controls and patients (Cabrera-Rubio *et al.*, 2012; Botero *et al.*, 2014). This is important because a reliable characterization of the upper respiratory tract microbiota will help to decipher how it may serve as the

“front-line” barrier for reducing pathogen access to the lungs. Yet, sputum and BAL do not yield the same results using different sampling methods in the same individual (Cabrera-Rubio *et al.*, 2012; Botero *et al.*, 2014). This issue already reduced the relevance of different findings obtained in individual studies (comparing for example saliva sample from controls to BAL from tuberculosis patients) and meta-analyses (pooling data from different studies having used different sampling methods) (Zhou *et al.*, 2015; Hong *et al.*, 2018; Eshetie and Van Soolingen, 2019). Thus, the characterization of the microbiota composition in the lungs still poses multiple challenges, and caution must be exercised in the interpretation of the available data in tuberculosis patients at this site.

Despite variability between subjects, one study report that some genera are found in all tuberculosis patients (*i.e.*, *Actinomyces*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Streptococcus*, and *Veillonella*), arguing that some changes in the microbiota may be strongly correlated with tuberculosis (Cheung *et al.*, 2013). In fact, comparison of studies using same type of samples between healthy controls and tuberculosis patients reveals that, while there may not be differences in the diversity of the respiratory microbiota, many taxa are differently represented between groups. For example, an increase in *Streptococcus species* in tuberculosis patients compared to controls has been observed in different studies (Wu *et al.*, 2013; Botero *et al.*, 2014; Nakhaee *et al.*, 2018). Interestingly, two studies also analyzed differences in fungi species that are abundant in the lungs and found an increase in *Candida albicans* in tuberculosis patients (Querido *et al.*, 2011; Botero *et al.*, 2014). Differences in viruses have also been observed in tuberculosis patients, suggesting that the virobiota and mycobiota should continue to be investigated in future studies (Dube *et al.*, 2016). Moreover, a recent study in non-human primates confirms that *M. tuberculosis* infection causes microbiota changes, in particular shortly after infection, which gradually decreased with time (Cadena *et al.*, 2018). These changes in microbiota composition may also occur within different part of lung tissue. A study based on tuberculosis patients with unilateral lesions found that the levels of *Porphyromonas* were increased in lesions along with *M. tuberculosis* (Zhou *et al.*, 2015). It is possible that lung remodeling during fibrosis or cavitation induces regional lung changes favoring colonization by certain species (Zhou *et al.*, 2015; O’Toole and Gautam, 2018). Hence, it appears that *M. tuberculosis*, as other respiratory diseases, drives an ecological reorganization of the lung microbiota during infection. This is a significant because such reorganization may allow the growth of pathobionts or entry of additional pathogens in the lungs, leading to future diseases. It is also possible that tuberculosis-induced dysbiosis is not only occurring in the lungs, as other respiratory diseases are associated with gut microbiota dysbiosis (Abrahamsson *et al.*, 2014; Bruzzese *et al.*, 2014; Groves *et al.*, 2018), as addressed below.

2. Gut microbiota dysbiosis in tuberculosis

As of today, only four studies have tried to decipher microbiota variations in the gut of tuberculosis patients compared to controls. They have observed some differences, such as a decrease in *Prevotella species* and an increase in *Roseburia* (Luo *et al.*, 2017; Maji *et al.*, 2018; Hu *et al.*, 2019). However, findings are again not homogeneous between studies. For instance, one study report a decrease in SCFA-producing bacteria in tuberculosis patients, while another one an increase in these species (Maji *et al.*, 2018; Hu *et al.*, 2019). Sampling of the gut microbiota is supposedly less variable (based on stool sample), but differences in DNA preparation or sequencing method may still lead to different results. In line with this, a study reveals that many species recovered by culturomic methods from a tuberculosis patient stool are not detected by sequencing (Dubourg *et al.*, 2013). As for the lung microbiota composition, it is possible that differences could depend on the time following infection. Indeed, in a mouse model, a rapid loss of diversity and a decreased in Clostridiales and Bacteroidales

are observed after infection, followed by recovery of microbiota diversity over time (Winglee *et al.*, 2014). A similar effect was observed using two different *M. tuberculosis* strains (H37Rv and CDC1552). However, this effect was reported to be lower in another study, using the C57BL/6 genetic background instead of BALBc mice (Namasivayam *et al.*, 2017). Therefore, tuberculosis likely induces alterations of both the respiratory and gut microbiota. These alterations may be through direct interaction or resulting from the high activation of the immune system that may target the microbiota as a side effect, but mechanisms this phenomenon remain unclear at the moment.

Better understanding of microbiota changes during tuberculosis is important because species from the lung or gut microbiota that highly change during tuberculosis could be used as biomarkers to improve tuberculosis diagnosis, which is still complicated (Tarashi *et al.*, 2018). To this date, these reports are preliminary and the low consistency between studies is a serious issue delaying progress in this field. Recent knowledge of the respiratory microbiota structure, and of the bias introduced by some sequencing methods, should allow the design of more robust and standardized human studies. The overall low number of subjects in all studies, and the fact that many of them were performed in the same geographical location (China), may also alter our perception of changes in microbiota composition during tuberculosis and should be taken into consideration (Adami and Cervantes, 2015). I will next address the need for the identification of indirect factors perturbing the microbiota composition (such as antibiotic use) during tuberculosis infection.

3. Microbiota dysbiosis mediated by anti-tuberculosis therapy

As mentioned in Section 2.1, the anti-tuberculosis therapy consists on a complex drug-regimen administered for a long time (Pai *et al.*, 2016a). Among the antibiotics used, many of them target bacterial components specific of mycobacteria (case of isoniazide, ethambutol or pyrazinamide for example). By contrast, rifampicin and some second line antibiotics are broad spectrum antimicrobials. It is therefore possible that in addition to *M. tuberculosis* infection, anti-tuberculosis treatment induces microbiota dysbiosis. In humans, this is difficult to assess and in most existing studies where patients are already undergoing anti-tuberculosis treatment. Two studies comparing tuberculosis patients at different disease stages (*i.e.*, under treatment, cured or recurrent tuberculosis), indicate an impact of the treatment on lung and gut microbiota. Treatment does not significantly disturb the overall gut microbiota diversity. In the lungs, microbiota diversity was decreased in patients experiencing treatment failure or recurrent tuberculosis compared to new patients, though *Pseudomonas species* were far more prominent (Wu *et al.*, 2013). While these studies do not clearly demonstrate that anti-tuberculosis drugs induce dysbiosis of the human gut and lung microbiota, some studies in animal models corroborate this hypothesis. Indeed, treatment of *M. tuberculosis* infected mice with isoniazid, rifampicin and pyrazinamide, induced a rapid but transient decrease in gut microbiota diversity associated with marked alteration of microbiota structure (with important decrease of Clostridiales); these changes were observed during the whole four months therapy course, and persisted at least three months after treatment cessation (Namasivayam *et al.*, 2017). Interestingly, many of the changes occurring were similar to those observed in mice treated with vancomycin, ampicillin, neomycin and metronidazole, classically used to induce broad microbiota dysbiosis. In this study, microbiota changes induced by the infection were minor compared to changes induced by the therapy, arguing this is an important parameter to consider. As expected, changes and in particular diversity reduction were mainly driven by the use of rifampicin. Surprisingly, administration of isoniazide or pyrazinamide, which are designed to specifically target mycobacteria, also induces a decrease in species of the

microbiota distant from mycobacteria and their combination lead to increased dysbiosis (Namasivayam *et al.*, 2017; Khan *et al.*, 2019).

The fact that microbiota composition is changing during treatment complicates the identification of microbiota species associated with tuberculosis development. However, changes induced by the treatment can also be useful for diagnostic purposes. Indeed, one study suggest that microbiota composition is changing in patients after cure, which may allow to detect treatment success (Wipperman *et al.*, 2017). Conversely, many studies highlight that dysbiosis of the microbiota can be a risk factor to develop diseases. Therefore, it is possible that microbiota alteration predisposes to *M. tuberculosis* infection or aggravate infection consequences, which is discussed in next section.

II. Alteration of the microbiota-immune system cross-talk: a risk factor for tuberculosis?

1. Tuberculosis infection outcome is influenced by microbiota composition

Recent studies based on animal models helped to determine the interaction between tuberculosis, the microbiota and the host. It seems that a bidirectional causal link exists between tuberculosis susceptibility and microbiota dysbiosis. In mice, microbiota dysbiosis by antibiotic treatment results in increased *M. tuberculosis* bacterial load in the lung, spleen and liver (Khan *et al.*, 2016; Dumas, Corral, *et al.*, 2018; Negi *et al.*, 2019). Similarly, a study in non-human primates found a correlation between the gut microbiota composition before infection and disease severity after *M. tuberculosis* infection, at the individual level. In this study, *Lachnospiraceae* and *Clostridiaceae* were enriched in susceptible animals, though some *Streptococcaceae* were decreased (Namasivayam *et al.*, 2019). Therefore, dysbiosis of the microbiota may lead to increased susceptibility to tuberculosis.

The microbiota influence on tuberculosis infection and progression can be through two mechanisms. First, it is possible that some species of the respiratory microbiota directly interact with *M. tuberculosis* mediating colonization resistance, as it is the case against *S. aureus* or *S. pneumoniae* (as detailed in Section 2 II. 3) (Iwase *et al.*, 2010; Bomar *et al.*, 2016). Medializations based on the studies comparing lung microbiota composition in human and non-human primates infected (or not) by *M. tuberculosis*, reveal this pathogen is in the center of many microbe-microbe interactions, and suggest that in human-anchoring species *Rothia mucilaginosa* may favor *M. tuberculosis* infection (Cadena *et al.*, 2018; Hong *et al.*, 2018). While experimental confirmations are needed, it is therefore very likely that, depending on its composition, the lung microbiota may favor or impede *M. tuberculosis* access to the lung. Similarly, *M. tuberculosis* may favor or inhibit growth of some microbiota species, leading to dysbiosis. Second, the lung and gut microbiota are able to modulate the lung immune system. Some recent studies suggest that dysbiosis of the microbiota alter the immune response to tuberculosis with deleterious outcomes.

2. Microbiota-immune system cross-talk during tuberculosis

a) Microbiota dysbiosis alters the immune response during tuberculosis

Different models inducing dysbiosis of the microbiota (at least in the gut) highlight the implication of the microbiota in different part of the immune response to tuberculosis. The microbiota seems, for example, to be implicated in innate cell activation. A study published this year reveals that after microbiota disruption by isoniazid and pyrazinamide, alveolar macrophage metabolism is highly altered, leading to reduced *M. tuberculosis* killing, an early phenomenon that seems to be independent of T cells (Khan *et al.*, 2019). Interestingly, a study by my team also found an early increased pathogen burden in mice treated with a broad spectrum antibiotic combination (vancomycin, ampicillin, metronidazole and neomycin), which was associated with a default in MAIT cell presence in the lungs (Dumas, Corral, *et al.*, 2018). Some studies suggest that MAIT cells present in the airway mucosa are activated after detection of *M. tuberculosis* in the airway by epithelial cells, and they can improve killing by alveolar macrophages through TNF- α or IFN- γ production (Gold *et al.*, 2010; Harriff *et al.*, 2014; Lerner, Borel and Gutierrez, 2015). It is, therefore, possible that at steady state, the healthy

microbiota is a source of ligand activating MAIT cells that will improve macrophage activation, but this requires further investigations. Other mouse-based studies reveal that the microbiota modulates the T cell compartment. Indeed, broad antibiotic-mediated dysbiosis reduced activation of Th1 and Th17, and increased the Foxp3⁺ Treg-induction of immunomodulation associated with increased survival of *M. tuberculosis* in the lungs and more granulomatous regions formation (Khan *et al.*, 2016; Negi *et al.*, 2019). Altogether, while preliminary, these studies strongly support the hypothesis of microbiota ability to alter different components of the immune response to tuberculosis. While a eubiotic microbiota may improve this response, its dysbiosis clearly impaired it at the detriment of the host.

In the study by Khan and colleagues, administration of the broad-spectrum antibiotic rifampicin had few impact on pathogen growth compared to the combination of isoniazid and pyrazinamide, while rifampicin alone induced higher dysbiosis (Khan *et al.*, 2019). This suggests, as shown in other contexts, that the role of the microbiota in the immune response to tuberculosis may be driven by some (but not all) species of the lung or gut microbiota having specific immunomodulatory effects, or effects important for the maintenance or restriction of species having these immunomodulatory effects. For example, a recent study shows that abundance of *Haemophilus species* in the BAL of tuberculosis patients correlated with expression of T-bet in BAL T cells; it is unknown at the moment if this effect is protective or deleterious regarding disease outcome (Nakhaee *et al.*, 2018). In addition, studies focused on co-infection models with *Helicobacter species*⁵³, considered as pathobionts, suggest the strong effect one bacterial species can have on the immune response to *M. tuberculosis*. Indeed, the presence of *Helicobacter pylori* in humans and non-human primates infected with *M. tuberculosis* correlates with protection against tuberculosis pathology; an effect mediated by increased of Th1 specific for *M. tuberculosis* (Perry *et al.*, 2010). By contrast, colonization of the gut microbiota with *H. hepaticus* increases mice susceptibility to *M. tuberculosis* infection characterized by higher pathogen burden, and increased immunopathology, leading ultimately to increased mortality (Majlessi *et al.*, 2017). This effect was mediated by impaired development of anti-tuberculosis immune response characterized by drastic accumulation of activated lung T cells leading to a huge increase in pro-inflammatory cytokine (*e.g.*, IL-6 or IL-1 β). In another mouse model, colonization of *Helicobacter hepaticus* reduces the development of specific T cells following immunization with the subunit vaccine Ag85A, leading again to susceptibility to infection (due this time to too low activation of T cell response) (Arnold *et al.*, 2015). In fact, the strong immunomodulatory effect of *H. hepaticus* may not be a direct one, as its colonization induces dysbiosis of the gut microbiota, enriching it in *Bacteroidaceae* and reducing the presence of *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae* and *Prevotellaceae*, which are families comprising immunomodulatory strains (as exemplified in Section I and in the next paragraph) (Majlessi *et al.*, 2017). Further identification of such species having direct or indirect beneficial (or deleterious) effect on the immune response to tuberculosis (and characterization of the mechanisms involved) may allow the design of microbiota-targeting approaches to improve tuberculosis treatment.

⁵³ Natural colonization with *Helicobacter species* is observed in the liver or gastrointestinal tract of many mice colonies and humans. While infection with *Helicobacter species* such as *Helicobacter pylori* or *Helicobacter hepaticus* is associated with development of diseases such as liver cancer and IBD it can be beneficial in other contexts as infection with *Helicobacter pylori* is associated with protection against infection by other pathogen and asthma, suggesting altogether that these bacteria may be pathobionts of the microbiota.

b) Mechanisms involved in the microbiota-driven alteration of the immune response to tuberculosis

The influence of microbiota on the immune response to tuberculosis may be mediated both by SCFA production and antigen recognition by innate immune cells (as it is the case in other contexts depicted in Section 1.II and III). An *in vitro* study suggests that SCFA induce IL-10 production by human leukocytes that eventually decreases the *M. tuberculosis*-induced pro-inflammatory cytokine response, such as TNF- α , IL-1 β and IL-17 (Lachmandas *et al.*, 2016). Importantly, it is possible that altered SCFA production is one link between susceptibility to tuberculosis and some risk factors. Indeed, microbiota dysbiosis is also implicated in tuberculosis risk factors such HIV, type-2 diabetes or malnutrition, which are discussed in Section 2.I.4 (Monira *et al.*, 2011; Wang *et al.*, 2012; Remely *et al.*, 2014; Subramanian *et al.*, 2014; Blanton *et al.*, 2016; Segal *et al.*, 2017). Microbiota dysbiosis in these diseases share some common features. For instance, the frequency of *Candida albicans* is increased in the oral microbiota of patients with tuberculosis, HIV, or type-2 diabetes (Belazi *et al.*, 2005; Back-Brito *et al.*, 2009). Interestingly, in HIV patients, an increase in pulmonary anaerobes (*e.g.*, *Prevotella*), which produce SCFA, correlates with increased induction of *M. tuberculosis*-specific Treg upon antigen stimulation. These induced Treg by suppressing IFN- γ and IL-17 production may provoke susceptibility to tuberculosis in the context of co-infection with HIV (Segal *et al.*, 2017). SCFA are reduced in type-2 diabetes patients (Wang *et al.*, 2012; Remely *et al.*, 2014). Though the mechanism may be different compared to that in type-2 diabetes patients, mice fed a high fat diet causing a reduction in Bacteroidetes, develop lung damages more rapidly upon infection, and are poorly protected by BCG vaccine (Arias *et al.*, 2019). While preliminary, these results may unravel new perspectives to prevent tuberculosis by reducing co-morbidity parameters influenced by microbiota dysbiosis.

One study published this year using a model of microbiota dysbiosis, reveals the protective effect of PRR stimulation by the microbiota. Upon vancomycin, neomycin and metronidazole administration, the authors show these mice (that are unable to restrict *M. tuberculosis* growth) exhibit altered *M. tuberculosis*-specific T cell response characterized by reduced activation of Th1 and Th17 and increased in Foxp3⁺ Treg induction. This effect is driven by altered Mincle-mediated activation of DC leading to impaired T cell priming following *M. tuberculosis* infection (Negi *et al.*, 2019). Importantly, in this model, *Lactobacillus* and *Bacteroides* species are highly decreased during dysbiosis, while *Enterococcus* are increased. Oral administration of *Lactobacillus plantarum* in mice treated with the antibiotics restore the ratio of effector T cells to Treg induction, resulting in the improved control of *M. tuberculosis* burden to the level of mice without dysbiosis (Negi *et al.*, 2019). In addition to deciphering mechanisms linking microbiota dysbiosis and altered immune response to tuberculosis, this study suggest that a probiotic strategy may reverse the deleterious effects induced by antibiotics on the host microbiota and immune system.

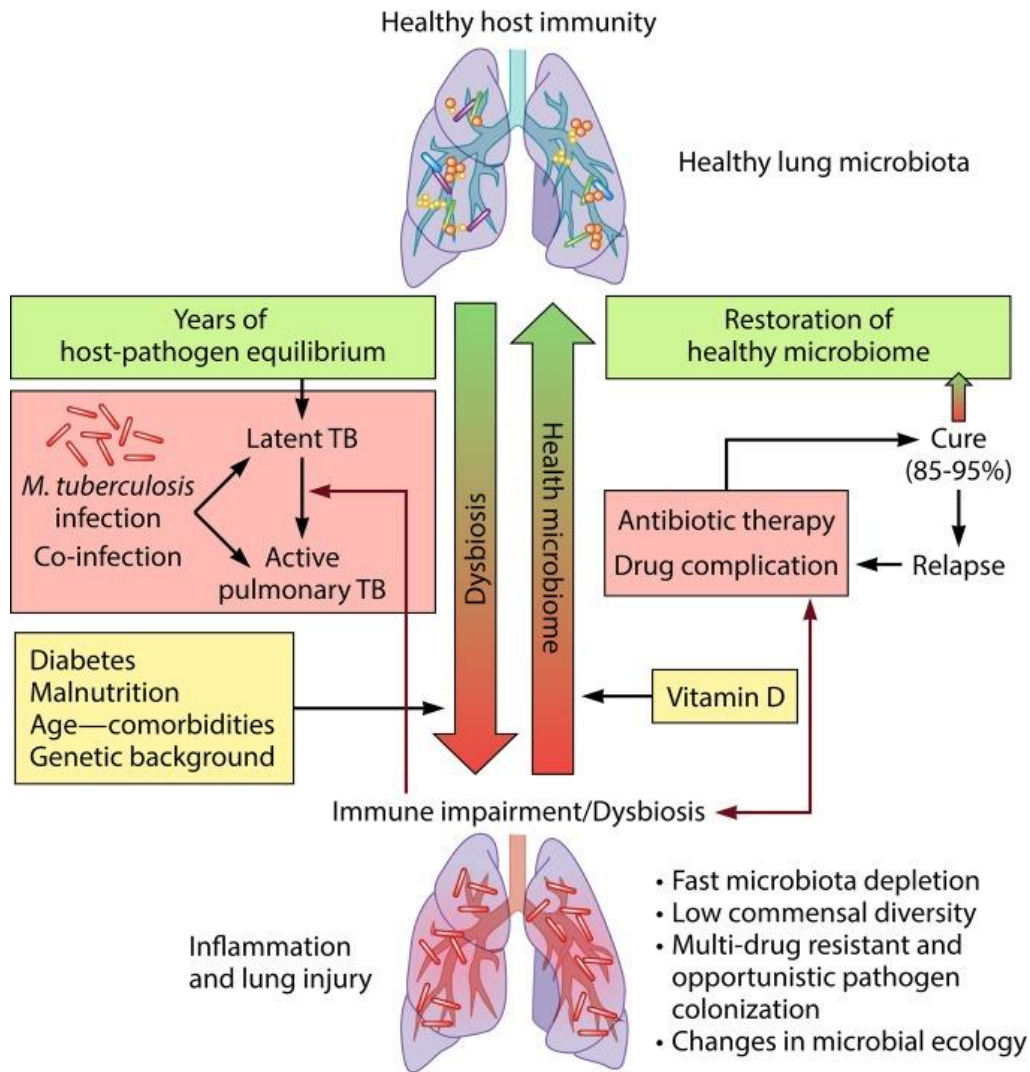


Figure 29: The host immunity and microbiota interaction is important to tuberculosis. Transition from a healthy balance between host immunity and lung microbiota towards dysbiosis can be driven by TB infection and TB therapy. In the healthy state (top), host immunity and microbiota interact with each other to maintain a balance (green box on the left). Due to genetic and/or environment factors (e.g. co-infection with HIV), latent TB state may progress toward active TB (red box). This leads to dysbiosis of lung microbiota (bottom), where colonization resistance to pathogens is weakened, leading to increased infection. Under this scenario, the immune system is less capable to eliminate or contain the pathogen growth at first, and then over-reacts to cause active disease and lung injury. The genetic make-up of the host and comorbidities like diabetes and malnutrition (yellow box) can influence disease progression and dysbiosis. While antibiotic treatment for TB may change the landscape of microbial ecology, it certainly targets *M. tuberculosis* during active TB. The decrease of pathogen burden then causes a drop in the inflammatory response by the immune system. The restoration of a healthy host-associated microbiome and the immune system should occur over time, when active tuberculosis transitions back to its latent form. Microbiota-based strategies from lung origin may complement this antibiotic treatment, and its beneficial effects should be explored with regards of re-establishing the good balance between lung immunity and local resident microbes (green box on the right). Figure and legends adapted from (Hong *et al.*, 2016).

III. Implications for the prevention and treatment of tuberculosis

In the last years, multiple studies have analyzed the possible interactions between the microbiota, *M. tuberculosis* and the host immune response. While the results are mostly preliminary, they certainly point out that such interactions exist. First, either directly through competition or cooperation with other members of the microbiota, and/or through its capacity to modulate the immune response, *M. tuberculosis* affects the microbiota composition both in the respiratory tract and the gut. Second, microbiota dysbiosis predisposes the host to infection by *M. tuberculosis* and aggravates the disease outcome. This dysbiosis may be mediated by previous *M. tuberculosis* infection, HIV infection (or other pathogens), type 2 diabetes, antibiotic use (including anti-tuberculosis drugs), diet or any other environmental or genetic factors (Figure 29). Therefore, the microbiota composition is part of the factors explaining susceptibility to tuberculosis. It may partly explain why many of the patients' contacts do not develop tuberculosis, and among those who are infected, why all patients do not develop active tuberculosis.

Importantly, deleterious interactions mediated by microbiota dysbiosis may be prevented or resolved by microbiota-targeting strategies. This approach may be particularly beneficial in children treated for tuberculosis that may, in addition to susceptibility to tuberculosis, develop an impaired immune system (O'Toole and Gautam, 2018). However, substantial work is needed to establish underlying principles involved in the interactions between the microbiota, *M. tuberculosis* and the immune system, before the development of microbiota-targeting strategies could be envisaged. For instance, current studies do not allow the clear establishment of which microbiota strains are biomarkers of tuberculosis infection, progression or cure. Likewise, mechanisms linking microbiota components to immune response alterations are still sparse. For instance, using probiotics to induce higher pro-inflammatory response may not be the only possible way they would improve protection against tuberculosis, as induction of immunological tolerance toward *M. tuberculosis* using environmental mycobacteria improves the infection outcome (Cardona *et al.*, 2016). In addition, even if one study suggests that probiotics can restore immune response to *M. tuberculosis*, altered by microbiota dysbiosis, it is unknown if they can improve it in patients having a "normal" microbiota composition. Otherwise, the interaction between the lung microbiota and the local immune system is not described at all. Such comprehension is needed to design efficient probiotics that may be composed of lung microbiota strains. Eventually, immunomodulatory microbiota strains, in particular from respiratory origin, could be, in the future, genetically engineered to express *M. tuberculosis*-derived antigens and improved mucosal vaccines.

In conclusion, in most cases, upon *M. tuberculosis* infection, the immune response may lead either to pathogen elimination or granuloma-based containment, which are two options that in theory ensures host survival and fitness. The microbiota may be implicated in the development of the immune response leading to these satisfying outcomes, as demonstrated by factors associated with dysbiosis that alter disease outcomes and render the host susceptible to reinfection or bacterial escape from containment. The host resistance mechanisms to control the bacillus, and how this pathogen has evolved to subvert and circumvent, have been an intense area of investigation since Robert Koch identification of the bacillus. By contrast, characterization of the disease tolerance mechanisms allowing symbionts from the microbiota to improve host survival in the tuberculosis context has only begun since 2012. As a whole, my thesis work argues that this must become a research priority if we wish to improve tuberculosis prevention and treatment, but also to exploit this microbial source of new candidates for host-directed therapies.

Thesis objectives

As detailed in the introduction, tuberculosis is still a major health issue today and the development of new preventive and therapeutic strategies are urgently needed, in particular to treat emerging *M. tuberculosis* resistant strains. In addition to new antibiotics, the development of host-directed therapies seems promising to improve the balance between resistance and tolerance mechanism of the anti-tuberculosis immune response. The immune system is influenced by many factors, such as the host genetics, co-infections and/or life habits. Recent findings have shown that one of the factors altering the most the immune system is the composition of the microbiota; this “forgotten organ” is also affected by the factors influencing tuberculosis, thus representing a link between environmental factors and immune activation. Implication of the gut microbiota in immune responses have been demonstrated in several diseases originating in the gut and lungs. While poorly described, it also appears that the lung microbiota could alter them. In addition, administration of some microbiota components, such as probiotics, improve both pathogen clearance and reduce immunopathology during respiratory infections.

Yet, at the beginning of this thesis (October 2015), few studies had investigated the implication of the microbiota in tuberculosis. Some suggested that the respiratory microbiota from tuberculosis patients differs from that of healthy individuals (Querido *et al.*, 2011; Cui *et al.*, 2012; Cheung *et al.*, 2013; Wu *et al.*, 2013; Botero *et al.*, 2014; Zhou *et al.*, 2015), and another study in mice had shown that *M. tuberculosis* infection causes gut microbiota dysbiosis (Winglee *et al.*, 2014). These reports suggested that *M. tuberculosis* induces a shift in the composition of the local and distal microbiota. However, at that time, it was unknown if the microbiota was altering tuberculosis outcome. To answer this question, two complementary approaches were developed in parallel in my team. While another colleague PhD student, Alexia Dumas, investigated if microbiota dysbiosis altered the immune response to tuberculosis and disease outcome, I tried to decipher if specific microbiota components could modify the immune response during tuberculosis. In addition, to improve knowledge on microbiota-immune cells interaction, the aim of my PhD project was to assess if microbiota-based strategies could be applied to this disease.

The lab of Dr. Olivier Neyrolles, where I performed my PhD, has a recognized expertise in the field of tuberculosis. In particular, we possess at my institute the BSL3 infrastructures and the expertise needed to study *M. tuberculosis* infection in cellular and mouse models. However, we did not have experience with microbiota studies. Therefore, at the beginning of my PhD thesis, we sought a collaboration with Drs. Muriel Thomas, Luis Bermudez-Humaran and Philippe Langella at the MICALIS institute (INRA, Jouy-en-Josas, France), which form a team unit recognized as one of the pioneers in France to investigate the use of commensal bacteria as probiotics. In addition of their advice, this collaboration gave me access to different bacterial strains from gut or lung origin that were used in different part of my thesis work.

To answer my thesis objective, I hypothesized that the immune response to tuberculosis could be naturally affected by gut strains through a gut-lung axis, potentially mediated by production of SCFA (as supported in the literature for other diseases), or lung strains through a local effect (unknown in the field). I started three related projects with different levels of novelty and probability of success, as described below.

- (1) Effect mediated by SCFA.** Macrophages are key immune cell in the etiology of tuberculosis, and their dual role as an effector and target cell for *M. tuberculosis* mainly determines the outcome of this disease. As detailed in Chapter 1 section II, SCFA had been shown to reduce inflammation in asthma or colitis, and to modulate lung myeloid cell activation (Trompette *et al.*, 2014). However, a few years ago, while oral administration of probiotics have been shown to improve respiratory pathogen clearance, the mechanisms involved were unknown (Racedo *et al.*, 2006; Khailova *et al.*, 2013). For this objective, I hypothesized that SCFA could improve macrophage microbicidal properties in the context of tuberculosis. To this end, I developed and adapted different *in vitro* models to condition primary human monocyte-derived macrophages with butyrate, propionate or acetate, which were then infected by the H37Rv strain of *M. tuberculosis*; the intracellular load of the pathogen was assessed in a time-course analysis. Among the different tested protocols, the one that was originally developed by the group of Dr. Powrie (Schulthess *et al.*, 2019), consisting on monocyte conditioning with butyrate during their differentiation towards macrophages, yielded a change in macrophage phenotype that lowers the intracellular burden of *M. tuberculosis*. However, these results were not reproduced in a second set of experiments in human macrophages or murine bone marrow derived macrophages. In addition, I also tested the effect of Butyrate administration *in vivo*. In a preliminary experiment where Butyrate was delivered in water supplementation, before and during infection with *M. tuberculosis* of SPF C57BL/6 mice, I observed no effect on the bacillus load in both the lungs and spleen, or in the granulomatous region formation in the lungs at day 21 or 42 post-infection. During my thesis, different studies were published eventually demonstrating that SCFA indeed improves pathogen clearance by macrophages (Schulthess *et al.*, 2019). This includes a study showing that *Klebsiella pneumoniae* susceptibility in GPR43-deficient mice is partly mediated by an impairment in phagocytosis and killing by alveolar macrophages, suggesting a protective role for SCFA in respiratory infections (Galvão *et al.*, 2018). Another study showed that phenylbutyrate, a chemical derivative of butyrate also having histone deacetylase inhibitory properties, inhibits *M. tuberculosis* growth *in vitro* and modulates macrophage phenotype decreasing the bacillus load after infection (Coussens, Wilkinson and Martineau, 2015). While these studies corroborate my original hypothesis, they also reduced novelty of this project. Based on these events, we decided not pursuit this project, and its preliminary data will not be presented or discussed for the rest of this PhD thesis.
- (2) Distal effect of *bona fide* probiotics involved in the gut-lung axis.** Independent of the effect of SCFA on macrophages, I hypothesized that the gut microbiota affects the immune response to tuberculosis, and that oral administration of probiotic strains improves disease outcome through the gut-lung axis. In this part of the project, I focused on two *bona fide* probiotic strains: *Lactobacillus plantarum* VEL12238 and *Lactobacillus casei* BL23, which were provided to us by our collaborators from the Langella team. These strains belong to species with known capacities to reduce pathogen load and inflammation in other respiratory infections (Racedo *et al.*, 2006; Kechaou *et al.*, 2013). In preliminary experiments, administration of either strain before and after infection with *M. tuberculosis* SPF C57BL/6 mice did not modulate the bacillus load in the lungs, dissemination to the spleen, or granulomatous region formation in the lungs 30 days post-infection. While we cannot exclude that the gut-lung axis does not influence tuberculosis, it is probable that other known gut probiotic strains could improve disease outcome. In addition, we cannot conclude that the tested strains could not influence tuberculosis under other settings. Indeed, oral administration of *Lactobacillus plantarum* MTCC 2621 was recently shown to reduce *M. tuberculosis* burden in mice pre-treated with antibiotics, arguing the effects induced by this species may be compensated in our SPF (microbiota-competent) mouse model (Negi *et al.*, 2019). Again, based on time limit and economic resources, it was decided not further pursue this project.

(3) Local effect of lung microbiota members. At the beginning of my PhD thesis, it was clear that commensal bacteria were present in the respiratory tract, and composition of this microbiota in health and disease was beginning to be comprehended (as detailed in Section 1.III). One study suggested that the respiratory microbiota was implicated in lung immune cell priming, suggesting that it may play a role in respiratory health and diseases (Gollwitzer *et al.*, 2014). In many diseases in which microbiota dysbiosis was observed, it was later found that healthy gut microbiota modulates immune functions to protect against disease and that modulation of the gut microbiota can improve immune response and thus disease outcome. Respiratory microbiota composition in tuberculosis patients seemed to be disturbed compared to healthy individuals (see Section III). Based on this evidence, I hypothesized that the respiratory microbiota modulates the local immune system, and that modification of its composition could alter the immune response leading to susceptibility in tuberculosis. Above all, I predicted that administration of specific pulmonary strains, isolated from the healthy respiratory microbiota, could improve lung immunity to alter the tuberculosis outcome in favor of the host. Before I started, Dr. Aude Remot from the MICALIS institute had isolated and cultivated 20 bacterial strains from neonatal SPF mice lungs containing *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Listeria*, *Lactobacillus*, *Escherichia coli*, and *Proteus mirabilis* strains (Remot *et al.*, 2017). Few collections of lung microbiota bacteria have been generated so far (Sibley *et al.*, 2011; Yun *et al.*, 2014). Our collaboration with the MICALIS institute gave me access to these strains that I decided to use as a tool to assess immunomodulatory properties of the lung microbiota, considering that some of them may represent attractive candidates for microbiota-based therapies. Previously, Dr. Aude Remot had shown that these strains were able to modulate lung epithelium cytokine production with different profiles. Based on these strong encouraging results, my objectives for this project were to assess whether such strains could modulate the lung immune response *in vivo*, and if so, to determine their influence in tuberculosis disease outcome. Due to the high novelty of this project, and promising preliminary data obtained during my second year as PhD student, I decided to focus on this project. The following sections of this PhD thesis will describe the compilation of methods and results in this project, and the discussion and perspectives deriving from my doctoral work.

Scientific contributions

This thesis project is part of a research collaboration effort aiming at developing microbiota-targeted strategies to improve the prevention and treatment of respiratory infectious disease. In particular, the objective of my PhD project was to assess if microbiota components could modulate the immune response during *M. tuberculosis* infection. The strategy chosen to answer this question was based on the development of *in vitro* and *in vivo* models characterized by exogenous administration of different microbiota components being: (i) known probiotic *Lactobacillus species* representative of the gut microbiota, (ii) known metabolites produced by the gut microbiota, and (iii) recently isolated commensals from the lung microbiota. The research work performed during my PhD at the IPBS in the team of Dr. Olivier Neyrolles, and under the supervision of Dr. Geanncarlo Lugo-Villarino, led to some significant scientific contributions.

During my third year of thesis, I participated in the bibliographical research and writing of this mini-review with my colleague PhD student, now Dr. Alexia Dumas. In particular, I was in charge of the probiotic chapter.

- **Mini review:** “The role of the lung microbiota and the gut-lung axis in respiratory infectious diseases”. Dumas A, Bernard L, Poquet Y, Lugo-Villarino G, Neyrolles O. *Cell Microbiol.* 2018. 20(12): e12966. doi: [10.1111/cmi.12966](https://doi.org/10.1111/cmi.12966) (See Appendix 1)

Results obtained during the three first years of my PhD suggested that one of the commensal strains isolated from the lung microbiota could modulate the lung immune compartment and reduced leukocyte infiltration associated with tuberculosis. The use of this strain as a probiotic in the context of tuberculosis (and other respiratory diseases) is in the process of being patented. A provisional application was filed in France containing key results that I obtained during this PhD. A copy of this document (in French) is included in Appendix 2.

Importantly, as detailed in the Results chapter, the commensal strain patented was first identified as a *Lactobacillus animalis/spp* based on sequencing of regions of the 16S rDNA. However, the analysis of its complete genome sequence recently revealed that it is in fact a *Lactobacillus murinus* strain. Therefore, the strain referred as to *L. animalis* in the patent is in fact the same strain as the one described in this thesis manuscript as *L. murinus* strain, which was deposited at the Pasteur collection under the number **CNCM I-5314**.

- **Patent FR1903364:** “Treatment of respiratory diseases using the bacteria *Lactobacillus animalis*”. Bernard L, Lugo-Villarino G, Neyrolles O, Thomas M, Remot A, Langella P. *Provisional application filed March 29, 2019.* (See Appendix 2)
- **Original research article:** “Pulmonary *Lactobacillus murinus* induces Foxp3⁺RORγt⁺ regulatory T cells and reduces Tuberculosis-associated lung inflammation”. Bernard-Raichon L, Colom A, Namouchi A, Cescato M, Garnier H, Corral D, Dumas A, Ghebrendrias N, Guilleton P, Hudrisier D, Cougoule C, Remot A, Langella P, Thomas M, Neyrolles O*, Lugo-Villarino G*[†]. *In preparation.* (*Equal contribution; [†]Corresponding author)

Attributions

The work presented in this thesis would not have been possible without the main contributions of the people listed in this section. When not annotated otherwise, people mentioned belong to the team “Mycobacterial interaction with host cells” led by Dr. Olivier Neyrolles at IPBS.

- All bacterial strains used in this study were provided by our collaborators from the MICALIS institute: Drs. Muriel Thomas, Luis Bermudez-Humaran and Philippe Langella (INRA, Jouy-en-Josas, France). In particular, the pulmonary commensals were isolated by Dr. Aude Remot (INRA, Jouy-en-Josas, France). Data presented in [Figure 33](#) and [Table 6](#) were also generated by Dr. Aude Remot.
- *Lactobacillus murinus* CNCM I-5314 DNA was extracted with the help of Dr. Alexia Dumas. Sequencing was performed by the DNA sequencing GeT-PlaGe (INRA, Castanet-Tolosan, France). Genome assembly, annotation, and comparisons to other genomes were performed by Dr. Amine Namouchi, as well as preparation of [Figure 46](#) (CEES, Oslo, Norway).
- Margot Cescato and Hugo Garnier performed their Master 1 internship under my co-supervision along with Dr. Geanncarlo Lugo-Villarino. They both participated in experimentations involving non-infected mice presented in [Figures 35, 36](#) for Margaux Cescato, and [Figures 44, 45, 47](#) as well as experimentations using heat-killed *L. murinus* mentioned in the discussion for Hugo Garnier.
- Pauline Guilleton was a former Master 2 intern student and set-up culture of some of the strains used in this study.
- André Colom and Dr. Geanncarlo Lugo-Villarino helped me in most *in-vivo* experiments, with bacterial administrations and organ preparation for analyses.
- Histological preparation including paraffin embedding, lung slicing, coloring, and scanning were realized by Florence Capilla, Christine Salon or Annie Alloy of the Purpan histo-pathology platform Anexplo Genotoul Toulouse. Histological analyses were performed with the help of Dr. Céline Cougoule (IPBS - CNRS) and Dr. Talal Al Saati (Purpan histo-pathology platform Anexplo Genotoul Toulouse).
- Flow-cytometry analyses, and in particular the ones involving bacteria detection ([Figure 34](#)), were performed with the help of Emmanuelle Näser (Tri Genotoul platform, Toulouse).
- RT-qPCR were performed with the help of Dan Corral and Maeva Dupont.
- *Lactobacillus murinus* bacterial components (lipopolisaccharide, lipids, RNA and proteins) mentioned in the discussion were prepared with the help of Dr. Jérôme Nigou (IPBS).
- Breeding, housing and assessment of mice well-being used in all experiments was performed by the Anexplo Genotoul staff, in particular by Céline Berrone and Flavie Moreau (in the BSL-3 facility), and Gregory Marsal (in the conventional animal facility).
- Natsinet Ghebrendrias and Yves-Marie Boudehen were highly involved in experiments belonging to my alternative projects that were halted during my thesis for strategic purposes and thus not described in this manuscript.

Material & Methods

I. Bacterial strains, growth and preparation for inoculation

1. Commensal bacteria

Different commensal bacteria isolated from mouse lung homogenates, as described in (Remot 2017 ISME), were provided by the laboratory of Dr. Muriel Thomas (INRA, Jouy-en-Josas, France). Among them, the strains tested in this study, three *Lactobacillus*, two *Staphylococcus* and a *Neisseria* strains are listed in [Table 2](#).

Table 2: Pulmonary bacterial strain growth conditions

Bacterial strain	Medium	Growth condition		Generation time	Concordance factor
		Pre-culture	Culture		
<i>Lactobacillus murinus</i> (CNCM I-5314)	MRS	1/10, ON, 30°C - agitation	1/100, 3-4 h, 37°C - agitation	40 min	1 uDO \approx 2.10 ⁸ CFU/mL
1 h				1 uDO \approx 2.10 ⁸ CFU/mL	
1 h 20 min				1 uDO \approx 2,3.10 ⁸ CFU/mL	
<i>Neisseria mucosa</i>	BHI	1/10, ON, 30°C + agitation	1/100, 3-4 h, 37°C + agitation	40 min	1 uDO \approx 1.10 ⁸ CFU/mL
<i>Staphylococcus sciuri</i> (CNCM I-4970)				30 min	1 uDO \approx 2.10 ⁸ CFU/mL
<i>Staphylococcus epidermidis</i>				45 min	1 uDO \approx 0,6.10 ⁸ CFU/mL
<i>Mycobacterium tuberculosis</i> (H37Rv)	7H9	1/10, 1-2 weeks, 37°C - agitation	1/100, 1 week, 37°C - agitation	24 h	1 uDO \approx 2.5.10 ⁸ CFU/mL

It is worth to mention that the three *Lactobacillus species*, were deposited at the French National Collection of Microorganism Cultures (CNCM). As indicated in [Table 2](#), these strains were cultivated in MRS (BD Difco Lactobacilli MRS Broth #288130) or BHI (BD Bacto Brain Heart Infusion #237500) media. 15% agar containing media was used to cultivate the strains in solid culture for bacterial load determination. Since the *Lactobacillus species* are facultative anaerobe, they were cultivated without agitation, in tubes full of culture medium. For solid culture, the plates containing the bacterial strains were sealed in a plastic Ziploc bags.

Aliquots received from our collaborators were cultured in a large media volume for ~7 h to obtain important quantities of bacteria. These bacterial cultures were frozen in 15% glycerol (Promega #H5433) at -80°C, constituting the “master” bank. Before each experiment, an aliquot from the master stock was thawed, and similarly cultured to generate a “working” bank stored at -80°C.

L. murinus growth curve

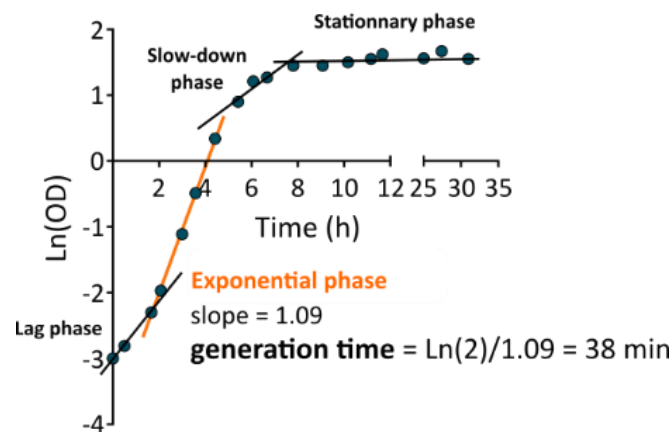


Figure 30: Determination of bacteria characteristics based on growth curves. Bacterial growth was studied for each strain on fresh cultures inoculated with 1/100 overnight pre-culture by repeated OD and CFU measurements. The representation of natural logarithm (Ln) of OD_{600nm} measurements, as function of time, allow linearization of OD that helps to identify growth phases by changes in curve slope, as exemplified here for *L. murinus*. Each point represents the medium of two duplicated OD measures from one among two independent experiments. These phases are: the lag phase (corresponding to a slow bacterial growth due to bacteria adaptation to the environment), the exponential phase (corresponding to growth in optimal conditions), the slow-down phase (characterized by a progressive reduction of growth caused by nutrient scarcity and increase in bacterial death due to waste accumulation), the stationary phase (characterized by an equilibrium between bacterial replication and death) and the decline phase (corresponding to superior death compared to replication, but not observed within the experiment duration). Slope during exponential growth was determined by linear regression. This value was used to determine the generation time of each strain in optimal conditions. During exponential phase, few bacteria are dying. Thus, correspondence between medium turbidity (measured by OD) and concentration in live bacteria is the highest in this phase, and few variations of this factor are observed within.

Bacterial growth characteristics were determined after performing bacterial growth follow up. Turbidity and bacterial concentration were assessed at regular time interval. Turbidity of the culture was estimated by spectrophotometric measurements of optical density (OD) at 600 nm. Colony Forming Unit (CFU) assay was used to determine the bacterial concentration (in CFU/ml) by serial dilution of culture in PBS, and incubation on agar medium for 48 h at 37°C. OD measurements were used to graph the strains growth curves, as represented for the *Lactobacillus murinus* in Figure 30. This allowed me to determine the different phases of bacterial growth and generation time. Comparison of OD and CFU count was then used to determine a concordance factor for each strain that permitted during experiments to estimate bacterial concentration in the culture at the time of measure based on OD value. These factors are calculated as follow.

$$\text{Generation time} = \frac{\text{Ln}(2)}{\text{Growth curve slope during exponential growth}}$$

$$\text{Concordance factor} = \frac{\text{OD}}{\text{Bacterial concentration at the same time point}}$$

For example, I determined that for *L. murinus*, the generation time was around 40 min, and that during exponential growth, 1 OD correspond approximatively to 2.10⁸ CFU/mL. Based on these results, OD measures were used to prepare bacterial suspensions in PBS at the required concentration.

During mouse-based experiments, for each bacteria administration time-point, a pre-culture was prepared by diluting a new aliquot from the working stock in the appropriate medium, and incubated overnight. This pre-culture was then diluted 1/100, and incubated at 37°C for 3-4 h.

Bacterial cultures in exponential phase were thus used to prepare the inoculum. Appropriate bacterial culture volume, to prepare a common inoculum for all mice of the same treatment group, was estimated based on OD measurements and concordance factor. Bacteria were harvested by centrifugation at 1550 xg at 4°C for 5 min, washed twice in phosphate buffered saline (PBS) (Gibco™ DPBS, no calcium, no magnesium #14190169), and resuspended in PBS before administration to mice. CFU assay was performed to verify a posteriori that the concentration in the inoculum was correct.

For some experiments, heat-inactivated bacteria were obtained by incubation of a part of the inoculum 30 min at 70°C, a pasteurization process conserving bacterial component integrity (Plovier *et al.*, 2017). Other experiments were performed with labelled bacteria. Accordingly, CFSE stained bacteria were obtained after incubation of the inoculum in 100 µg/mL CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen™ #C34554) for 10 min at 37°C, three washes in PBS 10% FBS (Fetal Bovine Serum) (PAN-BIOTECH 3302-P281501), and resuspension in PBS for administration (Ueckert *et al.*, 1997).

2. Mycobacterium tuberculosis

All experimentation using M. tuberculosis were carried out in BSL-3 laboratories and animal facility. M. tuberculosis (lab strain H37Rv) was grown in 7H9 (BD Difco Middlebrook 7H9 Broth #271310) supplemented with ADC (BD Difco™ BBL™ Middlebrook ADC Enrichment #11718173) at 10%, Glycerol at 0.5% and Tween 20 (EUROMEDEX #2001-B) at 0.05% as described in Table 2. A fresh culture in exponential growth was used to prepare each inoculum for infection. Bacteria were harvested by centrifugation at 1550 xg at 4°C for 15 min, and washed in PBS twice. *M. tuberculosis* growth is characterized by the formation of large aggregates or clumps; leukocytes infected by clumps are hardly able to control the infection (or are susceptible to cell death) compared to those infected with

separated bacteria (Brambilla *et al.*, 2016). To limit infection variation between animals, bacterial suspensions were homogenized by passage through a blunted needle (BIORAD #3551174) loaded into a syringe. Remaining aggregates were precipitated using a centrifugation at low speed (75 xg for 5 min). Bacterial concentration was then estimated by OD measurements at 600 nm with the concordance factor: 1 uDO \approx 2.5.10⁸ CFU/mL. An inoculum at 5.10⁴ CFU/mL was prepared for mice infection. To verify a posteriori its concentration, the inoculum was spread on 7H10 Agar medium (BD Difco Middlebrook 7H10 Agar #262710) supplemented with peptone (Fischer Scientific #1207-3775), OADC at 10% (BD Difco™ BBL™ Middlebrook OADC Enrichment # BD 211886) and Glycerol at 0.5%.

3. *Lactobacillus murinus* CNCM I 5314: whole genome sequencing

DNA extraction. DNA from lactobacilli is difficult to extract. Using a classic protocol recommended in the DNeasy® Blood & Tissue kit (Qiagen), the extraction failed. However, after adaptation of this protocol based on those tested for *L. casei* DNA extraction (Alimolaei Biologicals 2017), DNA from *L. murinus* was successfully extracted. Briefly, cell pellet from a 15 mL *L. murinus* culture in exponential growth was collected after centrifugation at 1550 xg for 5 min and frozen at -20°C. It was then resuspended in a solution containing 30 mM NaCl, 2mM EDTA (pH=8.0). Bacterial suspension was centrifuged, and cell pellet was resuspended in lysis buffer (20mM Tris HCl pH 8.0, 2 mM EDTA, 1.2% Triton® X-100) supplemented with lysozyme (20mg/mL, Sigma-Aldrich #L6876) for 2 h at 37°C. Proteinase K (DNeasy® Blood & Tissue kit) and RNase A were added to the lysate and incubated for another 1 h at 55°C. Same volume of the AL buffer (DNeasy® Blood & Tissue kit) was added to the lysate, vortexed and incubated 30 min at 56°C. After incubation, another volume of ethanol 100% was added. DNA was obtained from this suspension using DNeasy Mini spin columns (Qiagen) following the manufacturer's instructions.

DNA sequencing. DNA libraries preparation and sequencing were performed by the DNA sequencing platform GeT-PlaGe (INRA, Castanet-Tolosan, France). Briefly, DNA was fragmented via sonication to 200 to 1000 base pairs (bp). Fragments were then ligated to Illumina adapters. After sample quality assessment, DNA fragments were sequenced on an Illumina HiSeq 3000.

Whole genome assembly and annotation. Raw sequence reads were pre-processed using fastp, version 0.19.4 (Chen *et al.*, 2018) to remove illumina adaptors and low-quality reads. Filtered reads were assembled using Unicycler version, v0.4.7 (Wick *et al.*, 2017). The assembly quality was assessed using QUAST v5.0.2, b7350347c (Gurevich *et al.*, 2013) and the assembled genome was visualized using Bandage v0.8.1 (Wick *et al.*, 2015). The genome annotation was made using Prokka v1.13 (Seemann, 2014).

Edit distance calculation. The edit distance reflects the number of differences between the sequenced reads and the genomic sequence used as a reference. To compute the edit distance, the information stored in the bam file under the tag "NM:i" is analysed. An edit distance of 0 means that the reads are identical to the reference sequence. The tool BAMstats was used to compute the edit distance.

Evolutionary analyses of orthologous groups. Evolutionary analyses and comparative genomics of the new sequenced and assembled genome were performed using *L. animalis* and *L. murinus*. *L. lactis* was used as outgroup. To avoid any orthology discrepancies due to differences related to annotations methods, all selected genomes were re-annotated using Prokka v1.13. Orthofinder (v2.2.7) was used to identify orthologous sequences.

II. Assessment of immune system modulation by commensal bacteria in Mouse models

1. Mouse models

a) Ethic requirements and general considerations

All mouse-based experiments were performed in animal facilities (including the IPBS BSL3 animal facility) that met all legal and ethical requirements in France, and by qualified personnel in such a way to prevent excessive use of animals and minimize their suffering and discomfort. Animal care and experimentation were consistent with the French guidelines, and were approved by the Ministry of Higher Education and Research (Agreement APAFIS 5704). Six- to eight-week-old female SPF C57BL/6 mice were purchased from Charles River Laboratories. Five to thirteen mice per group were used in each experiment. Mice were sacrificed by cervical dislocation under anesthesia (4% isoflurane – Vetflurane Virbac Denmark) or by intraperitoneal administration of pentobarbital (Doléthal® Vétoquinol) at lethal dosing.

b) Microbiota inoculation in mice

To assess the direct effect of lung microbiota to local lung leukocyte populations, 10^7 CFU of lung commensal bacteria (or mock) in 20 μ L of PBS were administered intranasally (*i.n.*) to each mouse; bacterial suspension was deposited at the animal nostril, and aspirated by the animal under anesthesia three times a week during two weeks (for a total of seven administration) before infection or sacrifice. In the context of infection with *M. tuberculosis*, lung microbiota administration was pursued thereafter twice per week until sacrifice time point. In some experiments, lung commensal bacteria administration was only started after infection, and repeated twice a week until sacrifice, as indicated in figure legends.

To assess the indirect (gut-lung axis) effect of gut commensals to distal lung leukocyte populations, 10^9 CFU of commensal in 200 μ L of PBS were administered intragastrically (*i.g.*) to the mice every day during 10 day before sacrifice.

c) Infection with *M. tuberculosis*

In some experiments, mice were infected (*i.n.*) with 10^3 CFU of the H37Rv strain of *M. tuberculosis* under 4% isoflurane anesthesia. Mice were sacrificed at day 21, 30 or 42 post-infection, as indicated in figure legends.

2. Histological analyses

Because the intranasal infection does not lead to homogenized dispersion of *M. tuberculosis* in the lungs, we could not split the left and right lungs to perform simultaneously the histological and CFU analyses for each mouse. Therefore, specific mouse groups (3-6 mice per group) were dedicated exclusively to histological analyses. After mice were sacrificed by intraperitoneal administration of pentobarbital at lethal dosing, the lungs were inflated with 10% formol solution (Formalin solution, Sigma-Aldrich #HT501128-4L), and stored for 5 days at 4°C. This solution is proven to inactivate *M. tuberculosis*. Fixed lungs were then included in paraffin, and 5 μ m horizontal slices were done using a microtome. Some slices were stained with haematoxylin and eosin stain (HE) that stains cell nuclei in purple and cytoplasm in light pink. Stained slices were numerated using a Panoramic 250 scanner (3DHISTECH).

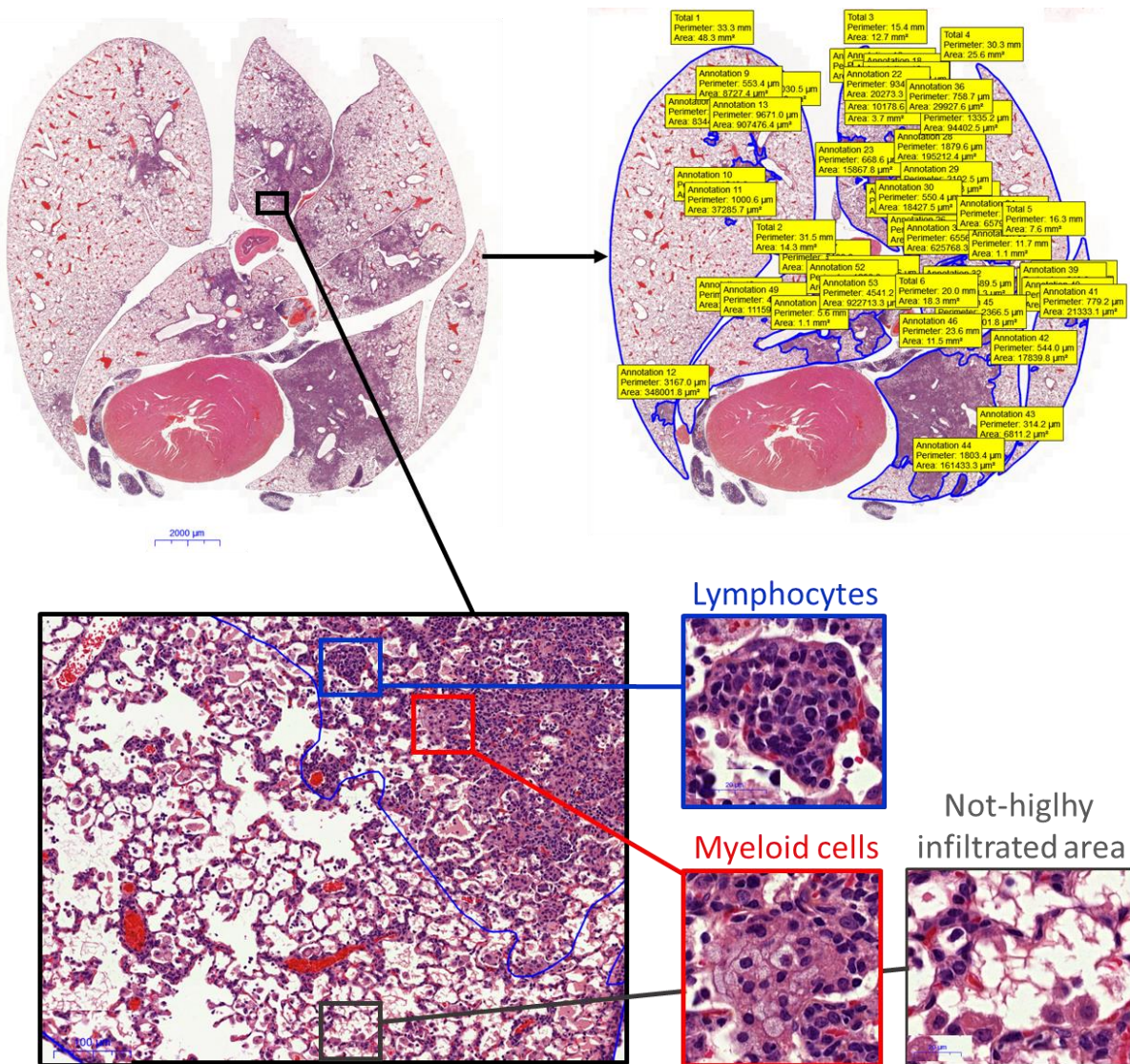


Figure 31: Quantification of leucocyte-infiltrated areas in *M. tuberculosis*-infected mouse lungs (related to Figures 34, 37 and 42). HE stained lung paraffin-embedded slices were quantified and analyzed using the CaseViewer software. Area of each lung lobe was delimited and called “Total” (top right). Lymphocyte (blue) and myeloid (red frame) rich areas, characterized by a strong purple staining were determined as infiltrated area (top right, “Annotation 1”, and so on) in comparison to the white-light pink area (grey), which is considered as non-infiltrated (even if alveolar macrophages can also be distinguished in these areas).

Leukocyte infiltration was quantified using the software CaseViewer (3DHISTECH), as exemplified in Figure 31. One lung slice was quantified per mice. Lung area considered not be infiltrated was mainly composed of the airway space that is visible in white-light pink in HE stained slices, whereas that with leukocyte infiltration (and in particular lymphocyte infiltration) was measured as the dramatic increase of the number of purple stained nuclei, reflected by a distinguishable purple area. As illustrated in Figure 31, the total lung area was determined by the addition of the areas of each lobe; the murine right lung is composed of 4 lobes (*i.e.*, superior, middle, inferior and post-caval), and the left lung by a single large lobe divided in regions (*i.e.*, upper, middle and lower). Purple-infiltrated areas were then delimited for each lobe. Area infiltrated by lymphocyte or myeloid cells were all quantified as infiltrated areas without distinction. The area of the total lung and infiltrated zone were extracted from CaseViewer, and the proportion of infiltration determined as:

$$\% \text{ of leukocyte infiltration} = \frac{\text{Sum of infiltrated areas}}{\text{Sum of total lung area}} \times 100.$$

3. Cellular and molecular analyses

Lungs from five to eight mice were prepared individually into cell suspensions, and different analyses were performed: immune-phenotyping by flow cytometry, lung cytokine content by ELISA, RNA gene profiling by qPCR analyses, and *M. tuberculosis* growth scoring by CFU assay. For flow cytometry analyses, given that lung homogenates are highly contaminated by blood cells, mice were injected intravenously (*i.v.*) with 2 µg of anti-CD45.2 antibody (BD Biosciences # 563685) 5 min before sacrifice (Patel *et al.*, 2015). This protocol allowed the staining of all leukocytes from the blood, but not those coming from the actual lung tissue. Entire lungs, spleen and colon (only in non-infected mice) were aseptically collected after sacrifice by cervical dislocation (followed by exsanguination).

a) Cell suspension preparation

Lung homogenate preparation. Lungs were collected in C-tubes (Miltenyi #130096334), homogenized with a gentleMACS dissociator (m_lung_01 cycle three times), incubated 30 min at 37°C with collagenase D (2 mg/mL, Roche #11088882001) and DNase I (0,1mg/mL, Roche #10104159001), and homogenized again with the gentleMACS dissociator (m_lung_02 cycle). When mice were infected, a part of this cell suspension was used for *M. tuberculosis* bacterial load determination as described later. The homogenate was passed through a 70 µm cell strainer (ClearLine #141379C) to get rid of tissue and cell debris.

Spleen and lymph nodes preparation. The spleens were individually collected from infected mice and only used for *M. tuberculosis* growth assessment. They were placed in M-tubes (Miltenyi #130096335), and homogenized with the gentleMACS dissociator (m_spleen_02 cycle twice). Alternatively, the spleens and mesenteric lymph nodes (MLN) were collected from non-infected mice and used for flow cytometry analyses. In this case, they were crushed up using a sterile syringe plunger on to 70 µm cell strainer, and washed thereafter to recover the debris-free homogenate.

Red Blood cell lysis. After centrifugation of lung and spleen cell suspensions at 330 xg for 5min, cell pellets were resuspended in 1 mL PBS (lung supernatant was save at this step for cytokine measurement). 5 mL of lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.2)), was added on cell suspension for 5 min. Lysis was stopped by the addition of RPMI medium containing 10% FBS. After centrifugation cell pellet was 40 µm (ClearLine #141378C) filtered to eliminate lysed red blood cell aggregates. Part of it was saved for RNA analysis whereas the rest was used for flow cytometry analysis.

Table 3: List of antibodies used for extracellular cell stainings (related to Figures 35-42, 43-45, and 47).

Antibody	Fluorochrome	Clone	Supplier
Anti-CD45.2	BV711	104	BD Biosciences
Anti-CD45.2	AF700	104	Biolegend
Anti-TCR- β	FITC	H57-597	Biolegend
Anti-TCR- β	AF700	H57-597	Biolegend
Anti-CD3	APC-Cy7	17A2	Biolegend
Anti-CD3	FITC	17A2	Biolegend
Anti-CD8	FITC	53-6.7	Biolegend
Anti-CD4	BV786	RM4-5	BD Biosciences
Anti-CD4	BV421	GK1.5	Biolegend
Anti-CD25	PE	PC61	BD Pharmingen
Anti-ICOS	BV786	C398.4A	Biolegend
Anti-CCR6	BV605	29-2L17	Biolegend
Anti-PD-1	BV711	J43 (RUO)	BD Biosciences
Anti-CD11b	BV650	M1/70	Biolegend
Anti-CD11c	PE-Cy7	N418	Biolegend
Anti-CD11c	AF700	N418	Biolegend
Anti-MerTK	PE	2B10C42	Biolegend
Anti-CD64	PE Dazzle	X54-5/7.1	Biolegend
Anti-F4/80	AF647	BM8	Biolegend
Anti-MHCII	APC-Cy7	M5/114.15.2	Biolegend
Anti-Siglec-F	BV786	E50-2440	BD Pharmigen
Anti-Siglec-F	PerCP Cy5.5	E50-2440	BD Biosciences
Anti-CD24	BV421	M1/69	Biolegend
Anti-CD103	BV605	2E7	Biolegend
Anti-Ly6G	AF700	1A8	BD
Anti-Ly6C	Pacific Blue	HK1.4	Biolegend
Anti-CD80	PE Dazzle	16-10A1	Biolegend
Anti-CD86	PE	GL1	eBioscience
Anti-CD206	BV421	C068C2	Biolegend
Anti-PD-L1	APC	10F.9G2	Biolegend
Anti-CD40	APC	44986	Biolegend
Anti-CD16/32	APC-Cy7	93	Biolegend

AF: Alexa Fluor, APC: Allophycocyanin, BV: Brilliant Violet, Cy: Cyanine, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PerCP: Peridinin-Chlorophyll-protein

b) Flow cytometry

Cell stimulation. To analyze cytokine production by CD4⁺ T cells, a fraction of cell suspension from lung homogenates was stimulated for 4 h at 37°C with 5% CO₂ in RPMI medium (RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES, Fisher #11544526) supplemented with 10% FBS, 50 ng/mL Phorbol Myristate Acetate (PMA, Sigma Aldrich #P8139-5MG), 500 ng/mL ionomycine (Sigma-Aldrich #I0634-1MG), Brefeldin A (GolgiPlug™ 1/1000, BD Biosciences #555029), and Monensin (GolgiStop™ 1/2000, BD Biosciences #554724) to segregate cytokines inside the cell. The remaining cell suspension was stored in Cell Staining Buffer (CSB, Biolegend #420201) at 4°C, and will be used for extracellular marker, and transcription factor staining. For some experiments, a fraction was also saved in CSB at 4°C for myeloid cell analysis.

Cell stainings. Cell staining were performed in V-bottom 96 well plates, centrifuged at 600 xg for 2 min (or 700 xg for 2 min after cell fixation). Cell suspensions are incubated 20-30 min at 4°C with a mixture containing an anti-CD16/32 antibody (TruStain FcX™, Biolegend #101320) to block Fc receptors (avoiding non-specific staining), a viability marker (live/dead fixable blue dead cell stain kit, Invitrogen™ #L34961), and appropriate extra-cellular antibodies diluted in CSB for which the

references are presented in Table 3. Cells were washed in CSB, fixed 30 min at room temperature (RT), and permeabilized 15 min in permeabilization buffer (Foxp3/transcription factor staining buffer set, eBioscience #00-5523-00). Cells were stained with an antibody set to detect intracellular factors (transcription factors and cytokines) presented in Table 4, and then diluted in Permeabilization buffer for 45 min at RT. In the context of *M. tuberculosis*-infected mice, the stained cell preparation was fixed for 2 h in PBS containing 4% ParaFormAldehyde (PFA) at RT to ensure pathogen killing.

Table 4: List of antibodies used for intracellular cell stainings (related to Figures 35-39, 41, 43-45, and 47)

Antibody	Fluorochrome	Clone	Supplier
Anti-Foxp3	AF 700	FJK-16s	eBioscience
Anti-Foxp3	APC	FJK-16s	eBioscience
Anti-RORγt	PE CF594	Q31 378	BD Biosciences
Anti-T-bet	PE Cy7	4B10	eBioscience
Anti-Ki67	AF 700	SolA15	eBioscience
Anti-CTLA-4	BV 605	UC10-4B9	Biolegend
Anti-Helios	APC-eFluor 780	22F6	eBioscience
Anti-TNF	PE Cy7	MP6-XT22	BD Biosciences
Anti-IFN-γ	BV 605	XMG1.2	Biolegend
Anti-IFN-γ	PE Dazzle	XMG1.2	Biolegend
Anti-IL-17	AF 647	C11-18H10	BD Biosciences
Anti-IL-17	PE	17B7	eBioscience
Anti-IL-10	FITC	JES5-16E3	BD Biosciences
Anti-LAP (TGF-β1)	BV 421	TW7-16B4	BD Biosciences

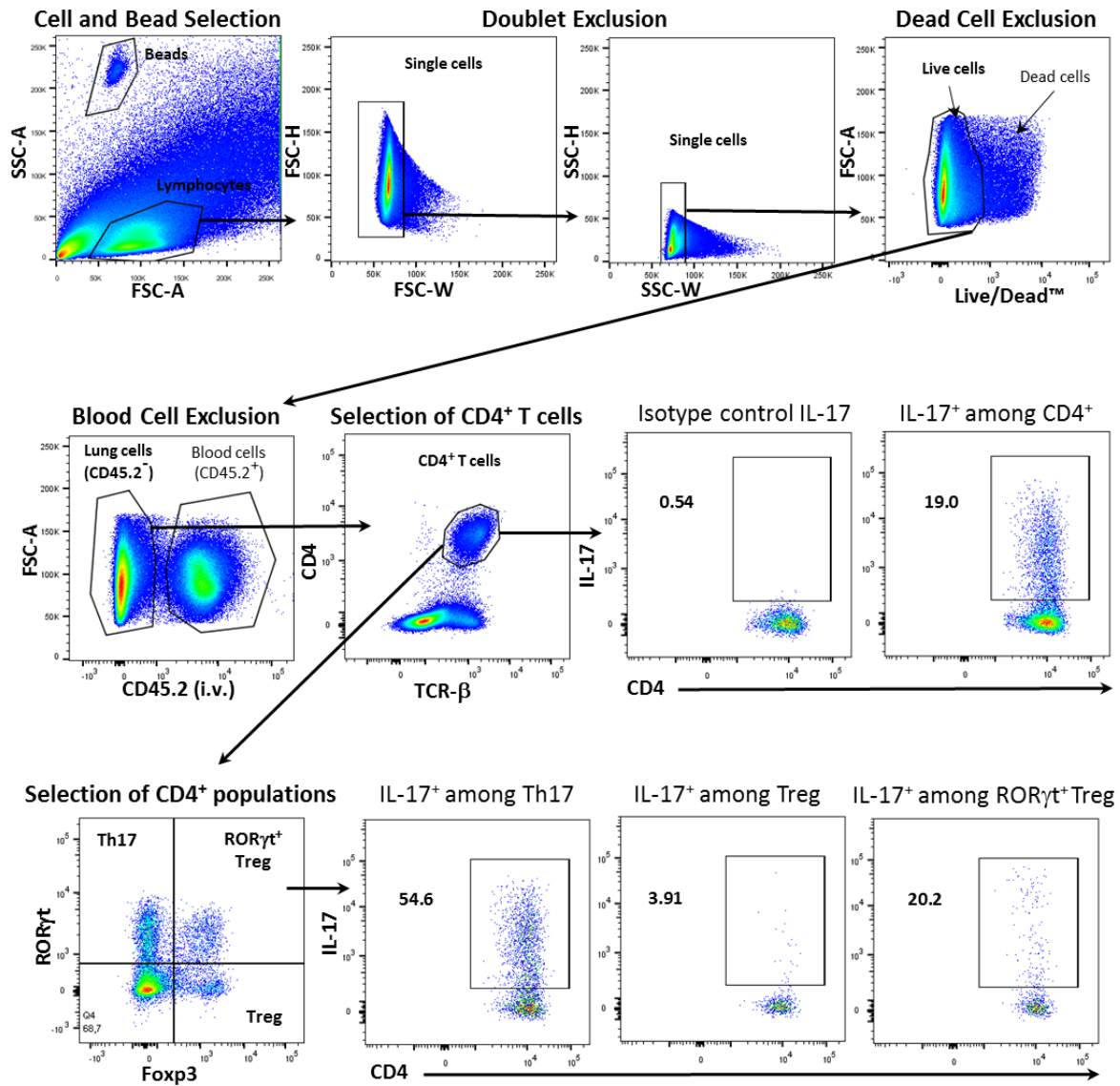


Figure 32: Gating strategy for the identification and characterization of live lung CD4⁺ T cell populations by flow cytometry (related to Figures 35-39, 43-45 and 47). Small and non-granulosed cells were selected based on the FSC-A and SSC-A parameters. Doublets were then excluded based on cell roundness using successive FSC-H vs FSC-W, and SSC-H vs SSC-W plots. Dead cells, stained with the Live/Dead™ fixable cell stain kit, were excluded. Likewise, contaminating cells from the blood were then excluded using CD45 labelling (an anti-CD45.2 intravenous injection 5 min prior mice sacrifice). Lung CD4⁺ T cells were selected on their expression of CD4 and TCR-β (or CD4 and CD3). Different CD4⁺ T cell subpopulations were identified by their expression of specific transcription factors (using intracellular cell staining). The expression of additional cell surface markers (or cytokine production) by total CD4⁺ T cell, or specific CD4⁺ T cell subpopulations, was assessed using isotype controls or FMO (Fluorescence Minus One) controls with the same gating for all conditions. As an illustrating example, IL-17-producing CD4⁺ T cells were chosen.

Flow cytometry analyses. Just before acquisition, 10-20 μL counting beads (Molecular Probes™ CountBright™ Absolute Counting Beads #11570066) were added to the cell suspension. Staining were acquired with a LSRII or Fortessa flow cytometer (BD Biosciences), and analyzed with the FlowJo V10 software. As described in [Figure 32](#), doublets, dead cells, and blood cells (excepted in some experiments, as indicated in figure legends) were excluded. CD4^+ T cells were selected based on their expression of TCR- β (or CD3) and CD4. Different CD4^+ T cell subpopulations were then identified based on their intracellular expression of specific transcription factors: T-bet for Th1 cells, ROR γt for Th17, Foxp3 for Treg, and both ROR γt and Foxp3 for ROR γt^+ Treg. The presence of these CD4^+ T cell subpopulations was assessed as a percentage of the total CD4^+ T cell gate, and as an absolute number. Their characterization was then based on expression of extracellular markers and transcription factors (in non-stimulated cells), as well as cytokines (in stimulated cells).

To determine the absolute cell number, number of events in the “Beads” and “Lymphocytes” gates were extracted, as well as frequencies of each population, among the “Lymphocytes” gate. Cell number in the “Lymphocytes” gate was calculated as:

$$\text{Lymphocytes cell number} = \frac{\text{nb of events in the "Lymphocytes" gate}}{\text{nb of events in the "Beads" gate}} \times \text{Bead concentration}$$

This number was adjusted, according to the proportion of lung cell suspension dedicated to this analysis, to obtain the “Total lymphocytes cell number” in the whole lung. It was then used to calculate the total number of cell within all cell populations from the same mice as:

$$\text{Population cell number} = \frac{\text{Total lymphocytes cell number} \times \text{Population frequency}}{100}$$

c) *M. tuberculosis* burden in lung and spleen

A fraction of the lung and spleen cell suspension was used to evaluate *M. tuberculosis* bacterial load. To this end, CFU assay was performed. Briefly, cell suspension was serially diluted in PBS, and spread on 7H10 Agar medium supplemented with peptone, OADC at 10%, Glycerol at 0.5%, and incubated at 37°C. Three weeks later CFU were counted, and global lung or spleen burden estimated for each mouse.

d) Cytokine concentration measurement

Supernatants from lung homogenates were 0.2 μm filtered twice (using Millex® filtration units, Merck Millipore #GPSLGPX13NK) to ensure exclusion of *M. tuberculosis*, and kept samples at -80°C. IL-6, TNF, IFN- γ , and IL-10 were dosed by ELISA using BD OptEIA™ Sets (#555240, 555268, 555138, 555252).

e) RT-qPCR

A part of lung cell suspension was used to analyze gene profile RNA expression. To this end, cell pellets were resuspended in TRIzol™ reagent (Invitrogen™ #15596018), and samples were stored at -80°C for at least 48 h (a procedure that also inactivates *M. tuberculosis*). RNeasy spin columns (RNeasy mini kit, Qiaen #74106) were then used according to the manufacturer’s instructions to extract RNA. RNA was then reverse-transcribed into cDNA using M-MLV Reverse transcriptase (Invitrogen™ 28025013). RT-qPCR reactions were performed using gene-targeted primers (Table 5), and a 7,500 RT-PCR System. Data were analyzed using the 7,500 Software version v2.3 (Applied Biosystems). Values were normalized using the β -actin housekeeping gene, and expressed as a fold change between experimental samples (*L. murinus*-treated mice) relative to control samples (PBS-treated mice).

Table 5: List of primers used for qPCR (related to Figure 42).

Genes	Primers	
<i>il-17A</i>	Forward primer (5'-3')	CATGGGATTACAACATCACTCGA
	Reverse primer (5'-3')	TGCTGAATGGCGACGGA
<i>il-10</i>	Mm_Il10_1_SG QuantiTect Primer Assay (Qiagen #GT00106169)	

il-17A and *il-10* encode for the cytokines IL-17A and IL-10 respectively.

III. Statistical analyses

Statistical analyses were performed using the software GraphPad Prism 7. In each graph, dots represent individual mice; a minimum of four mice per group were used in each experiment, repeated independently at least twice. Results from either one representative experiment or pooled experiments are presented as indicated in each figure legend. When samples followed a normal distribution, the mean value for each group was represented by the bar, and Student's t-test (when only two groups were compared), or One-Way ANOVA followed by Holm-Sidak's post-test (to compare more groups) were applied to compare each treated group to the PBS-control mice. When it was not the case (in particular when too few mice were compared), the bar represents the median of each group, and Mann-Whitney test or Kruskal-Wallis followed by Dunn's post-test were applied. A significant difference was represented by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$. $P < 0.2$ were indicated by their values.

Table 6: Description of the 20 strains isolated from the pulmonary microbiota (from Remot, ISME, 2017)

	Family	species	Gram	Catalase	Methode of identification	significant ex vivo effect (co-culture with GF lung explants, PCLS)		significant in vivo effect (HDM model)
						increase	decrease	
1		<i>sciuri</i> (CNCM I 4970)	+	+	16S Sequencing, API Staph, Mass spectrometry	IL-5, IL-10, IL-12p70, IL-17a, TSLP	-	exacerbative
2		<i>aureus</i>	+	+	16S Sequencing, API Staph	Non significant	-	
3		<i>lugdunensis</i>	+	+	16S Sequencing, API Staph	IL-4, IL-17a, TSLP	-	
4	Staphylococcaceae	<i>haemolyticus</i>	+	+	16S Sequencing, API Staph	Non significant	-	
5		<i>xylosus</i>	+	+	16S Sequencing, API Staph	Non significant	-	
6		<i>epidermidis</i>	+	+	16S Sequencing, API Staph	Non significant	-	
7		<i>pasteuri</i>	+	+	16S Sequencing	Non significant	-	
8		<i>lentus</i>	+	+	16S Sequencing	Non significant	-	
9		<i>dysgalactiae</i>	+	-	16S Sequencing, API20Strep	Non significant	-	
10	Streptococcaceae	<i>salivarius</i>	+	-	16S Sequencing, API20Strep	Non significant	-	
11		<i>mitis</i> / spp	+	-	16S Sequencing	Non significant	-	
12		<i>faecalis</i> (CNCM I 4969)	+	-	16S Sequencing, API20Strep, Mass spectrometry	IL-12p70	TSLP	protective
13	Enterococcaceae	spp/ <i>haemolyticus</i>	+	-	16S Sequencing	Non significant	-	
14		<i>rhamnosus</i> /spp (CNCM I 4967)	+	-	16S Sequencing, Mass spectrometry	IL-10, IL-12p70, IFNg	-	no effect
15	Lactobacillaceae	<i>salivarius</i> /spp (CNCM I 4968)	+	-	16S Sequencing, Mass spectrometry	IL-4, IL-5, IL-12p70, IL-17a, IFNg	-	
16		<i>animalis</i> /spp	+	-	16S Sequencing	Non significant	-	
17	Clostridiaceae	<i>Clostridium</i> / spp	+	-	16S Sequencing	Non significant	-	
18	Enterobacteriaceae	<i>E. coli</i>	-	+	16S Sequencing, API20E	IL-17a, IFNg	-	
19		<i>Proteus mirabilis</i>	-	+	16S Sequencing, API20E	IL-6, TNFa, IFNg, TSLP	-	
20	Listeriaceae	<i>Listeria</i> spp	-	+	16S Sequencing	Non significant	-	

Results

I. Pulmonary commensal lactobacilli modulate the local immune system in naïve and *M. tuberculosis* infected mice

1. Previous characterization of the pulmonary commensal bacteria bank

At the beginning of this PhD, our collaborators from the MICALIS institute have been able to isolate 20 bacterial strains from neonatal SPF mice lungs, containing members of the families *Staphylococcaceae*, *Streptococcaceae*, *Enterococcaceae*, *Listeriaceae*, *Lactobacillaceae*, *Clostridiaceae* and *Enterobacteriaceae* (as listed in [Table 6](#)). They next investigated if some of these strains were able to influence asthma outcome. To this end, they first selected promising candidates using an *ex-vivo* screening based on co-culture of each bacterial strain with mouse lung explants (200 µm lung slices obtained from germ-free mice lungs and viable in culture). In particular, the *Enterococcus faecalis* CNCM I-4969 strain was selected based on its unique ability to decrease the secretion by the lung explants of Thymic stromal lymphopoietin (TSLP), a cytokine driving asthma by orienting T cell maturation by APC towards a Th2 profile ([Table 1](#) and (Remot *et al.*, 2017)). By contrast, *S. sciuri* CNCM I-4970 was selected for its strong induction of pro-inflammatory cytokine secretion, including TSLP, IL-12, IL-17 or IL-5. Interestingly, when administered intranasally (*i.n.*) to neonates before and after asthma induction, *E. faecalis* reduced weight loss, eosinophil infiltration, IL-5 secretion and epithelium thickening, whereas *S. sciuri* increased weight loss and did not modify the other parameters ([Figure 33](#) and (Remot *et al.*, 2017)). These data demonstrated that the bank of strains used in our study contains commensals with the potential to modulate respiratory disease severity.

2. Rationale for candidate selection

When my PhD project started in October 2015, it was not known yet if the intranasal administration of the MICALIS pulmonary bacterial strains could modify the lung immune system. Results from the co-culture with mouse lung explants led me to hypothesize that these pulmonary bacteria have immunomodulatory properties that could be strain dependent, as it is the case for commensal strains from other origin (Youn *et al.*, 2012; Tomosada *et al.*, 2013). I chose to test this hypothesis in SPF mice harboring a complex microbiota (rather than in mono-colonized mice, for example) to identify strains with dominant effects. Indeed, I inferred that if a strain's immunomodulatory effect could be negated by the effect of the microbiota, it would then have poor probability to improve the tuberculosis treatment if given as a probiotic. Another parameter I kept in mind is the fact that the study of *M. tuberculosis* infection is long, constrainable and expensive. Therefore, I decided to first assess the impact of isolated pulmonary commensal strains in non-infected mice, which was previously a successful approach achieved in the context of skin probiotic identification by the team of Yasmine Belkaid (Naik *et al.*, 2015).

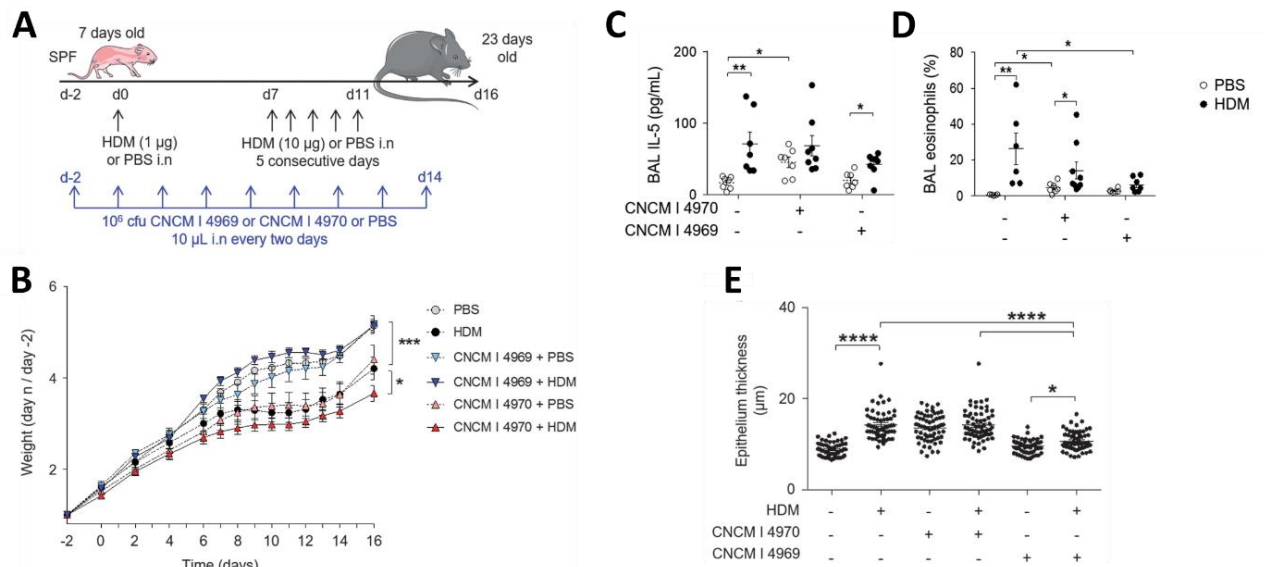


Figure 33: Characterization of strains from the pulmonary microbiota bank in an asthma model (adapted from Remot, ISME, 2017). **A:** Experimental design: 5-day-old C57BL/6 pups received intranasally 10⁶ bacteria (*Enterococcus faecalis* CNCM I-4969 or *Staphylococcus sciuri* CNCM I-4970) (or PBS) every two days before and after induction of house dust mite allergic asthma. **B:** Growth curves are expressed as the mean \pm s.e.m. of individual weights (normalized to the initial weight on two days before infection, day -2) for $n \geq 5$ mice per group and represent one of three independent experiments ($n=5-10$ mice per group). Tukey's multiple comparison test, repeated measures One-Way ANOVA was used for comparison of the growth curves. **C:** Bronchoalveolar lavage (BAL) IL-5 levels were measured by enzyme-linked immunosorbent assay (ELISA). **D:** BAL cells were enumerated, cytocentrifuged and stained with May-Gründwald-Giemsa. Eosinophils were enumerated and expressed as the percentage of total BAL cells. Data in C and D are shown individually and as the mean \pm s.e.m. All data represent one of three independent experiments ($n=5-10$ mice per group). **E:** Murine lung sections were stained with hematoxylin-eosin-saffron, or periodic acid-Schiff and alcian blue. Epithelium thickness was measured using Case Viewer software. In total, 100 measures per group are plotted on the graph. They correspond to the analysis of four mice per group. Five representative bronchi were selected for each mouse and four measurements were performed per bronchus (up, down, left, right side). All data represent one of two independent experiments ($n=5-10$ mice per group).

A final parameter was to prioritize the safer class I bacteria present in our bank, in view of future application to humans. Indeed, as discussed in Section 1.1 of the introduction, the microbiota (and our bacterial bank), is not only composed of safe symbiotic bacteria, but also of pathobionts, such as *Staphylococcus aureus*, which has the potential to cause disease if over-represented (Brugger, Bomar and Lemon, 2016).

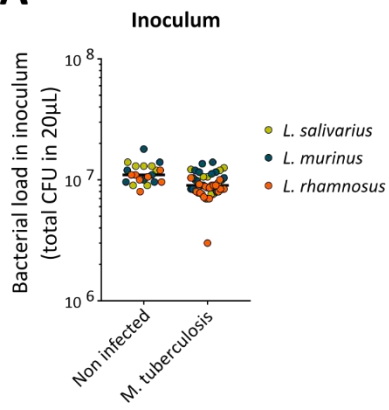
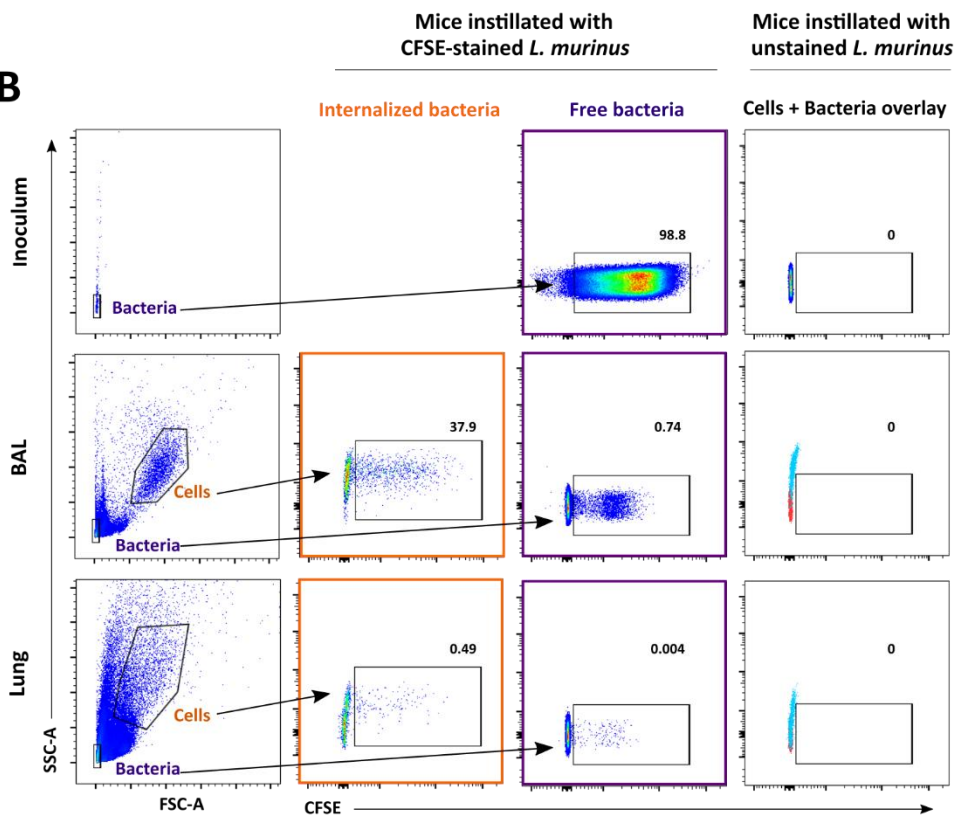
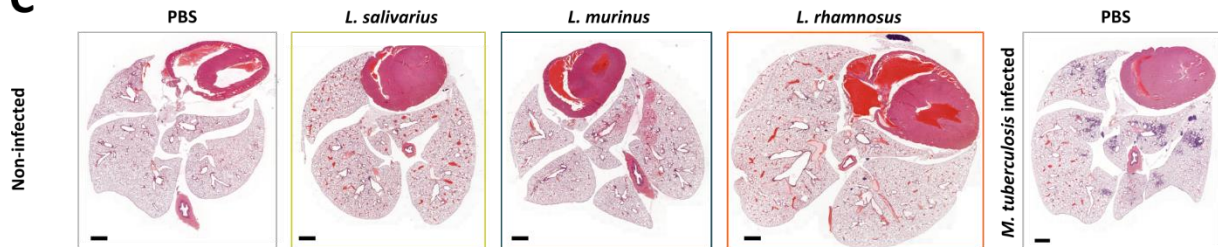
Among the 20 bacterial strains isolated from the pulmonary microbiota, six were safer class I strain listed in red in Table 6 and detailed in the Table 2. Among these, I first focused on the three *Lactobacillus species* having a GRAS status and easy to grow in culture in order to set up the bacterial assessment model. From here on, the results with these three strains are presented.

3. Set-up of an *in vivo* model to study pulmonary commensal bacteria influence on local immunity

As a first step, I set-up a model to administer the pulmonary commensal bacteria *i.n.*, adapted from classic gavage protocols (Kechaou *et al.*, 2013). I first cultured the lactobacilli and monitored their growth to determine their characteristics, such as generation time and a concordance factor between optical density (OD) and bacterial concentration, as detailed in the Material and Methods chapter (Section I.1). The OD/CFU concordance factor for each strain was used to prepare bacterial suspensions in PBS at the required density. Classic protocols used to identify gut probiotic bacteria use about 10^9 CFU per mouse every day for 10 days for bacterial administration by gavage. Since anesthesia is needed for *i.n.* administration, I chose an every two day administration protocol to limit the animal suffering, as described in other studies (Pellaton *et al.*, 2012; Remot *et al.*, 2017). Due to too high inoculum viscosity, I could not reproduce the *i.n.* administration of 10^9 (or even 10^8) bacteria to the mice that was described elsewhere (Pellaton *et al.*, 2012; Park *et al.*, 2013). I opted eventually for an inoculum containing 10^7 bacteria that was easily up taken by the mice; this is one log higher than the dose adopted by our collaborators in their recent publication (Remot *et al.*, 2017). Each inoculum was verified by counting CFU on agar-medium (Figure 34A).

I also verified that the *i.n.* administration resulted in delivery of bacteria to the lungs. To do so, a portion of the *L. murinus* inoculum containing 10^7 bacteria per mouse was stained with the fluorescent dye CFSE⁵⁴. Mice received either stained or unstained bacteria *i.n.* 2 hours before sacrifice. Broncho-alveolar lavage fluid (BALF) and lungs were recovered from each mouse and analyzed by flow cytometry using adapted threshold for FSC and SSC settings to detect and separate bacteria from cells (Figure 34B). In BALF, CFSE-labelled bacteria were detected both in the gate with low FSC and low SSC corresponding to free bacteria, and in the “Cells” gate that correspond to bacteria phagocytosed by alveolar macrophages. This detection was not due to cell auto-fluorescence, as we were not able to observe any positive events in the two gates from mice receiving unstained bacteria.

⁵⁴ Carboxyfluorescein succinimidyl ester (CFSE) is a dye that can penetrate inside living cells (or bacteria) and covalently bind intracellular components, which allow a long-term staining of cells. It will be diluted by a two fold factor any time the stained cells divide. Dilution of the staining in a cellular population can be visualized by FACS allowing to follow cell proliferation.

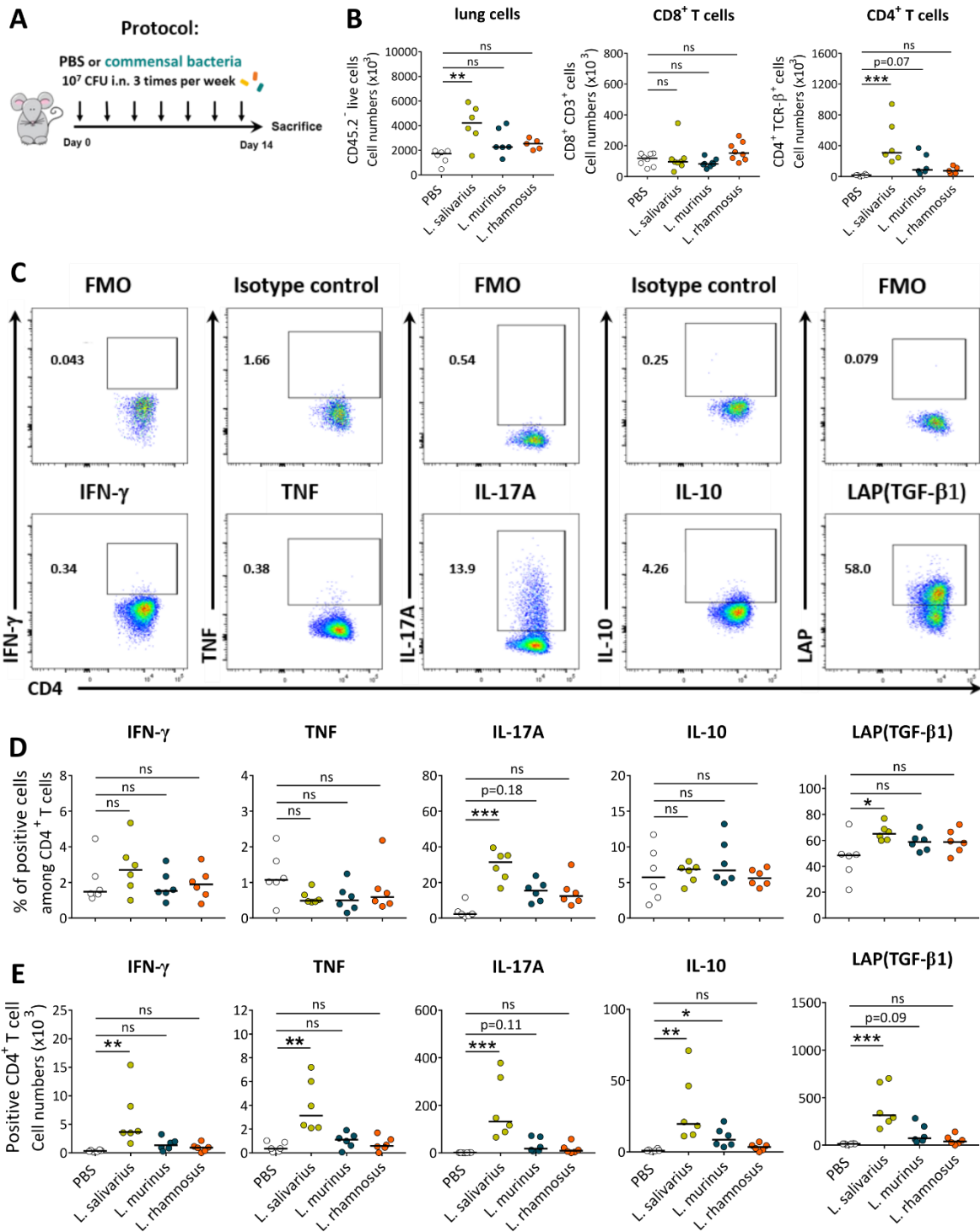
A**B****C**

Detection of positive events in lung homogenates was more difficult given that there is a higher frequency of cellular debris from this sample, and thus fewer events of interest were recorded. Yet, it seems that positive bacteria (that may come from the alveolar space) were also present in this organ. Therefore, I validated that *i.n.* instillation of 10^7 bacteria in 20 μL of PBS under isoflurane anesthesia is an appropriate protocol to deliver bacteria in the lungs.

Concerning the readout for immunomodulation by my pulmonary bacterial strain candidates, I chose the T cell compartment. Indeed, lymphocytes are less plastic than myeloid cells, requiring a strong stimulus with a lasting effect. As previously mentioned, a similar strategy was used by the team of Yasmine Belkaid to identify skin probiotics interacting with myeloid cells that subsequently primed protective CD8^+ T cell response (Naik, Nature, 2015). To allow the priming of T cells in draining lymph nodes and their recruitment to the lungs, I decided to perform the delivery of bacteria during 15 days before the end point. To verify that bacterial instillations are not harmful to animals, I first performed a kinetic analysis for body temperature and weight; there were no differences between the experimental and control groups (even in long-term experiments) (data not shown).

I then confirmed that the repeated administration of the lactobacilli does not induce lung inflammation, as measured by histological analyses. As shown in [Figure 34C](#), there is no apparent leukocyte infiltration (noticeable by purple coloration by hematoxylin and eosin staining) in any mice having received the commensal bacteria as compared to that from *M. tuberculosis* infected mice. Therefore, I concluded that the *i.n.* delivery of the pulmonary commensal bacterial strains is well tolerated.

Figure 34: Characterization of the lung commensal *Lactobacillus* strains and set-up of their intranasal administrations. Inocula of each strain were prepared from fresh cultures in exponential phase. 10^7 CFU are administered intranasally three times a week to SPF C57BL/6 mice for 15 days, which are then sacrificed (“Non-infected”) or infected with *M. tuberculosis*. For infected mice, lung commensal bacteria are again intranasally delivered two times a week following infection, and the mice are sacrificed at day 42 post-infection. After each instillation, the bacterial load is verified by spreading the inoculum on solid medium and colony scoring. **A:** As examples, the bacterial load within 20 μL (estimated by colony scoring) from each inoculum used for a whole experiment in the non-infected mice (left) and in the *M. tuberculosis* infected mice (right) models, comparing PBS to: *Lactobacillus salivarius* (in yellow), *Lactobacillus murinus* (in blue) and *Lactobacillus rhamnosus* (in orange). Each dot represents an individual inoculum. **B:** To assess delivery of *L. murinus* to the lung after intranasal instillations, the *L. murinus* inoculum was stained with CFSE (or kept unstained). Inoculum, bronchoalveolar lavage (BAL) or total lungs, from mice having received CFSE-labelled or unstained *L. murinus* intranasally 2 h before sacrifice were analyzed by flow cytometry. Free bacteria and internalized bacteria were selected with FSC and SSC parameter values corresponding to size and granularity of bacteria or myeloid cells, respectively (left). CFSE staining was observed in both gates for mice having received stained *L. murinus* (middle panels) compared to those animals inoculated with unstained *L. murinus* (overlaid plots on the right). Representative plots of two independent experiments with one mouse per group are presented. **C:** In *M. tuberculosis* infected mice, leukocyte infiltration (purple area) was analyzed in pulmonary tissues at day 42 post-infection by histological hematoxylin and eosin staining (HE). Same analysis was performed in non-infected mice having received each *Lactobacillus* strain (individually) for 15 days *i.n.* in order to assess inflammatory status of lung tissue. A representative picture for each condition is shown. The black bars correspond to 5 mm.

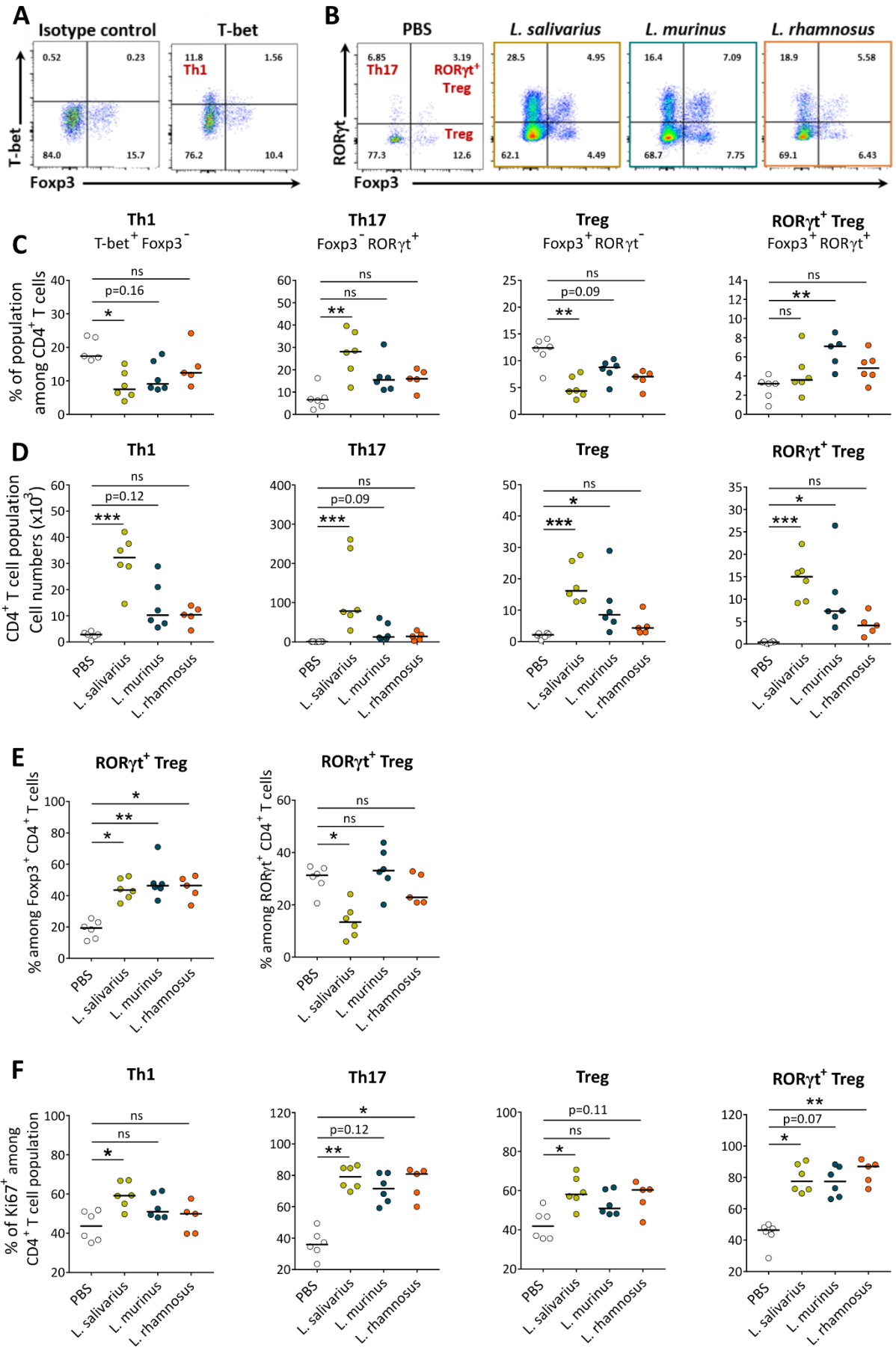


4. Pulmonary commensal lactobacilli modulate the CD4⁺ T cell lung compartment in naïve mice

For the first set of experiments, naïve mice received *i.n.* either PBS, *L. salivarius*, *L. murinus* or *L. rhamnosus*, every other day during 15 days (Figure 35A). Before sacrifice, mice were administered *i.v.* with an anti-CD45.2 antibody to exclude contaminating blood cells as described in (Patel *et al.*, 2015). With the exception of *L. salivarius*, which increased the number of CD4⁺ T cells, administration of the different bacteria did not change the total number of lung T cells (Figure 35B). It was previously described that some probiotic strains that do not modify the global T cell number, mediate their protective effect by changing lymphocyte phenotype or by changing equilibrium between pro- and anti-inflammatory subpopulations (H. W. Kim *et al.*, 2018). Thus, I characterized the CD4⁺ T cells more in details. To assess global changes in cytokine production, I performed intracellular cell staining of CD4⁺ T cells from lung homogenates stimulated with PMA and ionomycin (Figure 35C). As shown in Figure 35D-E, *L. salivarius* elicited a massive immunomodulatory effect in the CD4⁺ T cell compartment reflected in the total number of cells producing both pro-inflammatory (IL-17A, TNF and IFN- γ) and anti-inflammatory (TGF- β ⁵⁵ and IL-10) cytokines. By contrast, *L. murinus* showed a slight tendency to increase IL-17A, while *L. rhamnosus* did not modify cytokine production (Figure 35D-E).

Figure 35: Intranasal administration of pulmonary commensal *Lactobacillus* strains modulates the lung CD4⁺ T cell cytokine production in naïve mice. **A:** Experimental design: C57BL/6 naïve mice received intranasally 10⁷ CFU of murine pulmonary commensal bacteria (*L. salivarius* (yellow), *L. murinus* (blue), *L. rhamnosus* (orange)) in 20 μ L PBS or mock (white) three times per week during two weeks before sacrifice. **B:** Vertical scatter plots show total number of live lung cells (left), lung CD8⁺ T cells (middle) and lung CD4⁺ T cells (right), as quantified by flow-cytometry in lung homogenates. Blood-circulating and dead cells were excluded from all analyses. **C-E:** Part of the lung homogenates was stimulated for 4 h with PMA and ionomycin in presence of Brefeldin A to visualize intracellular cytokine production. Dot plots show the gating strategy used to select cytokine secreting lung CD4⁺ T cells (gated on CD4⁺CD3⁺ cells) based on intracellular cell staining, and using isotype or Fluorescence Minus One (FMO) controls (C). Vertical scatter plots indicate the frequencies (D) and total number (E) of cells producing IFN- γ , TNF, IL-17A, IL-10, or TGF- β 1. LAP (Latency Associated Protein) is the membrane-bound form of TGF- β . Each circle represents an individual mouse, while the black bar line is the median of each group. Experimental groups were compared to control within the same experiment using a Kruskal-Wallis test and Dunn's post-test, *P < 0.05; **P < 0.01; ***P < 0.001; a representative experiment is shown from two independent experiments.

⁵⁵ **TGF- β 1** production was assessed in all experiments indirectly by the detection of **LAP** (Latent Associated Peptide), with which it forms a propeptide that can be expressed at cell membrane or be clived to release the biologically active form of TGF- β 1.



To continue with the characterization of CD4⁺ T cells, I use additional markers to discriminate different CD4⁺ T cell populations. I focused on Th1, Th17 and Treg subsets, which are crucially involved in the immune response to tuberculosis (unlike the Th2 subset) and often modulated by probiotic administrations as described in the introduction. These subsets were identified through the expression of characteristic transcription factors: T-bet for Th1, ROR γ t for Th17, and Foxp3 for Treg (as shown in [Figure 36A, B](#)). While at first, I had considered all ROR γ t⁺ CD4⁺ T cells as Th17, and all Foxp3⁺ CD4⁺ T cells as Treg, I recently realized by representing CD4⁺ T cells in a ROR γ t *versus* Foxp3 dot plot that a fraction of these populations was expressing both transcription factors, in particular after commensal administration ([Figure 36B](#)).

As detailed in the introduction (Section 1.II.3), commensal bacteria have been shown to induce such double positive cells often named ROR γ t⁺ Treg in the gut that have Treg functions, (Sefik *et al.*, 2015; Yang *et al.*, 2016). In the lungs, there is one study that report the presence of this population (Lochner *et al.*, 2008), but until now, its modulation by the microbiota in this organ has never been addressed. Since the generation of gut ROR γ t⁺ Treg is dependent on the presence of local microbiota (Ivanov *et al.*, 2009; Atarashi *et al.*, 2011; Sefik *et al.*, 2015), I decided to pay special attention to this subset of Treg along with Th1, Th17 and classical Treg. Therefore, I went back to my previous flow cytometry data and reanalyze them considering this cell population (if the antibody panel allowed me to do so). In this aim, the term “Th17” used in this section includes CD4⁺ T cells expressing ROR γ t but not Foxp3, “Treg” refers to CD4⁺ T cells expressing Foxp3 but not ROR γ t, and “ROR γ t⁺ Treg” alluding to the double positive population ([Figure 36B](#)).

Figure 36: Intranasal administration of pulmonary commensal *Lactobacillus* strains modulates the lung CD4⁺ T cell populations in naïve mice. C57BL/6 naïve mice received intranasally 10⁷ CFU of murine pulmonary commensal bacteria (*Lactobacillus salivarius* (yellow), *Lactobacillus murinus* (blue), *Lactobacillus rhamnosus* (orange)) in 20 μ L of PBS or mock (PBS alone, white) three times per week during two weeks before sacrifice. Lung CD4⁺ T cells (expressing CD4 and TCR- β) were analyzed by flow cytometry after exclusion of dead and blood-circulating cells. **A, B:** Dot plots illustrate the gating strategy for lung CD4⁺ T cell subpopulation selection based on the intracellular staining of specific transcription factor expression: T-helper 1 (Th1), expressing T-bet; Th17, expressing ROR γ t; regulatory T cell (Treg), expressing Foxp3; and ROR γ t⁺ Treg, expressing ROR γ t and Foxp3. Positivity for T-bet was determined using an isotype control (A). As an example, representative dot plots for expression of ROR γ t and Foxp3 in each experimental group are presented (B). **C, D:** Vertical scatter plots indicate the corresponding percent frequencies (C) and absolute number (D) of the indicated lung CD4⁺ T cell subpopulations, according to specific bacterial (or mock) administration. **E:** Vertical scatter plots display the percentage of ROR γ t⁺ Treg frequency among total Foxp3⁺ (left) or ROR γ t⁺ (right) CD4⁺ T cells. **F:** Vertical scatter plots indicate the frequency of Ki67 positive cells among CD4⁺ T cell sub-populations. Each circle represents an individual mouse, while the black bar line is the median of each group. Experimental groups were compared to control within the same experiment; a representative experiment is shown from two independent experiments. A Kruskal-Wallis test and Dunn’s post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01; ***P < 0.001.

After administration in naïve mice, I observed that the three lactobacilli modulate CD4⁺ T cell populations. They all tended to induce a lower proportion of Th1 and conventional Treg, and an increased level of Th17 and RORγt⁺ Treg (Figure 36C). In the case of *L. salivarius*, there was a significant increase in the total cell number of all populations, mainly distinguished by the massive presence of Th17 (Figure 36D). *L. murinus* displayed a similar (albeit lower) tendency to induce all populations of CD4⁺ T cell in terms of cell numbers, with a significant effect on RORγt⁺ Treg (Figure 36D). By contrast, the immunomodulatory effect by *L. rhamnosus* was really low, characterized by small tendencies with no statistical significance (Figure 36C-D). RORγt⁺ Treg represented a high proportion of total Foxp3⁺ CD4⁺ T cells in the lungs, going from 20% in control mice to 40% in lactobacilli-treated mice (Figure 36E, left).

Of note, they were estimated to represent 40% in the colonic lamina propria in SPF mice by Sefik and colleagues (Sefik *et al.*, 2015), ranging from 15% to 80% in mice harboring minimal to complex microbiota (Yang *et al.*, 2016). In addition, we noticed that RORγt⁺ Treg represented an important proportion of lung RORγt⁺ CD4⁺ T cells, ranging up to 30% in all conditions except for the *L. salivarius*-treated mice where Th17 were massively augmented (Figure 36E, right). This is quite in accordance with the previous study reporting that RORγt⁺ Treg represented about 20% of total RORγt⁺ CD4⁺ T cells in the lungs (Lochner *et al.*, 2008). Moreover, I observed that the increase in CD4⁺ T cell populations seemed to be partly mediated by cell proliferation, as most lymphocytes expressed the proliferation marker Ki67 in lactobacilli-treated mice (Figure 36F). In particular, lung Th17 cells and RORγt⁺ Treg seemed highly proliferative in mice treated with all the lactobacilli strains, suggesting an important role for lung microbiota in the generation of these subsets.

This first set of experiments gave rise to different questions for my thesis project, including:

(i) Is the modulation of the lung CD4⁺ T cell compartment by lung *Lactobacillus* strains conserved during tuberculosis infection? If so, would these changes alter the disease outcome? These questions are addressed in subsection I.5.

(ii) Is the induction of some CD4⁺ T cell subsets associated with modulation of their phenotype and functions? This aspect is described in subsection III.1.

(iii) Are there additional lung commensals (other than *Lactobacillus species*) inducing modulations of the lung T cell compartment? This is assessed in subsection III.3.

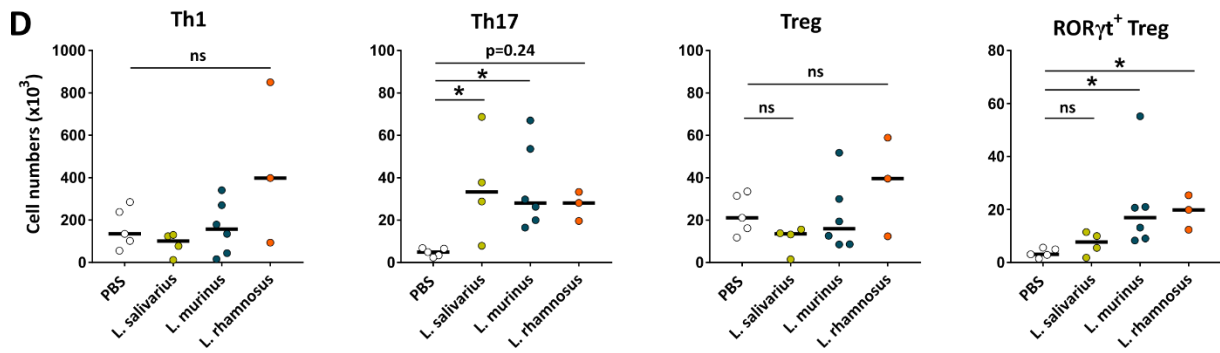
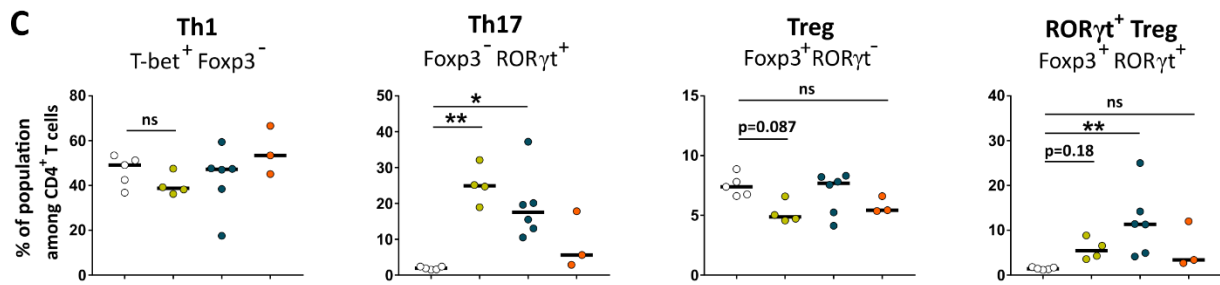
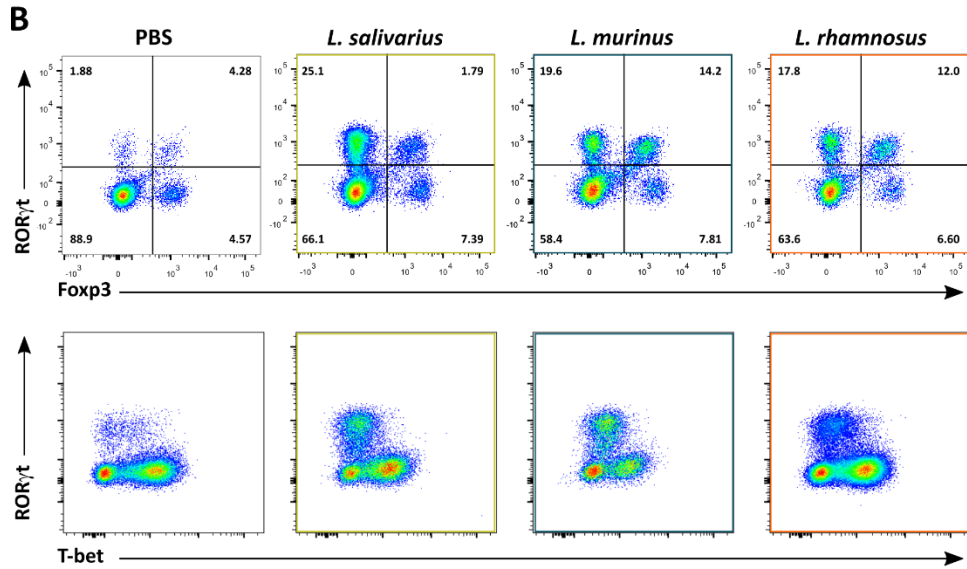
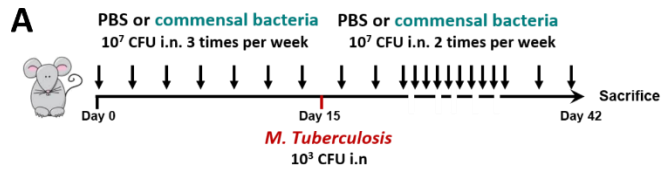
5. *L. murinus* administration decreases lung inflammation associated to *M. tuberculosis* infection associated with an increase in lung Th17 and ROR γ ⁺ Treg

To test the probiotic effect of pulmonary commensal lactobacilli in the context of tuberculosis, I adapted the administration protocol of commensals to the mouse model of *M. tuberculosis* infection previously used in our team and others (Troegeler *et al.*, 2017; Dumas, Corral, *et al.*, 2018). In this model that I define here as the “prophylactic” administration mode, mice received 10⁷ CFU of the *Lactobacillus* strains *i.n.* three times per week for 15 days, and were then infected (*i.n.*) with 1000 CFU of the H37Rv strain of *M. tuberculosis*. Thereafter, *i.n.* delivery of lactobacilli continued two times per week until end point at day 42 post-infection (Figure 37A).

As in the non-infected mice, all *Lactobacillus* strains displayed a tendency to increase Th17 and ROR γ ⁺ Treg. I observed again that *L. salivarius* and *L. murinus* were the strongest inducers of Th17 and ROR γ ⁺ Treg, respectively (Figure 37B-E). Of note, neither Th1 cells nor conventional Treg were significantly modulated by the lactobacilli administration in this model. As discussed in the introduction, Th17 role is not fully understood in tuberculosis, but they are certainly involved in tuberculosis immunopathology. Some studies in patients suggest that pathogenic Th17 are in fact Th1/Th17 cells; ROR γ ⁺ T-bet⁺ poly-functional cells producing both IL-17 and IFN- γ (Lyadova and Panteleev, 2015). In my hands, even though some ROR γ ⁺ cells slightly expressed T-bet in the control group, the ROR γ ⁺ population that increased with lactobacilli administration did not express this transcription factor (Figure 37B, bottom). This suggests a different role for the Th17 population induced by our strains, which is discussed in the Discussion section.

To assess if the immunomodulation induced by the pulmonary lactobacilli could affect the disease outcome, we looked at the pathogen burden and lung integrity at a late time point (42 days post-infection). The lactobacilli delivery to the lung neither modify *M. tuberculosis* loads in the lungs, nor its dissemination to the spleen (Figure 37F). This suggests that our *Lactobacillus* strains do not influence mechanisms involved in the control of this pathogen, and I infer that the increase in ROR γ ⁺ Treg does not inhibit the protective anti-bacterial immunity. While histological examination of the lungs did not reveal massive leukocyte infiltration in all animal groups, quantification of the occupied area by leukocyte infiltrates revealed that administration of *L. murinus* resulted in a striking reduction of this inflammatory parameter (Figure 37G). By contrast, while administration of *L. salivarius* followed the same decreasing trend (albeit not significant), *L. rhamnosus* failed to exert any effect as compared to the mock-treated group.

Based on these results, I decided to focus uniquely on *L. murinus* and to further characterize its effects in the context of tuberculosis.



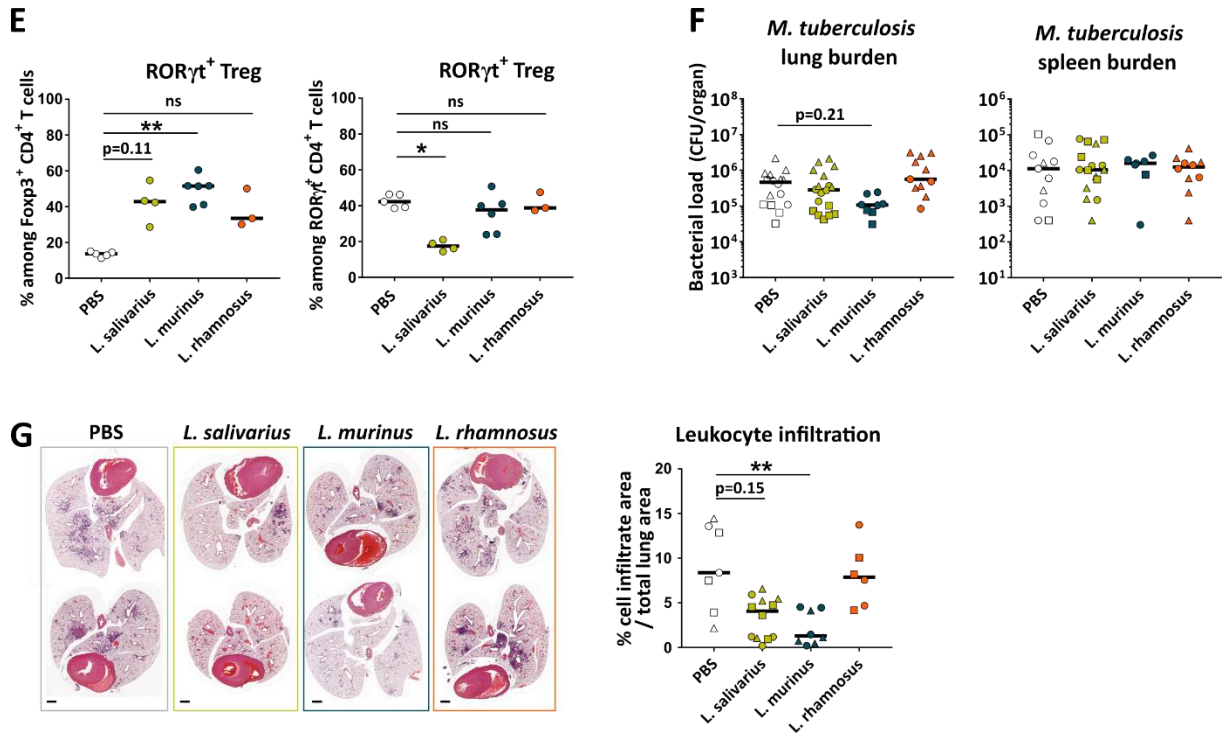
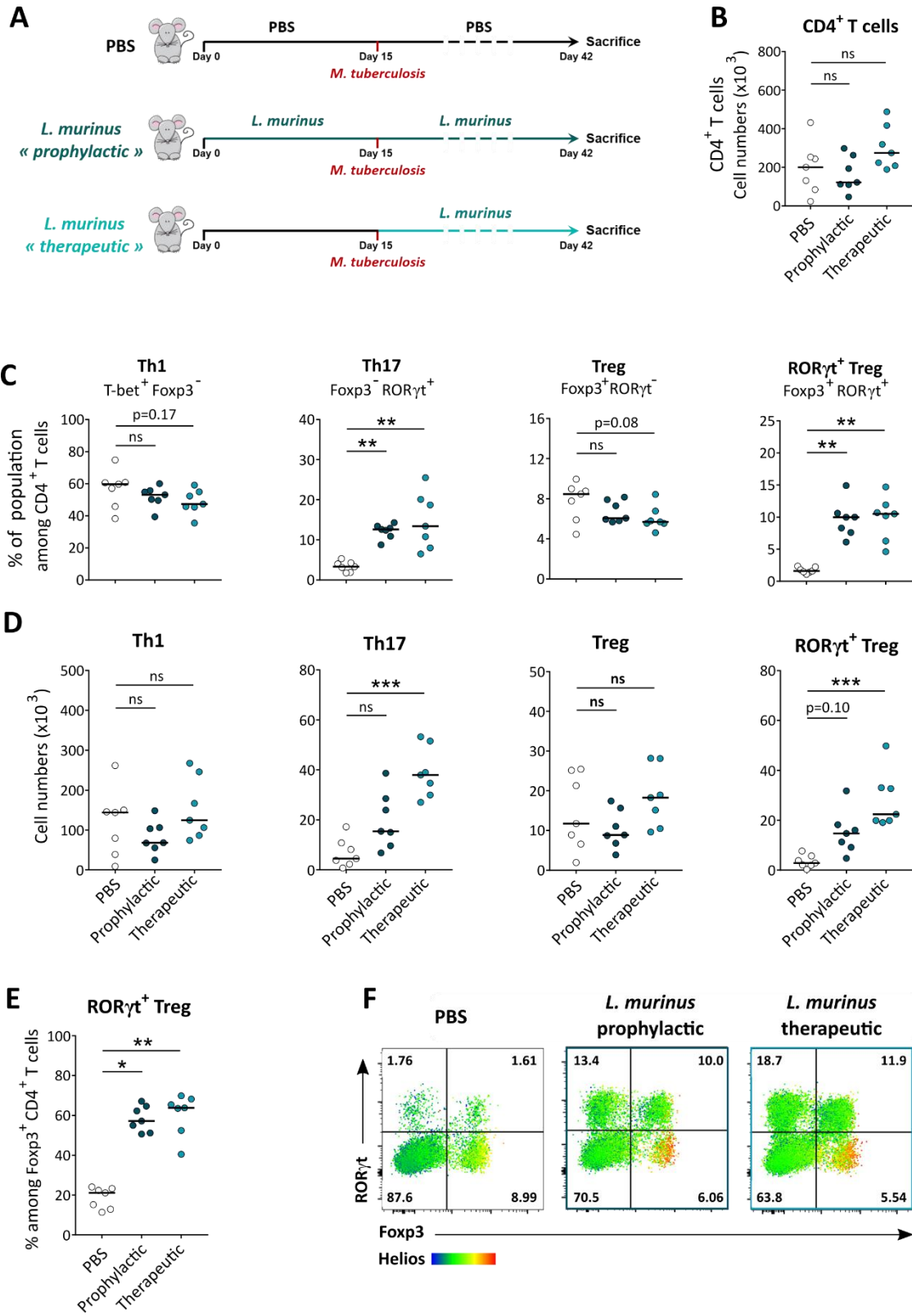


Figure 37: Prophylactic administration of *L. murinus* decreases lung inflammation associated to *M. tuberculosis* infection associated with an increase in lung Th17 and ROR γ t⁺ Treg. **A:** Experimental design. C57BL/6 naïve mice received intranasally (*i.n.*) 10⁷ CFU of murine pulmonary commensal bacteria (*Lactobacillus salivarius* (yellow), *Lactobacillus murinus* (blue), *Lactobacillus rhamnosus* (orange)) in 20 μ L of PBS or mock (PBS alone, white) three times per week during two weeks prior to *i.n.* infection with 10³ CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice a week with each lactobacilli strain or mock. At day 42 post-infection, mice were sacrificed for analyses. **B:** Representative dot plots for expression of ROR γ t and Foxp3 (top), or ROR γ t and T-bet (bottom), among CD4⁺ T cell in each experimental group. Dead and blood-circulating cells were excluded from the analysis before CD4⁺ T cell (CD4⁺ CD3⁺) selection. **C, D:** Vertical scatter plots display the percent frequencies (C) and absolute number (D) of the indicated lung CD4⁺ T cell subpopulations, according to the commensal (or mock) administration. **E:** Vertical scatter plot show the ROR γ t⁺ Treg frequency among total Foxp3⁺ CD4⁺ T cell (top) or total ROR γ t⁺ CD4⁺ T cell (bottom). **F:** Vertical scatter plots show the loads of *M. tuberculosis* in lung (left) or spleen (right) homogenates day 42 post-infection. **G:** Leukocyte infiltration detected by histological hematoxylin and eosin staining (left), and quantified as illustrated in vertical plot (right) in pulmonary tissue. On the left panel, the black bars on pictures correspond to 5 mm. **C-G:** Each circle represents an individual mouse. In C-E, only one experiment was performed. In F-G, three independent experiments are pooled (each experiment is represented by a different symbol). The black bar line represents the median of each group. A Kruskal-Wallis test and Dunn's post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01; ***P < 0.001.



II. Assessment of *L. murinus* potential as a probiotic candidate for tuberculosis

1. Prophylactic and therapeutic administration of *L. murinus* increase lung Th17 and ROR γ ⁺Treg during *M. tuberculosis* infection

To assess the potential of *L. murinus* as a microbiota-based therapy in *M. tuberculosis* infection, mice received the lactobacillus administration only after infection, which I termed as “*therapeutic*” mode, or as previously in a “*prophylactic*” mode (Figure 38A). Similar to the *prophylactic* administration, the *therapeutic* mode does not significantly change the total number of lung CD4⁺ T cells (Figure 38B), nor the levels of Th1 and conventional Treg (Figure 38C-D). By contrast, it increases lung Th17 and ROR γ ⁺ Treg compared to mock-treated ones inducing an even higher total numbers of these populations compared to the *prophylactic* mode (Figure 38C-E). In this experiment, the transcription factor Helios was added to our antibody panel. This allowed us to confirm that ROR γ ⁺ Treg present in the lungs, and increased by *L. murinus* administrations, were induced locally (and not in the thymus) because they expressed poor level of Helios compared to conventional Treg, as reflected by the color scale in Figure 38F.

Figure 38: Prophylactic and therapeutic administration of *L. murinus* increase lung Th17 and ROR γ ⁺Treg during *M. tuberculosis* infection. **A:** Experimental design. For the *prophylactic* administration, C57BL/6 mice received intranasally (*i.n.*) 10⁷ CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10³ CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice per week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). **B:** Vertical scatter plot showing the total number of live lung CD4⁺ T cells, as quantified by flow-cytometry in lung homogenate at day 42 post-infection, after blood-circulating and dead cell exclusion. **C-D:** Vertical scatter plots display the percent frequencies (C) and absolute number (D) of the indicated lung CD4⁺ T cell subpopulations, according to the indicated mode of *L. murinus* (or mock) administration. **E:** Vertical scatter plot show the ROR γ ⁺ Treg frequency among total Foxp3⁺ CD4⁺ T cell. **F:** Representative dot plots for expression of ROR γ , Foxp3, and Helios (color gradient from low (blue) to high (red) expression) among CD4⁺ T cells in each experimental group. Each circle represents an individual mouse. A representative experiment is shown from two independent experiments. The black bar line represents the median of each group. A Kruskal-Wallis test and Dunn’s post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01; ***P < 0.001.

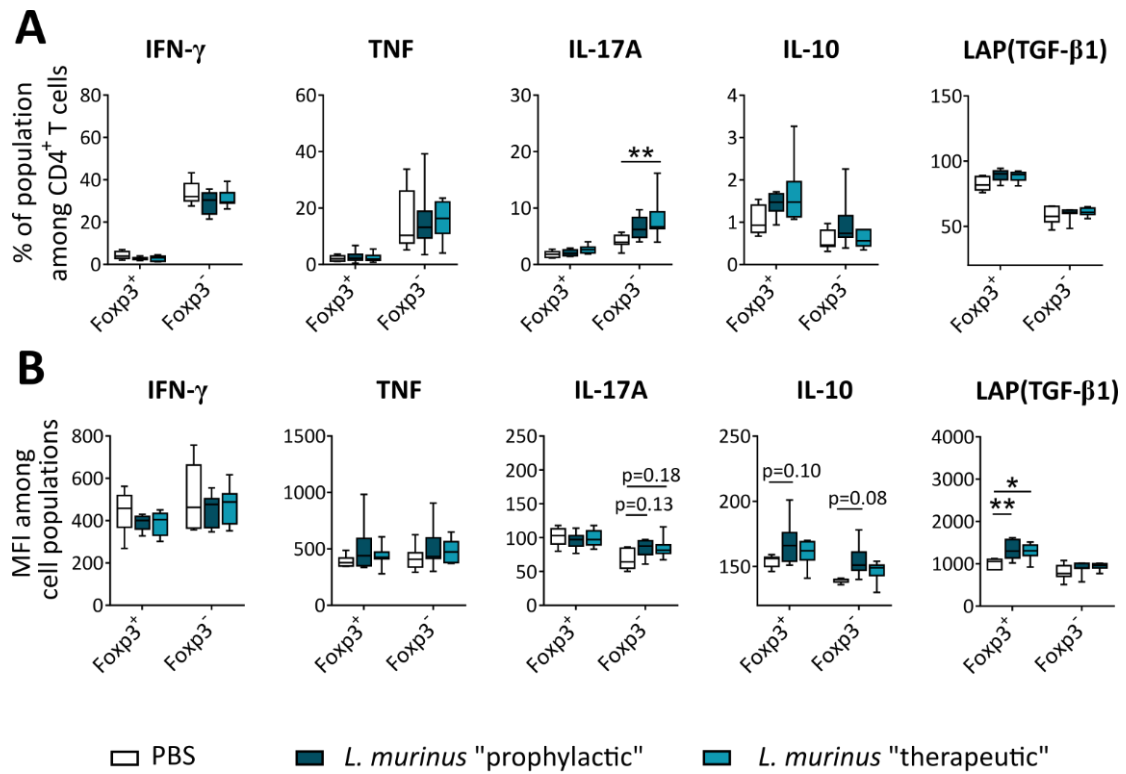


Figure 39: Prophylactic and therapeutic administration of *L. murinus* modifies IL-17A and TGF- β 1 expression in the lung CD4⁺ T cell compartment during *M. tuberculosis* infection. For the *prophylactic* administration, C57BL/6 mice received intranasally (*i.n.*) 10⁷ CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10³ CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice a week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). Mice were sacrificed at day 42 post-infection. Lung homogenates were then stimulated for 4 h with PMA and ionomycin in presence of Brefeldin A and Monensin. Vertical scatter plots display the percent frequencies (A) and Median of Fluorescence Intensity (MFI) (B) of the indicated cytokines produced by total Foxp3⁺ cells (or Foxp3⁻ cells) among lung CD4⁺ T cell, after blood-circulating and dead cell exclusion, and according to the indicated mode of commensal bacterial (or mock) administration. A representative experiment is shown from two independent experiments (n=7 mice per group). A Two-Way ANOVA test followed by Tukey multiple comparison test were performed to compare the mean of each group in each population (and between populations), *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Statistical differences between Foxp3⁺ and Foxp3⁻ are not represented.

To better characterize the immunomodulatory effect of *L. murinus*, we also analyzed the cytokine content in CD4⁺ T cells (Figure 39). Of note, the anti-ROR γ t antibody was absent from the flow cytometry panel used in these given experiments, and thus I was not able to assess the cytokine content specifically in Th17 and ROR γ t⁺ Treg. Nevertheless, I was able to discriminate cytokines produced by Foxp3⁺ (including conventional Treg and ROR γ t⁺ Treg) from Foxp3⁻ cells (Th17 and Th1 cells, among others). As expected, Foxp3⁺ cells produced less pro-inflammatory cytokines (IFN- γ , TNF, IL-17A) and more of the anti-inflammatory TGF- β 1 propeptide (in complex with LAP) than Foxp3⁻ cells (Figure 39A). The effect of *L. murinus* administration was quite evident (independent of the administration mode) in the higher percentage of CD4⁺ T cells that produced IL-17A among Foxp3⁻ cells (Figure 39A), and in the elevated intensity of LAP expression among Foxp3⁺ cells (Figure 39B). I also observed tendencies (albeit not significant) for *L. murinus* to lower the IFN- γ production in Foxp3⁻ cells and increase that of IL-10 expression in all CD4⁺ T cells (Figure 39 A-B).

Altogether, these results indicate that the mode of administration does not vary in the fashion *L. murinus* modulates the lung CD4⁺ T cell compartment, and showed that this pulmonary strain has a clear effect in the induction of Th17 and ROR γ t⁺ Treg.

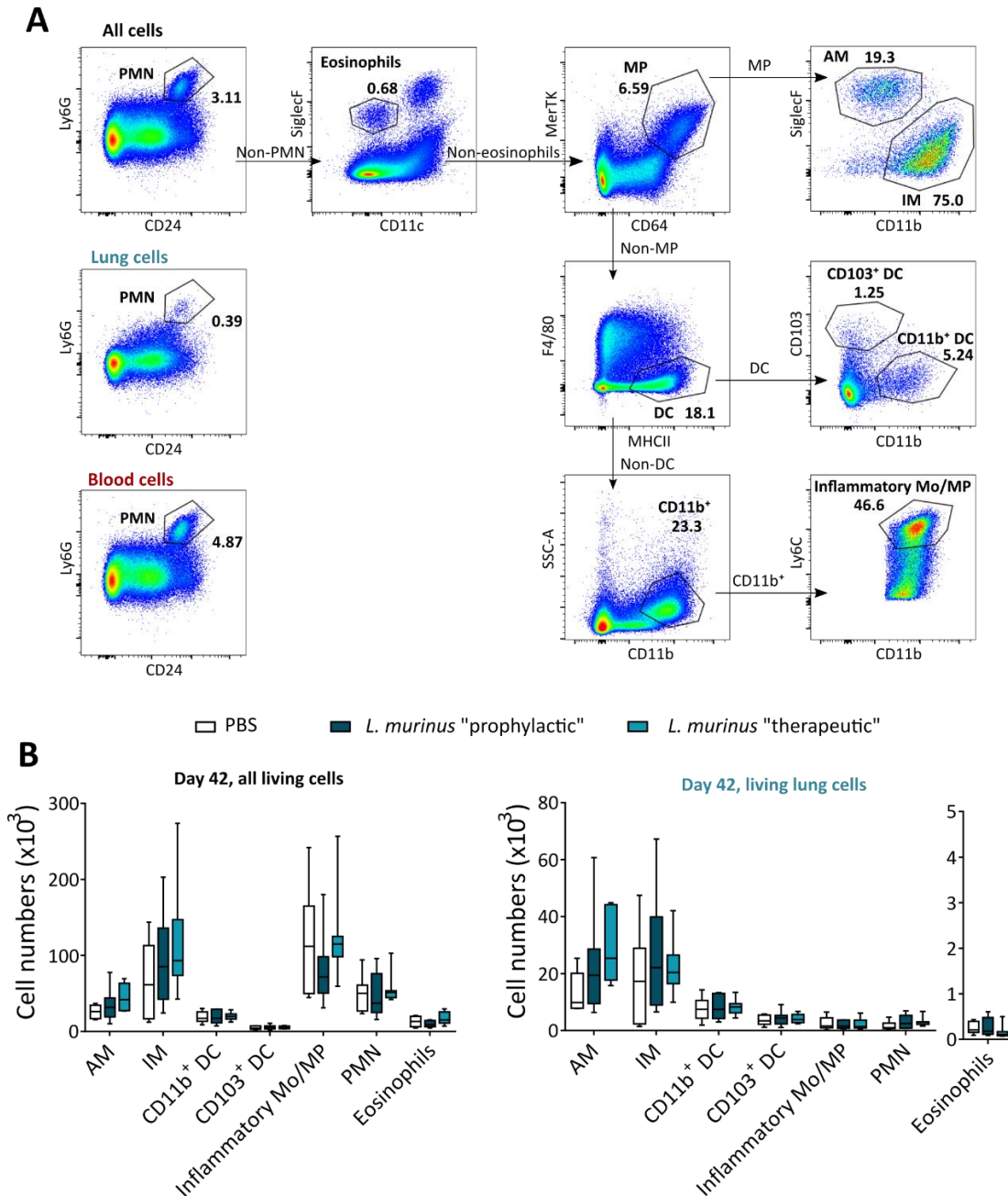
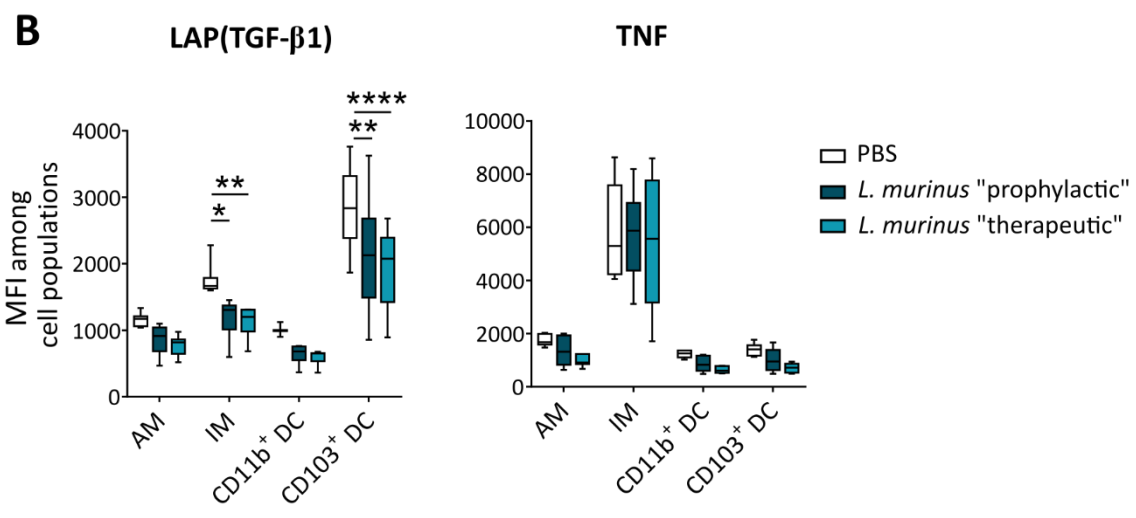
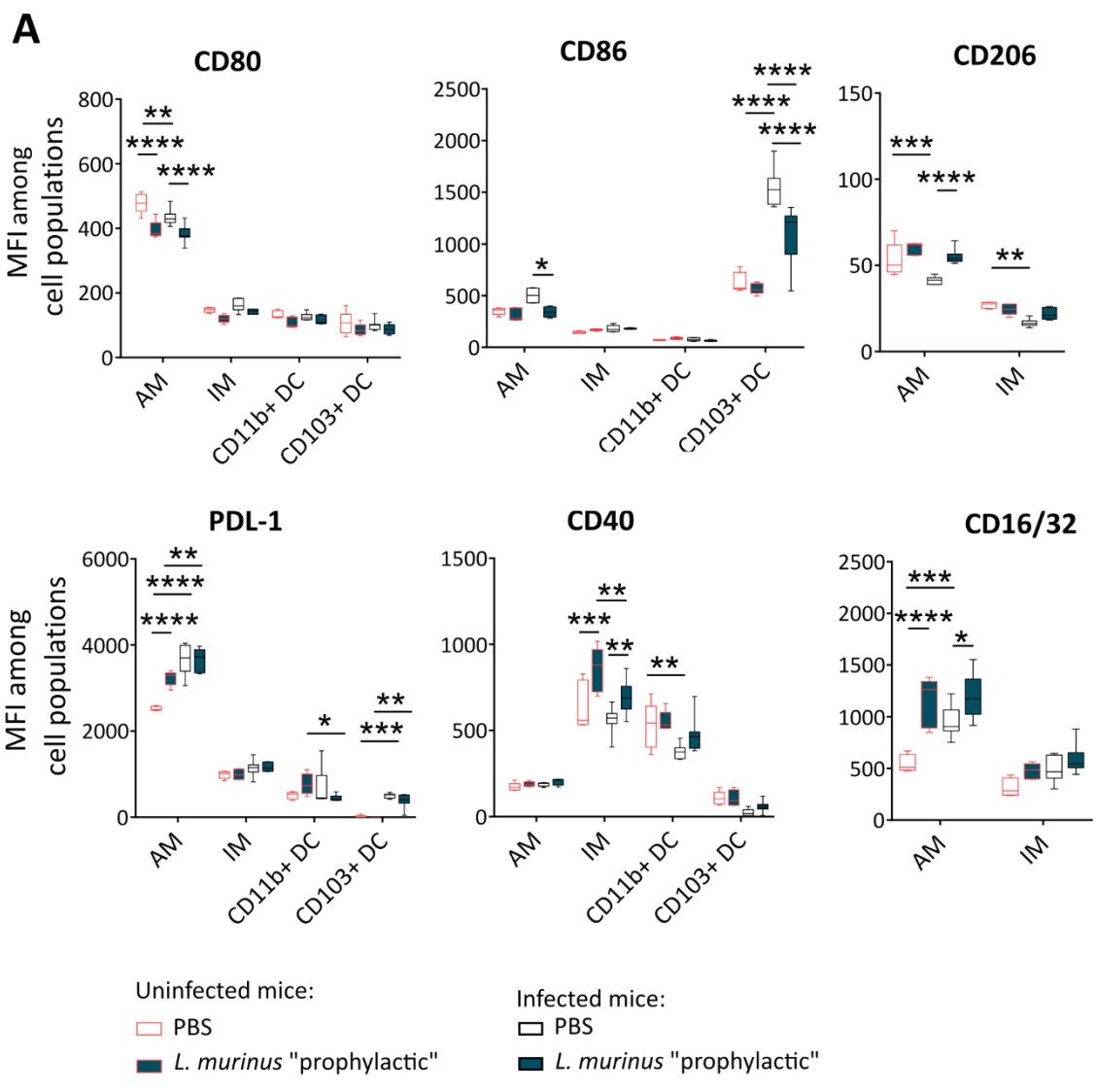


Figure 40: Prophylactic and therapeutic administration of *L. murinus* does not modify the lung myeloid cell number during *M. tuberculosis* infection. For the *prophylactic* administration, C57BL/6 mice received intranasally (*i.n.*) 10^7 CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10^3 CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice a week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). Mice were sacrificed at day 42 post-infection and received anti-CD45.2 antibody *i.v.* 5 min before sacrifice to discriminate cells from the blood. **A:** Gating strategy to assess the lung myeloid cell compartment after exclusion of doublets and dead cells regardless of the CD45.2 staining (All cells, black). Similar gating strategy was done for CD45.2⁺ (blood-circulating cells, red) and CD45⁻ (lung cells, blue); examples of the Ly6G versus CD24 gate are provided. PMN: polymorphonuclear cells, MP: macrophages, AM: alveolar macrophages, IM: interstitial macrophages, DC: dendritic cells, Mo/MP: monocyte/macrophages. **B:** Vertical scatter plots display the cell number of the myeloid cell compartment based on either "All Cells" (left) or "Lung Cells" (right) gating, according to the indicated mode of *L. murinus* (or mock) administration. A representative experiment is shown from two independent experiments ($n=7$ mice per group). No significant difference between experimental group was found using Kruskal-Wallis test and Dunn's post-test.

2. Prophylactic and therapeutic administration of *L. murinus* modulate the phenotype of lung macrophages and DCs at steady state and during *M. tuberculosis* infection

Since myeloid cells are key players in the immune response to *M. tuberculosis*, I decided to assess whether the myeloid compartment was modulated by *L. murinus* administration during infection. At 42 days post-infection, I identified lung neutrophils (PMN), eosinophils, alveolar (AM) and interstitial macrophages (IM), CD11b⁺ and CD103⁺ dendritic cells, and inflammatory monocytes/macrophages by flow cytometry, based on the markers proposed by the team of David Russell, among others (L. Huang *et al.*, 2018) (Figure 40A). Of note, according to the i.v. staining performed before mouse sacrifice to stain cells present in the blood circulation, most of the interstitial macrophages, inflammatory monocyte/macrophages and neutrophils, were present in the lung vascularization in comparison to lung tissue (Figure 40A and B). With or without exclusion of cells from the blood, I did not detect any differences in the total number of myeloid cells identified with the administration of *L. murinus*, independently of the mode of administration. It is possible that *L. murinus* had an effect on the myeloid compartment at an earlier time point (as myeloid cells are activated and recruited in the lungs during the first 10 days post-infection), but I can exclude a long-lasting effect lingering at the end time point. It can also be noted that despite increased level of IL-17A secreting CD4⁺ T cells, *L. murinus*-treated mice did not show a massive lung infiltration by neutrophils.

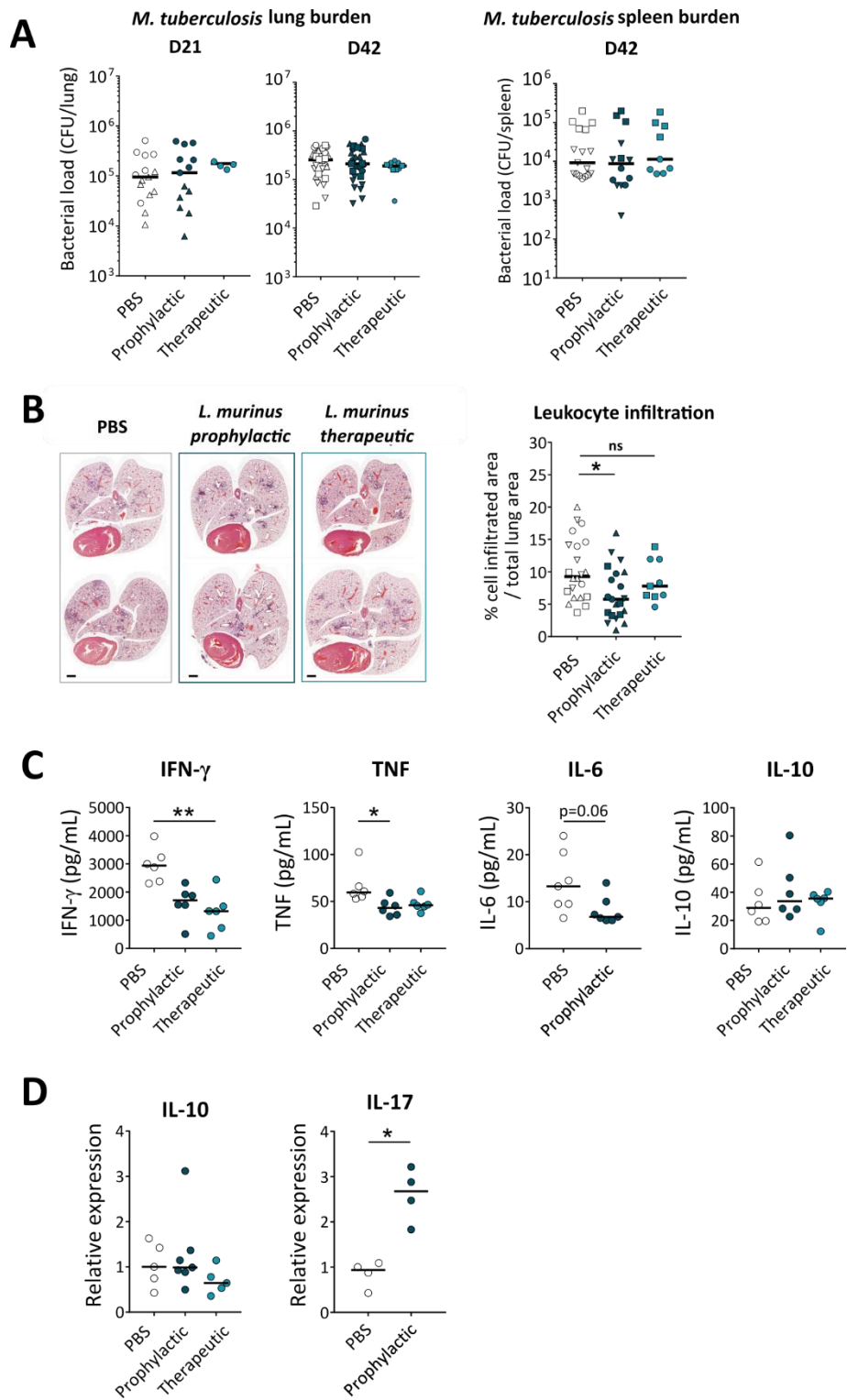


To further characterize the myeloid compartment, I compared the expression of a battery of extracellular markers in mice infected (or not), treated with *L. murinus* (*prophylactic mode*) (or not) (Figure 41A). Such analysis reveals that alveolar macrophages exhibited a decreased expression of pro-inflammatory macrophage (M1) markers (e.g., CD80 and CD86) and an increase in anti-inflammatory (M2) markers (e.g., CD206 and CD16/32) in mice treated with *L. murinus*, regardless of *M. tuberculosis* infection. The CD86 level was also decreased in CD103⁺ DC from *L. murinus*-treated mice. The inhibitory ligand PD-L1, involved in T cell exhaustion, was increased in alveolar macrophages and CD103⁺ DC from infected mice, and by *L. murinus* treatment in non-infected alveolar macrophages. CD40, involved in the induction of Th17 (Sia *et al.*, 2017), was expressed by interstitial macrophages and DC. Its expression in both cell types was reduced in *M. tuberculosis* infected mice compared to non-infected ones. Yet, it was increased in interstitial macrophages from all *L. murinus*-treated mice.

In another experiment comparing the different modes of *L. murinus* administration in *M. tuberculosis* infected mice, I assessed the cytokine production on LPS re-stimulated lung homogenates containing myeloid cells. IL-10 producing cells were not observed in this setting (data not shown). By contrast, TGF- β 1 production was detected in all APC and seemed to decrease upon treatment with *L. murinus*, independently of the administration mode. TNF expression, which was high in interstitial macrophages but not in other subsets, was not modulated by *L. murinus* administrations (Figure 41B).

Altogether, these findings indicate that while *L. murinus* do not modify the numbers of myeloid cells present in the lungs late during tuberculosis infection, it modulates their phenotype. It is clear, however, that more experiments need to be performed in order to fully understand the effects exerted by this *Lactobacillus* strain, in particular at earlier post-infection time points.

Figure 41: Prophylactic and therapeutic administration of *L. murinus* modulate the phenotype of lung macrophages and DC at steady state and during *M. tuberculosis* infection. For the *prophylactic* administration, C57BL/6 mice received intranasally (*i.n.*) 10⁷ CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10³ CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice a week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). Mice were sacrificed at day 42 post-infection. **A:** In this experiment, a group of mice were kept uninfected (red border), but they received the prophylactic mode of *L. murinus* administration (or PBS) as the infected mice (with the same treatment duration). Vertical scatter plots display Median of Fluorescence Intensity (MFI) of indicated extracellular markers expressed by the indicated lung macrophage or dendritic cell populations. Results from one experiment with n=5 (uninfected) or 7 (*M. tuberculosis* infected) mice per group **B:** Cytokine stainings were performed on lung homogenates stimulated with LPS for 4h, in presence of Brefeldin A and Monensin. Vertical scatter plots display MFI of the indicated cytokines expressed by the indicated lung macrophage or dendritic cell populations. Results from one experiment with n=7 mice per group. AM: alveolar macrophages, IM: interstitial macrophages, DC: dendritic cells. A Two-Way ANOVA test followed by Tukey multiple comparison test were performed to compare the mean of each group in each population (and between populations), *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.



3. Prophylactic and therapeutic administration of *L. murinus* modulate the lung inflammation associated to *M. tuberculosis* infection

To confirm the broad effect of the administration modes of *L. murinus* during *M. tuberculosis* infection, I measured in the same experiments, the pathogen burden, leukocyte infiltration and cytokine production in the lungs. The administration modes of *L. murinus* did not modify *M. tuberculosis* loads in the lungs or the incidence of bacterial dissemination to the spleen at day 21 or 42 post-infection (Figure 42A), suggesting again that this commensal bacterial strain does not affect the pathogen growth or its killing by the immune system. However, while the *prophylactic* mode clearly decreased leukocyte infiltration in the lung, as measured by histological staining, effect of the *therapeutic* mode was not significant (Figure 42B). More experiments with the therapeutic mode of administration may be required to assess whether it has a lower effect compared to the prophylactic mode or no effect at all on this parameter. Regarding cytokine production, I observed an overall decrease tendency in the level of pro-inflammatory cytokines IFN- γ , TNF and IL-6 in lung homogenate supernatants at day 42 post-infection, as measured by ELISA (Figure 42C). By contrast with the leukocyte infiltration, here, the effect was stronger with the therapeutic administration. There was no difference in the overall production of IL-10 in the lung (Figure 42C). This result was confirmed by a preliminary RT-qPCR analysis on total lung homogenates in which no difference was observed between mock and treated animals (Figure 42D). As for the other cytokines, I tried to measure the level of IL-17 by ELISA, but its abundance level was below the detection limit (data not shown). However, the RT-qPCR analysis revealed a clear increase of IL-17 expression in mice treated with the *prophylactic* administration of *L. murinus* (Figure 42D).

Collectively, these findings suggest that *L. murinus* has the capacity to reduce the lung inflammation associated with *M. tuberculosis* infection, without affecting the pathogen burden, and that *prophylactic* mode and *therapeutic* mode of administration may be different regarding this effect.

Figure 42: Prophylactic and therapeutic administration of *L. murinus* modulate the lung inflammation associated to *M. tuberculosis* infection in C57BL/6. For the *prophylactic* administration, C57BL/6 mice received intranasally (*i.n.*) 10^7 CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10^3 CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice per week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). **A:** Vertical scatter plots show the *M. tuberculosis* burden in lung (left) or spleen (right) homogenates at day 21 or 42 post-infection. **B:** Leukocyte infiltration detected by histological hematoxylin and eosin staining (left), and quantified as illustrated in vertical plot (right) in pulmonary tissue at day 42 post-infection. **C:** Vertical scatter plots illustrate the indicated cytokine concentration in lung homogenate supernatants at day 42 post-infection. **D:** Expression of IL-17 and IL-10 relative to β -actin in lung homogenates was measured by RT-qPCR at day 42 post-infection. Values are expressed as a fold change between *L. murinus*-treated mice relative to PBS-treated mice. Each circle represents an individual mouse. In A and B, three experiments are pooled (different symbols are used for each experiment). In C, a representative experiment is shown from two independent experiments. The black bar line represents the median of each group. A Kruskal-Wallis test and Dunn's post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01.

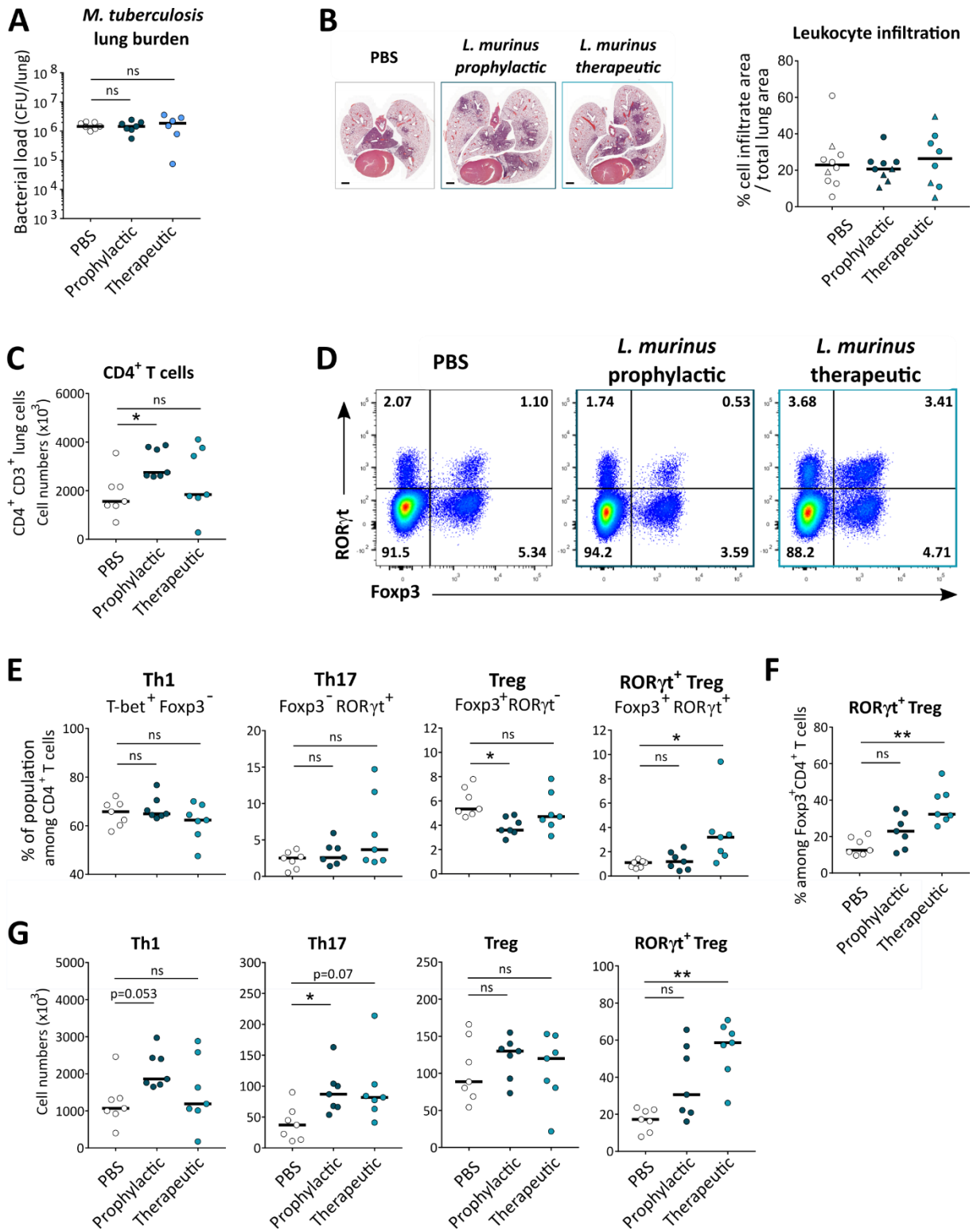
4. Prophylactic and therapeutic administration of *L. murinus* do not exert beneficial effects in 129/Sv mice that are susceptible to lung inflammation associated to *M. tuberculosis* infection

C57BL/6 mice are often used in the tuberculosis field to decipher mechanisms involved in the immune response, partly because many genetic tools are available on this background. However, there are not the best model to assess immunopathology associated to tuberculosis as they do not develop necrotic lung lesions as humans do (Kramnik and Beamer, 2016). As in this model I still observed a difference in leukocyte infiltration, I was wondering if it could lead to a decrease in immunopathology. To do so, we choose to repeat our experiment in 129/Sv mice, that are more susceptible to the disease, succumbing to uncontrolled inflammation leading to lung tissue damages (Medina and North, 1998; Dorhoi *et al.*, 2014).

To assess the protective effect of *L. murinus* in this mouse model, I applied the same protocols as previously described for the C57BL/6 model. In line with the literature, *M. tuberculosis* burden in the lung of 129Sv mice was higher compared to C57BL/6 mice, as well as the lung leukocyte infiltration (Figure 42A,B and Figure 43A,B). Indeed, large infiltrated areas, containing dense cell clusters were visible in the lung of these mice (Figure 43B), whereas small and diffuse cell clusters were usually observed in C57BL/6 mice (Figure 42B). This higher infiltration was reflected by a ten-fold increase in the cell number of all CD4⁺ T cell subsets, while the proportion between them was similar between both mouse models (Figure 38 and Figure 43D,E). As in C57BL/6 mice, *L. murinus* administration (independently of the mode) did not change *M. tuberculosis* bacterial loads at day 42 post-infection (Figure 43A). However, in the 129/Sv mice, I did not see any effect of *L. murinus* in lung leukocyte infiltration (Figure 43B).

Modulations of the CD4⁺ T cell compartment were also different between the two mouse models. In particular, in 129/Sv mice, while the *prophylactic* mode of administration of *L. murinus* increased the total number of lung CD4⁺ T cells, including Th1 and Th17, it failed to increase that of conventional and RORγt⁺ Treg (Figure 43C-G). In fact, while modulations of Th17 and RORγt⁺ Treg can be observed with both administration modes, there are clearly low compared to what was observed in the C57BL/6 mice (Figure 38). Regarding cytokine production, increased IFN-γ and IL-17A levels in lung CD4⁺ T cells were observed despite the high expression of the suppressive TGF-β in *L. murinus*-treated mice with the prophylactic mode, while the cytokine production was less impacted by the *therapeutic* mode (Figure 43H-I). Investigation of the impact of *L. murinus* on the myeloid compartment was not assessed in this mouse model.

These results indicate that the beneficial effects of *L. murinus* in the context of tuberculosis might be dependent on the mouse genetic background, as well as the immunomodulatory capacity of the CD4⁺ T cell compartment in the lung.



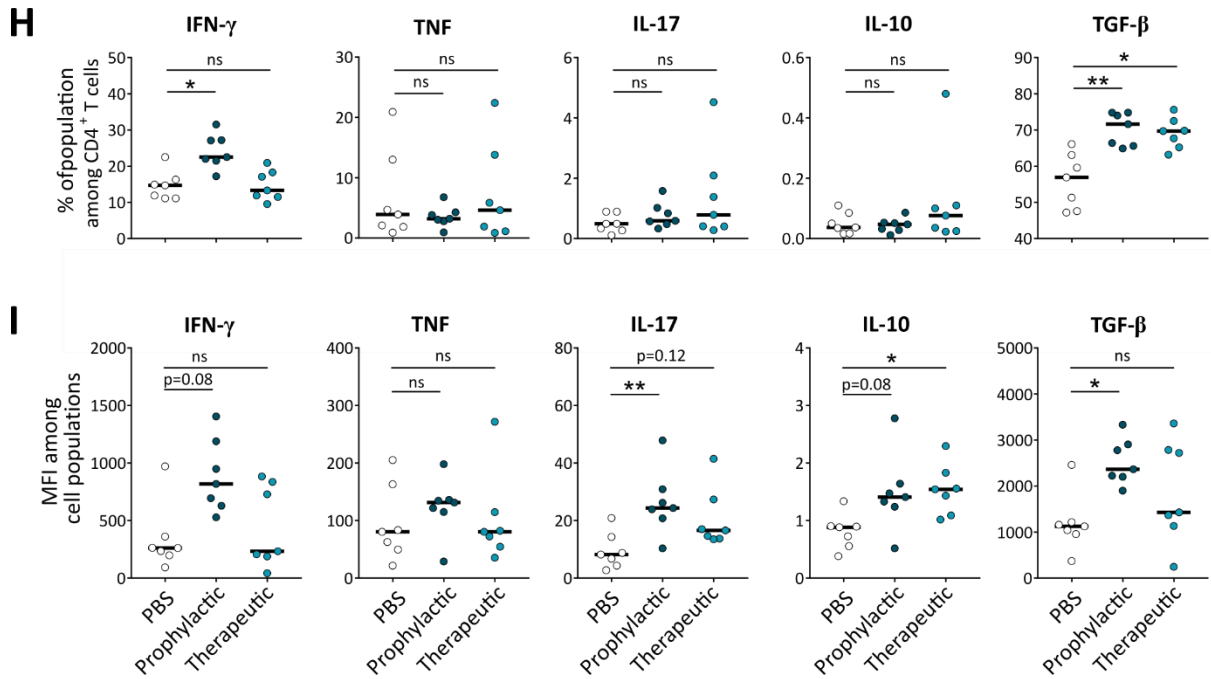
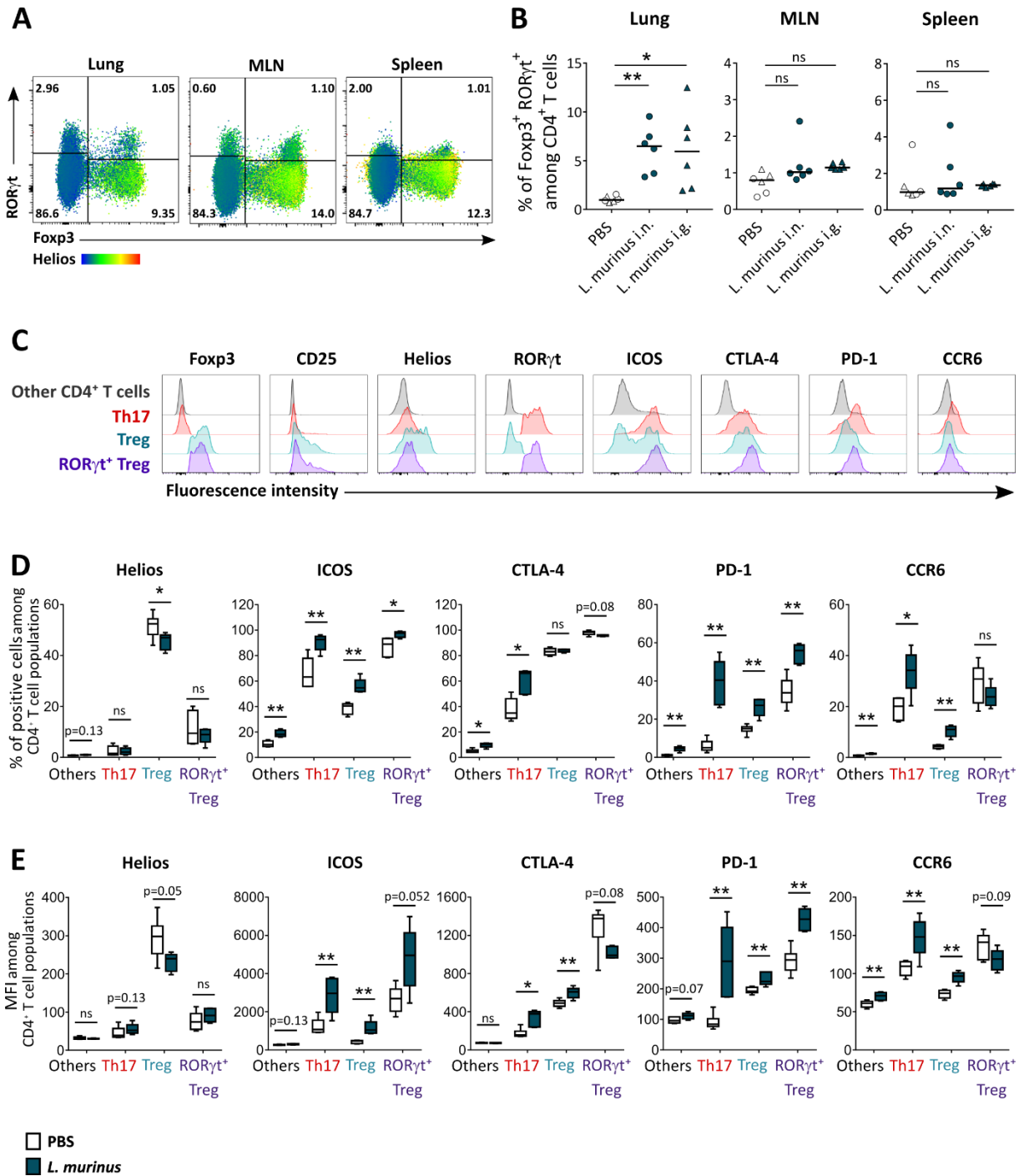


Figure 43: Prophylactic and therapeutic administration of *L. murinus* do not exert beneficial effects in mice susceptible to lung inflammation associated to *M. tuberculosis* infection. 129Sv mice received intranasally (*i.n.*) 10^7 CFU of *Lactobacillus murinus* in 20 μ L of PBS or mock (PBS alone, white) three times a week during two weeks prior to *i.n.* infection with 10^3 CFU of *M. tuberculosis* H37Rv. Thereafter, mice were inoculated twice a week post-infection to continue the *prophylactic* administration (dark blue), or as a starting point for the *therapeutic* administration (light blue). **A:** Vertical scatter plots show the *M. tuberculosis* loads in lung homogenates at day 42 post-infection. **B:** Leukocyte infiltration detected by histological hematoxylin and eosin staining (B), and quantified as illustrated in vertical plot (C) in pulmonary tissue at day 42 post-infection. In (B) the black bars represent 5 mm. **C:** Vertical scatter plots show total number of live lung CD4⁺ T cells, as quantified by flow-cytometry in lung homogenates at day 42 post-infection, after blood-circulating and dead cell exclusion. **D:** Representative dot plots for expression of ROR γ t and Foxp3 among CD4⁺ T cell in each experimental group. **E-F:** Vertical scatter plots display the percent frequencies (G) and absolute number (H) of the indicated lung CD4⁺ T cell subpopulations, according to specific bacterial (or mock) administration. **G:** Vertical scatter plot show the ROR γ t⁺ Treg frequency among total Foxp3⁺ CD4⁺ T cell. **H-I:** Lung homogenates were stimulated for 4 h with PMA and ionomycin in presence of Brefeldin A and Monensin. Vertical scatter plots display the percent frequencies (I) and absolute number (J) of CD4⁺ T cell producing the indicated cytokines, according to the indicated mode of commensal bacterial (or mock) administration. Each circle represents an individual mouse. A representative experiment is shown from two independent experiments (the two experiments are pooled in C). The black bar line represents the median of each group. A Kruskal-Wallis test and Dunn's post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01.



III. Characterization of *L. murinus* and additional pulmonary bacterial strains

1. Formal characterization of lung Th17 and ROR γ ⁺ Treg at steady state or after intranasal administration of pulmonary *L. murinus*

Among the few studies characterizing ROR γ ⁺ Treg, most of them reported ROR γ ⁺ Treg presence in intestinal and colonic lamina propria, and one reported that gavage with gut microbiota strains increased their presence in this mucosal site (Sefik *et al.*, 2015). To compare our results with these studies, I performed administration of *L. murinus* either i.n. or intragastrically (i.g.) and assessed the distribution of ROR γ ⁺ Treg in the lungs, colonic lamina propria, mesenteric lymph nodes and spleen. Unfortunately, I recovered few CD4⁺ T cell from the colonic lamina propria and was unable to carry out a proper assessment for this site. As previously described (Yang *et al.*, 2016), I observed that ROR γ ⁺ Treg were not a dominant CD4⁺ T cell subtype in non-mucosal sites, such as the spleen or mesenteric lymph nodes in SPF mice (Figure 44A, B). Importantly, both i.n. and i.g. administrations of *L. murinus* increased ROR γ ⁺ Treg levels in the lungs (but not in the spleen or lymph nodes) (Figure 44B). The i.n. administration resulted in a higher induction than the i.g. one (this difference was accentuated in another experiment) (Figure 44B). This result suggests that *L. murinus* may mediate immune regulation at the lungs, either distally through the gut-lung axis but also locally. This was shown with other *Lactobacillus* strains for example in (Youn *et al.*, 2012) but was not addressed using strains from lung origin before.

Figure 44: Formal characterization of lung Th17 and ROR γ ⁺ Treg at steady state or after intranasal administration of pulmonary *L. murinus*. C57BL/6 naïve mice received intranasal (i.n.) administration of 10⁷ CFU of *Lactobacillus murinus* in 20 μ L PBS (blue circles or boxes) or PBS alone (white circles or boxes) three times a week during two weeks before being sacrificed. Alternatively, other mouse groups received intragastric (i.g.) administration of 10⁹ CFU of *Lactobacillus murinus* in 200 μ L of PBS (blue triangles) or PBS alone (white triangles) every day during a 10-day period before being sacrificed (only in B). **A:** Representative expression of ROR γ ⁺, Foxp3 and Helios (color gradient from low (blue) to high (red) expression) in CD4⁺ T cells in the lung, mesenteric lymph node (MLN) or spleen, from mock-treated mice. **B:** Vertical scatter plots show ROR γ ⁺ Treg frequencies among CD4⁺ T cell in indicated organs, according to the indicated mode of *L. murinus* (or mock) administration. **C-E:** Representative expression (C), frequency (D) or Median of Fluorescence Intensity (MFI) (E) of indicated markers by lung CD4⁺ T cells populations: Foxp3⁻ ROR γ ⁻ (other CD4⁺ T cells), Foxp3⁻ ROR γ ⁺ (Th17), Foxp3⁺ ROR γ ⁻ (Treg), Foxp3⁺ ROR γ ⁺ (ROR γ ⁺ Treg), after intranasal administration of PBS or *L. murinus*. In B, Kruskal-Wallis test and Dunn's post-test were performed to compare the mean rank of each group to the mock group. In C and D, a Mann-Whitney t-test was performed to compare the median of each marker in the mock group to the *L. murinus*-treated group for each cell population. A representative experiment is shown from two independent experiments in each panel, (n=5-6 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001.

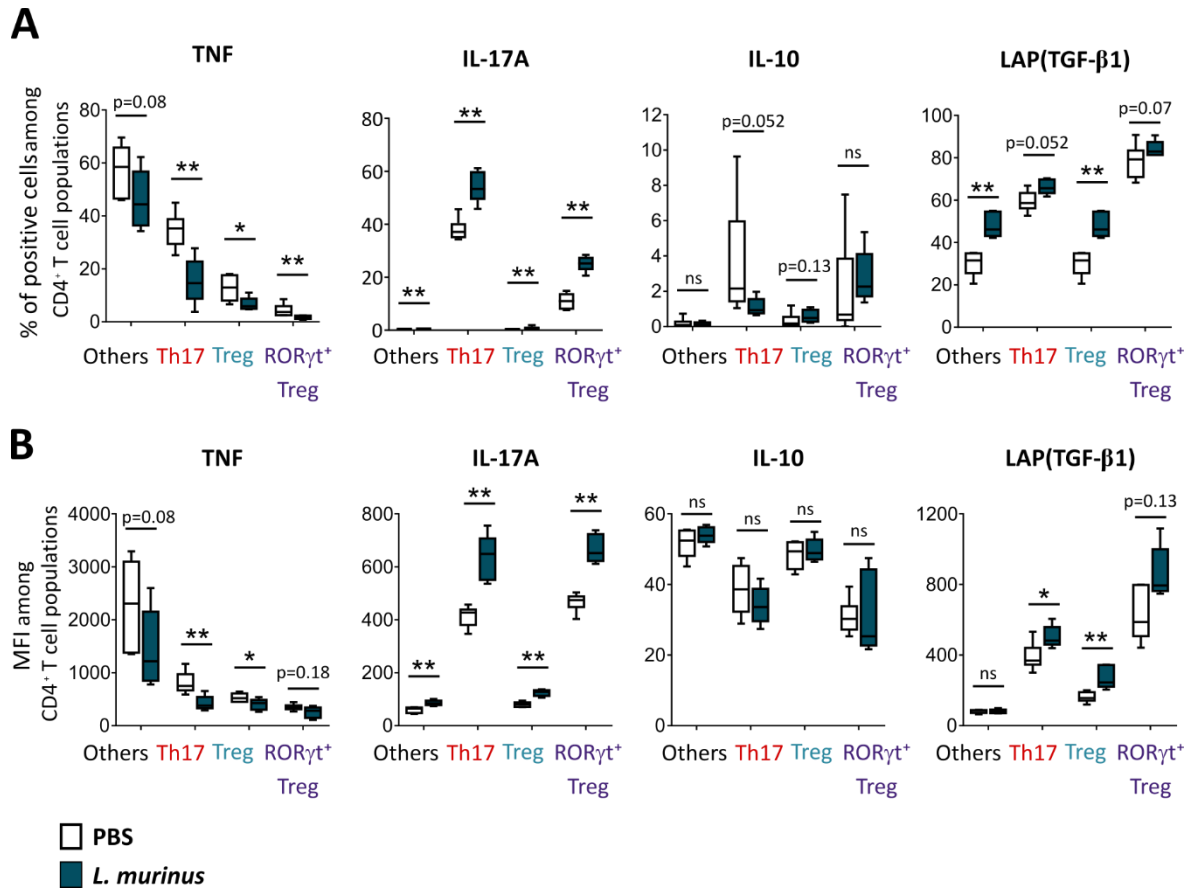


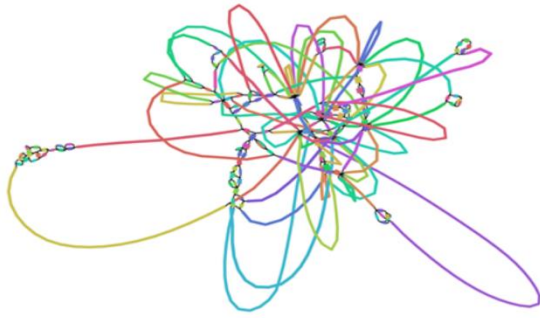
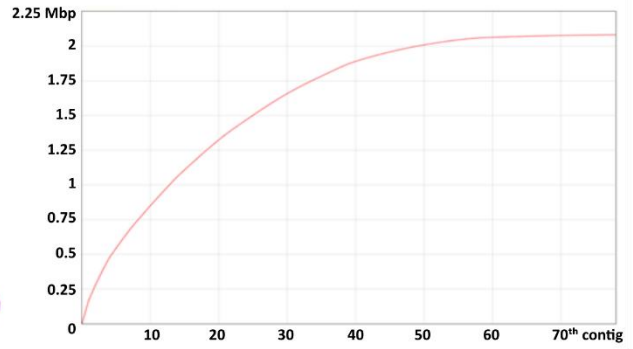
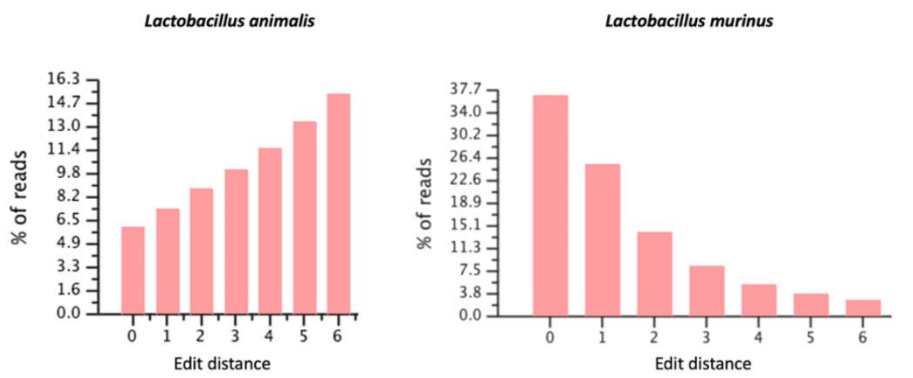
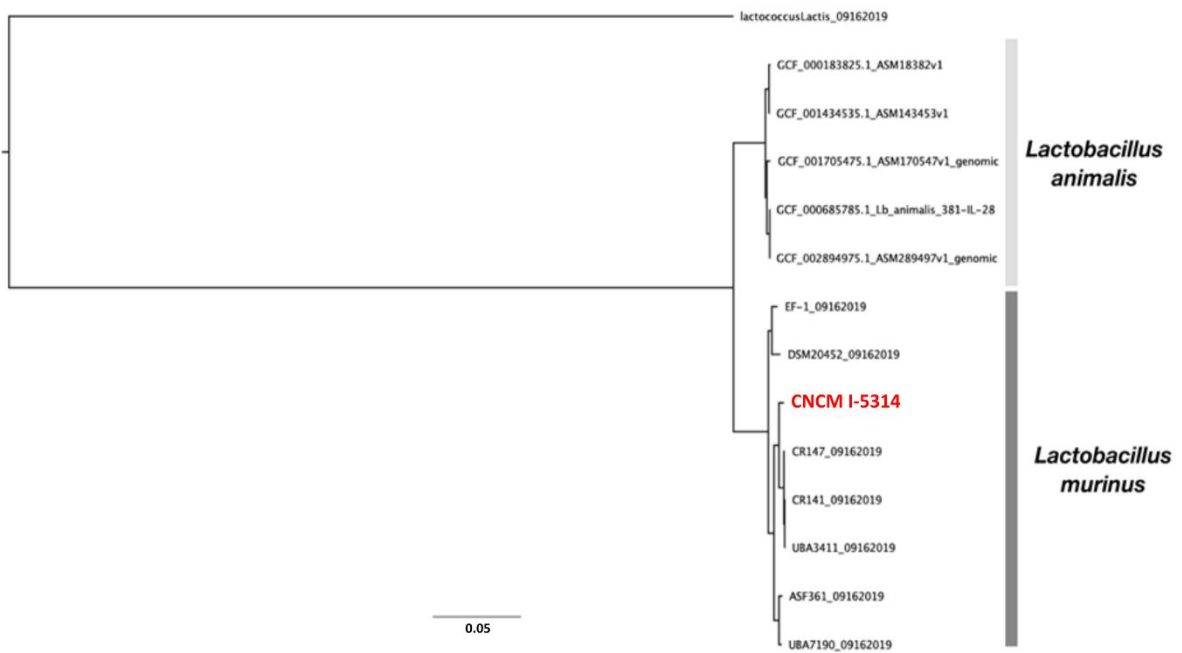
Figure 45: Formal characterization of the cytokine content in Th17 and RORγt⁺ Treg at steady state or after intranasal administration of a pulmonary *L. murinus*. C57BL/6 naïve mice received intranasal (*i.n.*) administration of PBS (white boxes) or 10⁷ CFU of *Lactobacillus murinus* (blue boxes) three times a week during two weeks before being sacrificed. Lung homogenates were stimulated for 4 h with PMA and ionomycine in presence of Brefeldin A and Monensin. **A, B:** Frequency (A) or Median of Fluorescence Intensity (MFI) (B) of indicated cytokines by lung CD4⁺ T cells populations: Foxp3⁻ RORγt⁻ (other CD4⁺ T cells), Foxp3⁻ RORγt⁺ (Th17), Foxp3⁺ RORγt⁻ (Treg), Foxp3⁺ RORγt⁺ (RORγt⁺ Treg), according to the indicated mode of commensal bacteria (or mock) administration. Blood-circulating cells were not excluded in this experiment. A Mann-Whitney t-test was performed to compare the median of each marker in the mock group to the *L. murinus*-treated group for each cell population. A representative experiment is shown from three independent experiments (n=5-6 mice per group). *P < 0.05; **P < 0.01; ***P < 0.001.

As described in the literature for ROR γ t⁺Treg from gut origin (Ohnmacht *et al.*, 2015; Sefik *et al.*, 2015; Yang *et al.*, 2016), lung ROR γ t⁺Treg expressed Foxp3 at a similar level than conventional Treg, and ROR γ t at similar level than Th17 (Figure 44C). In addition, lung ROR γ t⁺Treg expressed very low levels of Helios in comparison to conventional Tregs (Figure 44A, C-E). Moreover, these cells failed to express CD25 (Figure 44C), as reported in ROR γ t⁺Treg isolated from spleen and lymph node (Yang, mucosal immunol 2016). Of note, I found few levels of total Foxp3⁺ CD4⁺ T cells that express CD25 in the lungs in different experiments (Figure 44C). In terms of their activation status, I noticed that lung ROR γ t⁺Treg expressed high level of ICOS, which was even more increased by *L. murinus* administration (Figure 44 C-E). They also highly expressed the immune-suppressive marker CTLA-4, which was not modulated by *L. murinus* administration. Surprisingly, Th17 were found to express CTLA-4 in our model, which was further increased in *L. murinus*-treated mice (Figure 44 C-E). *L. murinus* also induced the expression of the suppressive marker PD-1 in the entire CD4⁺ T cell compartment, including Th17 and ROR γ t⁺Treg (Figure 44 C-E). As previously described, Th17 and ROR γ t⁺Treg expressed high levels of CCR6 (Lochner *et al.*, 2008). Except for ROR γ t⁺Treg, *L. murinus* enhanced the CCR6 expression levels in all CD4⁺ T cells (Figure 44 C-E).

In terms of cytokine production, Th17, Treg and ROR γ t⁺Treg expressed low levels of TNF in comparison to Th1 cells; administration of *L. murinus* dramatically decreased this pro-inflammatory cytokine across all CD4⁺ T cells subsets (Figure 45).

By contrast, production of IL-17A was massively increased in all CD4⁺ T cell subsets and, in particular, in Th17 and ROR γ t⁺Treg (Figure 45). Again, I observed very poor levels of CD4⁺ T cells expressing IL-10, and I failed to notice any effect induced by *L. murinus* on this cytokine. However, TGF- β 1 propeptide expression was generally increased in all CD4⁺ T cell subsets from *L. murinus*-treated mice, including Th17 cells. Of note, I report for the first time that lung ROR γ t⁺Treg expressed TGF- β 1 propeptide at the highest level (Figure 45).

Collectively, this characterization suggests that lung ROR γ t⁺Treg have an inhibitory phenotype that is further enhanced by pulmonary *L. murinus*. This is mirrored by the expansion of Th17 cells with anti-inflammatory properties that may participate in disease tolerance rather than resistance mechanisms.

A**B****C****D**

2. *Lactobacillus murinus* genome analysis

Given the highly interesting immunomodulatory properties of our *L. murinus* isolate along with the reduction of leukocyte infiltration observed in the C57BL/6 mice infected with *M. tuberculosis*, we sought the formal assessment of its identification to be crucial for its application as a probiotic strain. All lung strains used in this thesis have had been roughly identified at the time of their isolation by 16S PCR sequencing ((Remot *et al.*, 2017) and Table 6). With this method, our strain candidate had been previously designated as a *Lactobacillus animalis/species* (Remot *et al.*, 2017). Of note, it was reported that this technique does not always allow the precise identification of *Lactobacillus species* due to poor phylotype resolution of profiling approaches based on the 16S rRNA gene (carrying many recombination in these bacteria) (C. H. Huang *et al.*, 2018; Milani *et al.*, 2018; Seol *et al.*, 2019). To assess its identity using a whole genome sequencing strategy, we established a collaboration with Dr. Amine Namouchi. After testing several protocols as detailed in the Material and Methods (subsection I.3), I successfully extracted the DNA of this strain. After sequencing, the short Illumina reads were assembled in contigs (that are larger fragments) using Unicycler. The assembled genome obtained with this strategy is shown in Figure 46A on a representation obtained with the Bandage program. The Unicycler analysis had generated 124 contigs with size varying from 100 bp to 168 265 bp with a median size (or N50) of 51 475 bp (Figure 46B). The cumulative size of the assembled contigs was 2.1 Mbp, which is close to genome size obtained by other groups having sequenced whole genomes of *L. murinus* strains (Wannemuehler *et al.*, 2014; Fritz and Miller, 2017; Bassam, Milovic and Barbour, 2019). First comparisons of these contigs with published genomes of other *L. animalis* strains, suggested that our candidate strain's genome was quite different from the others (data not shown). In fact, a broader search reveals that contigs generated were better aligned with genomes of *L. murinus* strains (which is the closest species to *L. animalis*). This is exemplified in Figure 46C showing that the percentages of reads with an edit distance⁵⁶ of 0 is higher when aligning the reads against the genome from a *L. murinus* strain (~37.7%) than when aligning them against *L. animalis* (~5%). Furthermore, we performed evolutionary analyses and comparative genomics of the new sequenced and assembled genome of our strain with available genomes from *L. murinus* strains and *L. animalis* strains (in addition to a *Lactococcus lactis*, added to the analysis as an outgroup). This analysis confirmed that our *Lactobacillus species* CNCM I-5314 is in fact a *Lactobacillus murinus* and not a *Lactobacillus animalis*. This analysis also allowed the identification of the strains of *L. murinus* that are the closest to our isolate. For example, *L. murinus* CR147 strain is more relative to our strain than *L. murinus* ASF361. Identification of our isolate and of relative strains may be helpful in the future to further decipher our isolate mechanism of action as proposed in the discussion section.

Figure 46: *Lactobacillus murinus* genome analysis. (A) Graph representation of the *L. murinus* assembly genome using Bandage. (B) Size of contigs generated from read assembly using Unicycler. (C) The histograms of each sample dataset show the edit distance and the percentage of reads after alignment to *L. animalis* strain LL1 (left) (Pan *et al.*, 2018), and *L. murinus* strain CR147 (right) (Bassam, Milovic and Barbour, 2019). (D) Phylogenetic tree reconstruction based on the concatenation of all orthogroups identified after comparison of *L. animalis*, *L. murinus* and *L. lactis*. The sequenced genome cluster with all the included *L. murinus* isolates, which confirms the result of Edit distance analysis.

⁵⁶ The **edit distance** reflects differences between the sequenced reads and the genome sequence used as a reference.

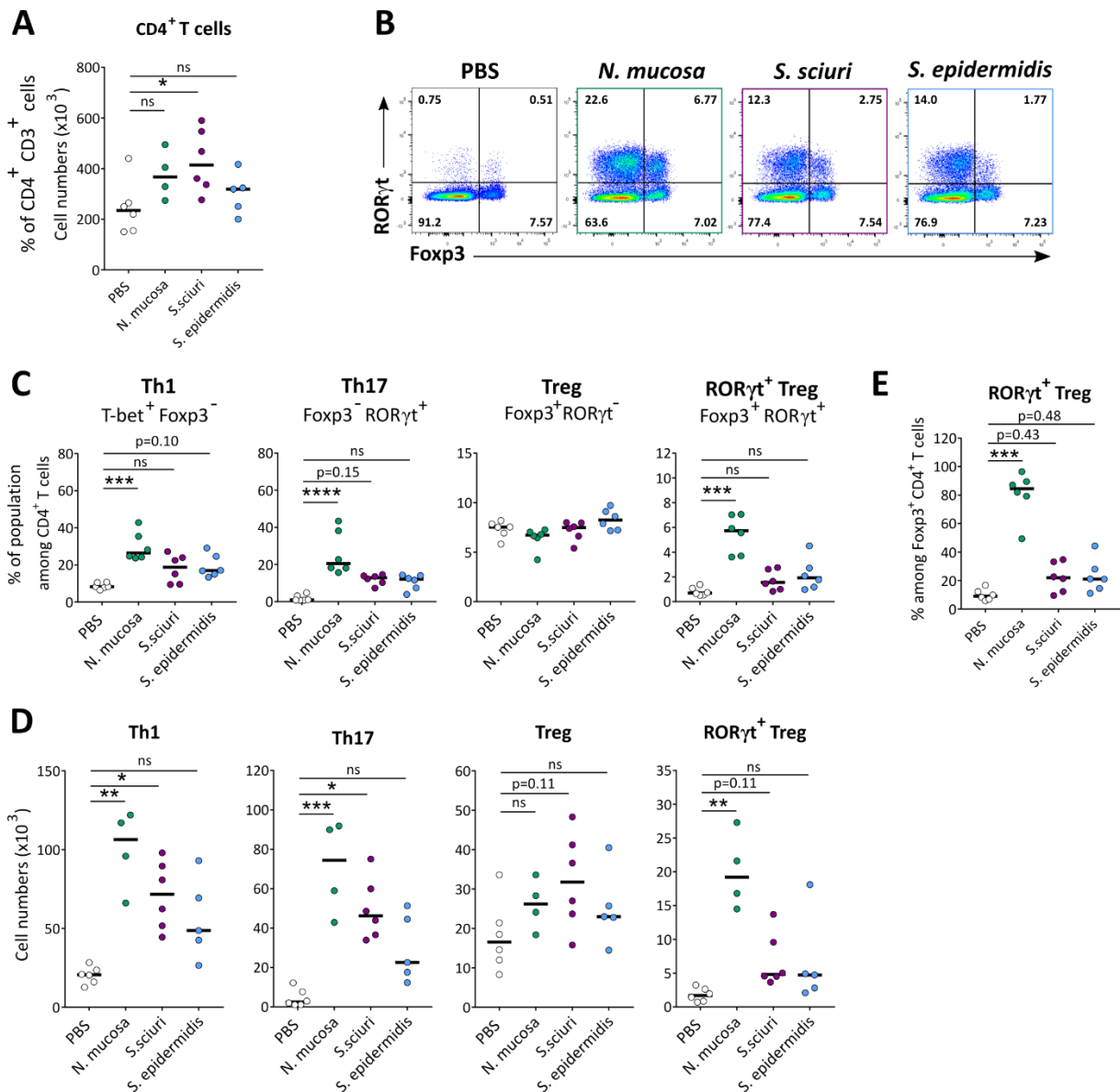


Figure 47: Intranasal administration of pulmonary commensal bacteria (other than lactobacilli) modulates the lung CD4⁺ T cell populations in naïve mice. Experimental design: C57BL/6 naïve mice received intranasally (*i.n.*) 10⁷ CFU of murine pulmonary commensal bacteria (*Neisseria mucosa* (green), *Staphylococcus sciuri* (purple), *Staphylococcus epidermidis* (light blue)) in 20 μL of PBS or mock (PBS alone, white) three times a week during two weeks before being sacrificed. CD4⁺ T cells populations were quantified by flow-cytometry in lung homogenates (gated on live, singlet cells, without the exclusion of cells from the blood). **A:** Vertical scatter plots show total number of CD4⁺ T cells. **B:** Representative dot plots for expression of RORγt and Foxp3 in CD4⁺ T cells in each of the indicated experimental group. **C, D:** Vertical scatter plots display the corresponding percent frequencies (C) and MFI (D) of the indicated lung CD4⁺ T cell subpopulations, according to the indicated commensal bacterial (or mock) administration. **E:** Vertical scatter plot show the RORγt⁺ Treg frequency among total Foxp3⁺ Treg population. Each circle represents an individual mouse, while the black bar line is the median of each group. Experimental groups were compared to control within the same experiment; two pooled experiment are presented (circles versus triangles represent different experiments). A Kruskal-Wallis test and Dunn's post-test were performed to compare the mean rank of each group to the PBS control group, *P < 0.05; **P < 0.01; ***P < 0.001.

3. Intranasal administration of pulmonary commensal bacteria (other than lactobacilli) modulates the lung CD4⁺ T cell populations in naïve mice

To find out if members of other families of lung commensal bacteria are able to induce lung ROR γ ⁺ Treg, I delivered intranasally *Neisseria mucosa*, *Staphylococcus sciuri* and *Staphylococcus epidermidis* strains (described in the [Table 2](#)) in naïve mice, as previously described for *L. murinus*. Administration of the strains increased the total CD4⁺ T cell number in the lungs of treated mice, which was significant for *S. sciuri* ([Figure 47A](#)). All strains had the tendency to increase lung ROR γ ⁺ Treg, including *N. mucosa* that exhibited the strongest effect ([Figure 47B-E](#)). Of note, this increase seemed higher than that induced with the lactobacilli. Likewise, I observed that these bacterial strains also induced an increase in lung Th17, which again was particularly high in the case of *N. mucosa* ([Figure 47B-E](#)). By contrast to the lactobacilli, these new strains also induced lung Th1 cells, and the total number of conventional lung Treg (but in lower proportion) ([Figure 47B-E](#)). Therefore, it appears that various pulmonary microbiota strains, similarly to strains from the gut, can modulate the local immune system with different intensities and profiles.

Discussion

As I have discussed throughout this manuscript, the microbiota is a key parameter of the host health. Alteration of its composition by environmental and genetic factors can impair responses of the host immune system to various challenges leading to aggravated outcomes in cases of infections, among others. Interestingly, this also seems to be the case in tuberculosis. However, the mechanisms involved in microbiota alteration of immune responses directed against pathogens is still poorly understood. This knowledge is crucial as it may allow the development of host-directed therapies targeting the microbiota with the potential to improve prevention and treatment of infection diseases, such as tuberculosis. Our results demonstrate that bacteria isolated from the lung microbiota (including *Lactobacillus species*) have the potential to modulate the local immune system and in particular, to induce Th17 and ROR γ ^t Treg. Importantly, induction of these cell populations by nasal administration of the *Lactobacillus species* is also observed in C57BL/6 mice infected with the H37Rv strain of *M. tuberculosis*. Of note, administration of the *Lactobacillus murinus* (CNCM I-5314) strain is associated with reduced lung leukocyte infiltration following *M. tuberculosis* infection. In this section, I will discuss how our results, and those from other teams, support the potential of *L. murinus* as a probiotic candidate and as a model microbiota strain to study interactions between the lung immune system and the respiratory microbiota.

Properties of *L. murinus* strains

The recent identification of the strain CNCM I-5314 as a *Lactobacillus murinus* strain allows comparison of our work with other studies describing this species. In human, *Lactobacillus species* are highly present in infants, playing key functions in host development. They are also present in the gastrointestinal tract of adults, in particular in the duodenum, but are not highly present in the lungs (Erb-Downward *et al.*, 2011; van der Gast *et al.*, 2011; Charlson, Diamond, *et al.*, 2012; Man, De Steenhuijsen PETERS and Bogaert, 2017; Tropini *et al.*, 2017). By contrast, *Lactobacillus species* were found to represent around 20% of detected species in the lungs of healthy SPF mice, and around 10% in wild-caught mice (Yun *et al.*, 2014). In particular, *L. murinus* has been described as a natural member of the gut microbiota in mice (Tang *et al.*, 2015; Wilck *et al.*, 2017). Isolation of this strain from the lungs of neonates mice by our collaborators suggests its presence at both sites (Remot *et al.*, 2017). Of note, *L. murinus* ASF361 strain is a component of the altered Schaedler flora (ASF), a bacterial mixture composed of 8 bacterial strains that are thought to represent the microbiota composition and functions of a normal microbiota (Brand *et al.*, 2015). Indeed, ASF administration compensate for most alterations observed in germ-free mice and is used by Taconic Farms as a basal inoculum introduced in all rederived mice.

Different studies have identified a decrease in *Lactobacillus species*, particularly *L. murinus*, in pathological conditions including gentamycin-mediated dysbiosis associated with susceptibility to pathobiont translocation, high salt diet associated with hypertension and autoimmunity, or colitis associated to *Lactobacillus species* depletion by AMP production by the host (Tang *et al.*, 2015; Wilck *et al.*, 2017; Singer *et al.*, 2019). On the contrary, *L. murinus* among other *Lactobacillus species* is increased in mice with lower gut permeability (mediated by vancomycin-mediated dysbiosis) and related lowered inflammation (by calorie restriction regimen in an inflammaging context) (Pan *et al.*,

2018; Singer *et al.*, 2019). In these models, administration of certain *L. murinus* strains (but not others) isolated from protected mice, recapitulates the protective effect, suggesting that some *L. murinus* strains have a strong potential as probiotics to prevent or ameliorates intestinal disorders.

Until now, no strains of *L. murinus* have been detected in humans (Wilck *et al.*, 2017). While it is still possible that strains of *L. murinus* are present and have not yet been detected, it is likely that *L. murinus* have poor fitness in this host. Therefore, administration of *L. murinus* in humans may not lead to establishment of this strain. Yet, this does not mean that this species is useless for microbiota-targeting therapies. For instance, it is probable that administration of *L. murinus* in humans will still yield beneficial interactions with human immune cells improving human health. Alternatively, if the ROR γ ⁺ Treg induced by *L. murinus* are responsible for diminishing the lung over-inflammation associated to tuberculosis in mice, then human lactobacilli strains (or strains from other families) can be screened for their capacity to modulate this cell population. Moreover, the identification of the *L. murinus*-derived components (*e.g.*, antigen, metabolite) exerting the beneficial effects in mice may further improve the screen for microbiota strains inducing immunomodulatory effect in humans (Tang *et al.*, 2015), and it may even foment the development of recombinant bacteria (suitable for administration in humans) carrying *L. murinus* antigens or producing same metabolites.

The capacity of *L. murinus* to engender health in the host seems mediated by different properties of *L. murinus*. First, it may influence colonization of mucosal sites by other species. In a study related to pathobiont translocation (due to neonate exposure to gentamycin), it was suggested that the protective effect exerted by *L. murinus* was mediated by the modulation of microbiota establishment during infancy (while not yet demonstrated). The intestine of neonates is aerobic, an environment tolerated by this species. Generation of fermentation products by *L. murinus* in this environment may change the oxygen tension in the intestine, favoring the colonization by other strains (Singer *et al.*, 2019). Such modulation of the environment by *Lactobacillus* species was shown in the vaginal mucosa, where their production of lactic acid maintains a low pH, influencing bacteria colonization (Turovskiy, Sutyak Noll and Chikindas, 2011). It is possible, while not addressed in our study that *L. murinus* CNCM I-5314 perform similar functions in the lung. Last, *L. murinus* may increase the disease tolerance mechanisms at mucosal sites, as different studies have described its immunomodulatory effect resulting in an overall inflammation decrease, which is in line with the results I generated in my thesis project in the lung context (Tang *et al.*, 2015; Lamas *et al.*, 2016; Wilck *et al.*, 2017; Pan *et al.*, 2018).

Deciphering the mechanisms involved in *L. murinus* induction of lung ROR γ ⁺ Treg

In our study, along with that by Tang and colleagues, the administration of *L. murinus* in mice results in Treg increase, and higher expression of TGF- β by these cells. While it was not clearly established, colonic Treg increased in their model are probably also ROR γ ⁺ Treg (Tang *et al.*, 2015). Interestingly, induction of ROR γ ⁺ Treg (and Th17) is not a feature shared by all *L. murinus* strains, as mice colonized by ASF (containing the *L. murinus* ASF361) have low levels of colonic Th17 and ROR γ ⁺ Treg (Ivanov *et al.*, 2009; Yang *et al.*, 2016). The fact that colonic ROR γ ⁺ Treg can be induced by different bacterial genera, but not by strains from the same species, raises the question about the mechanisms leading to their increase. This has been investigated in the gut and, while the mechanism is not fully understood, some factors have been determined. However, our study is the first one studying ROR γ ⁺ Treg induction in the lung and the mechanisms involved in our model may be slightly different to those already described.

As discussed in the introduction (Section 1.IV), the interaction of some *Lactobacillus* strains with APC result in Treg induction. While different mechanisms have been described depending on the strain used, they seem mostly to involve the recognition and binding of the lactobacilli by PRRs (such as DC-SIGN, TLR2, or NOD2), resulting in the induction of tolerogenic APC, and the secretion of retinoic acid, heme-oxygenase and/or IDO expression that favors the generation of Treg over other CD4⁺ T cell subtypes (Smits *et al.*, 2005; Foligne *et al.*, 2007; Fernandez *et al.*, 2011; Karimi *et al.*, 2012; Konieczna *et al.*, 2015). Importantly, Treg generated by tolerogenic APC in these experiments were not specific for the commensal having induced their generation; naïve T cells used in the assays were isolated from OTII mice⁵⁷ and were thus all specific for the OVA peptide. Interestingly, *L. murinus* induction of Treg in the gut was mediated by modulation of lamina propria APC that produced increased levels of IL-10 and TGF- β (Tang *et al.*, 2015). In other studies, APC production of IL-10 and TGF- β was also induced in the presence of different *Lactobacillus* species. While these cytokines were not linked to the APC capacity to induce Treg, they were involved in the protective anti-inflammatory effect exerted by the *Lactobacillus* species (Foligne *et al.*, 2007; Karimi *et al.*, 2012). Altogether, these studies suggest that some *L. murinus* strains can induce a tolerogenic profile in APC.

In our study, we did not demonstrate how *L. murinus* exerts its immunomodulatory effect in the lung, which could be direct or mediated by another commensal bacterial strain(s) favored by *L. murinus* administration. I propose to test the hypothesis of a direct effect through an *ex-vivo* co-culture system where the ROR γ t⁺ Treg induction may be driven from CD4⁺ T cells from OTII transgenic mice and OVA peptide-loaded APC that have been activated/conditioned by *L. murinus*. Alternatively, *L. murinus* co-culture with lung single-cell suspension may also increase ROR γ t⁺ Treg, which can be detected by flow cytometry. Indeed, a similar protocol with colonic cells leads to the increase of Foxp3, RORc and Tbx21 expression in CD4⁺ T cells after *L. murinus* stimulation (Tang *et al.*, 2015). Interestingly, this could be done in comparison with other pulmonary *Lactobacillus* strains, such as *L. rhamnosus*, which I showed has a poor capacity to induce lung ROR γ t⁺ Treg *in vivo*. To test the indirect effect, I would have carried out a 16S sequencing analysis of the lung microbiome of mice treated with *L. murinus*, *L. rhamnosus* (internal control) or mock (PBS), at different time points. In this manner, I could assess whether the administration of *L. murinus* results in dysbiosis of the local microbiome associated with the ROR γ t⁺ Treg generation. In parallel, I propose to test the capacity of *L. murinus* to induce lung ROR γ t⁺ Treg in the absence of other microbiota strains by either using axenic or antibiotic-treated mice. If this strain is still capable to induce lung ROR γ t⁺ Treg in either mouse model, then I can discard the potential indirect effect by favoring other microbiota communities.

For the rest of the discussion, I will assume that *L. murinus* has direct immunomodulatory properties. In this hypothesis, identifying PRR interacting with our *L. murinus* strain may be key to understand how this strain mediates important generation of ROR γ t⁺ Treg. It has been shown that APC (mouse bone marrow derived DC (BMDC) or human monocyte-derived DC) in co-cultured with *Lactobacillus* species are able to bind and phagocytose them, leading to their internalization in phagosomes (Foligne *et al.*, 2007; Konieczna *et al.*, 2015). However, the nature of the PRR in this process influenced the induction or not of the APC tolerogenic phenotype. Low expression of co-stimulatory molecules (CD80/CD86, CD40) and low production of cytokines (IL-10, IL-6, TNF- α , IL-12, IL-2) were also characteristic of the activation of the tolerogenic profile (Foligne *et al.*, 2007; Konieczna *et al.*, 2015). Of note, one study reported that a *L. murinus* strain bound poorly to DC-SIGN, induced the increase of co-stimulatory CD80/CD86 molecules on DC surface, and was not able to favor Treg induction over Th1 and Th17 (all

⁵⁷ T cells from the transgenic OTII mice have all a T cell receptor specific for chicken ovalbumin 323-339 peptide (OVA).

three subsets were induced) (Konieczna *et al.*, 2015). This is probably not the mechanism at play in the case of our *L. murinus* strain. While I observed that *L. murinus* administration induced the presence of lung Th17 (along with an anti-inflammatory phenotype), Th1 and classical Treg were not affected *in vivo*, suggesting a selective induction capacity. In addition, in a preliminary experiment, co-culture of *L. murinus* with BMDC did not modify DC-SIGN expression and poorly induced CD86 expression compared to *L. salivarius* (data not shown).

To identify the PRR responsible for recognition of our *L. murinus*, I propose the use of commercially available PRR (or adaptor molecules) reporter cell lines. Among PRR, I predict that our *L. murinus* strain may bind Mincle, which was previously shown to recognize a *L. plantarum*-derived glycolipid (Shah *et al.*, 2016; Chellappan *et al.*, 2019). Mincle recognition of this glycolipid was shown to reinforce the intestinal immune barrier *via* the induction of intestinal Th17- and ILC3-driven production of IL-17 and IL-22 (Martínez-López *et al.*, 2019). It was also associated with restored controlled of *M. tuberculosis* burden in infected mice treated with antibiotics *via* Th1 and Th17 induction (Negi *et al.*, 2019). Therefore, the potential binding of *L. murinus* to Mincle is likely to be responsible for the induction of Th17 and overall production of IL-17 observed in our model. While I cannot exclude that Mincle is also involved in the generation of ROR γ ⁺ Treg, *L. murinus* strain may bind to a different PRR. Hence, the identification of PRR(s) recognizing *L. murinus* may allow a better understanding of its immunomodulatory properties. In addition, at the end of this PhD, I was able, thanks to a collaboration with Dr. Jérôme Nigou (IPBS, Toulouse), to prepare fractions from our *L. murinus* strain containing either lipopolysaccharides, lipopolysaccharides with RNA, and proteins or lipids (not shown). The use of these fractions with the reporter cell lines may facilitate the identification of components triggering different PRR. Co-culture experiments of APC, primed with these *L. murinus*-derived components and loaded with a specific peptide (*e.g.*, OVA), and naïve transgenic T cells (*e.g.*, OTI-II), will likely facilitate the identification of the molecular factors involved in the induction of ROR γ ⁺ Treg. In this context, I performed intranasal administration of heat-killed *L. murinus* and compared its effect on the generation of lung CD4⁺ T cell subsets to administration with the live bacteria. Unfortunately, I obtained contradicting results in two independent experiments; one suggesting that the heat-killed bacteria poorly induced ROR γ ⁺ Treg and Th17 compared to mock (PBS) or live *L. murinus* treatment, while in the second one the induction of both populations was higher with the heat-killed than with the live strain (data not shown). Future experiments will be carried out to define this issue, and to test the different components of *L. murinus*.

If the above experiments demonstrate that heat-killed *L. murinus* cannot induce ROR γ ⁺ Treg (or only partially), it is also possible that the secretion of microbial-derived soluble factors (*e.g.*, metabolites) are responsible for this effect. Indeed, while *Lactobacillus species* do not produce SCFA, *L. murinus* CNCM I-5020 strain metabolizes tryptophan in indole derivatives that are AhR ligands (Lamas *et al.*, 2016). A lactobacilli mixture containing *L. murinus* CNCM I-5020 was involved in this pathway resulting in increased ILC3-production of IL-22, ultimately leading to AMP production and tissue repair (Lamas *et al.*, 2016). Also,IDO generation of such indole tryptophan derivatives by DC activates AhR activity, which is involved in the generation of Treg versus Th17 in the lungs (de Araújo *et al.*, 2017). Therefore, production of AhR ligands by our *L. murinus* strain could be a mechanism inducing ROR γ ⁺ Treg. Alternatively, it is also possible that the APC phenotype is modulated by cytokines produced by epithelial cells in contact with the bacteria. However, in the co-culture model using lung explants developed by our collaborators, no modification of cytokine production was reported in presence of *L. murinus*, unlike that obtained with *L. salivarius* and *L. rhamnosus* strains (Table 6).

Another approach to identify the mechanisms involved in the induction of lung ROR γ ⁺ Treg would be to characterize more in detail the interaction between *L. murinus* and the lung APC compartment. This

is a pertinent issue given that the interaction between commensals and lamina propria DC does not induce same phenotypes as exerted in spleen DC (Tang *et al.*, 2015). In addition, induction of iTreg in the lung is poorly characterized even though it is a key mechanism involved in the ability of pulmonary commensal microbes to generate a local tolerogenic environment during development (Gollwitzer *et al.*, 2014). The induction of lung iTreg providing tolerance to allergens is established by lung macrophages producing retinoic acid and TGF- β (Soroosh *et al.*, 2013). Because these factors have been implicated in generation of ROR γ ⁺ Treg, it is possible that this is also how commensal specific lung iTreg are induced (Lochner *et al.*, 2011; Ohnmacht *et al.*, 2015; Yang *et al.*, 2016; Al Nabhani *et al.*, 2019).

In this front, I provided the partial characterization of the lung myeloid compartment in mock- and *M. tuberculosis*-infected mice (day 42 post-infection) treated (or not) with *L. murinus*. In general, I did not observe any significant changes in terms of total cell number from the major myeloid-derived populations, such as neutrophils, eosinophils, monocytes, macrophages or DC. Intriguingly, however, alveolar macrophages exhibited an anti-inflammatory phenotype characterized by CD16^{hi}CD206^{hi}PDL-1^{hi}CD86^{low}CD80^{low} (Figure 41). This cell-surface marker signature, including the high ratio between PDL-1 and CD86, has been previously associated to immuno-suppressive macrophages that downregulate the capacity of CD4⁺ T cells to proliferate and produce IFN- γ (Lastrucci *et al.*, 2015). The establishment of this cell-surface marker signature in alveolar macrophages appears to be independent of the *M. tuberculosis* infection, as those from mock-infected mice displayed a similar phenotype. Moreover, this phenotype seems specific to alveolar macrophages, as other cells including interstitial macrophages fail to acquire it. Of course, while these results point towards alveolar macrophages as the main APC modulated by *L. murinus*, and the main candidate responsible for the induction of ROR γ ⁺ Treg, I cannot discard that other APCs are probably involved. In fact, the characterization performed at day 42 post-infection is definitely a late time point to observe the true effects of *L. murinus* within the myeloid compartment. Thus, future experiments will assess the myeloid compartment at different early time points of *L. murinus* administration and during *M. tuberculosis* infection. I predict that the DC compartment might be heavily modulated during the first week following *L. murinus* administration.

If it is the case, I propose to combine *ex vivo* and *in vivo* approaches to characterize *L. murinus* modulation of APC phenotype in order to investigate the mechanisms leading to ROR γ ⁺ Treg generation. While the use of lung cells is challenging because few APC can be sorted from the lungs, it is still worth the trouble as mucosal APC do not exactly behave as spleen or bone marrow APC (Tang *et al.*, 2015). In fact, it could be interesting to compare APC from different compartments to assess if *L. murinus* modulations are tissue-specific, and thus improving our understanding of microbiota regulation of the immune system at mucosal sites. Characterization of lung APC sorted from mice (treated with *L. murinus* or mock) by qPCR (targeting RALDH enzymes, IDO, AhR or TGF- β), may facilitate to determine which of them (and how) are modulated by *L. murinus* (Soroosh *et al.*, 2013). This approach should be complemented by co-culture of *ex vivo* *L. murinus*-induced lung APC (loaded *in vitro* with OVA) and naïve T cells (from OTII mice) to characterize their capacity to differentiate ROR γ ⁺ Treg (and Th17) generation (by qPCR, FACS phenotyping and ELISA). Alternatively, adoptive transfer of lung APC, loaded with *L. murinus* (either sorted from *L. murinus* treated mice or *ex-vivo* co-cultured), into untreated mice should lead to the generation of ROR γ ⁺ Treg, if our hypothesis is true.

This type of characterization may lead to the identification of molecular pathway involved in the induction of Treg. Blocking these pathways with blocking antibodies (e.g., anti-TGF- β), or with inhibitors of the molecules identified (e.g., retinoic acid receptor inhibitor), or by using APC from mice deficient for such factors (e.g., IDO1^{-/-} or AHR^{-/-} mice), may prove critical to demonstrate their

pertinence (de Araújo *et al.*, 2017; Al Nabhani *et al.*, 2019). Such type of approaches were successful to show, for example, the protective effect of Treg generated by BMDC loaded with *Lactobacillus rhamnosus* during a TNBS-induced colitis model (Foligne *et al.*, 2007). The use of live, heat-killed, isolated bacterial components or supernatants from *L. murinus* cultures, may also shed light on which bacterial component are involved in activating these molecular pathways. Likewise, the use of alternative *L. murinus* strains (for example isolated from other sites such as the gut, from different mouse strains or from mouse models of different respiratory diseases), might help to decipher if all members of this species are capable to induce lung ROR γ ⁺ Treg using the same or different mechanisms. Finally, I provided evidence that other lactobacilli, as well as other pulmonary bacterial strains (e.g., *N. mucosa*), are also able to induce lung ROR γ ⁺ Treg; the use of these strains may provide further clues to understand whether specific (or shared) mechanisms are involved in the generation of ROR γ ⁺ Treg among lung microbiota strains.

Characterization of resident and *L. murinus*-induced lung ROR γ ⁺ Treg

Lung ROR γ ⁺ Treg have been previously identified by Lochner and colleagues (Lochner *et al.*, 2008). Yet, they were probably previously observed by other groups that characterized the lung Treg population without assessing their expression of ROR γ , as I show that most Helios^{lo} Treg were indeed ROR γ ⁺ Treg. Indeed, one of the main contributions of thesis work is the first formal characterization of lung resident ROR γ ⁺ Treg as Helios^{lo}CD25^{lo}ICOS^{int}CTLA-4^{hi}PD-1^{int}CCR6^{hi}IL-17^{int}TGF- β 1^{hi}IL-10^{lo}TNF^{lo}, whose total numbers are increased and phenotype is further accentuated (Helios^{lo}CD25^{lo}ICOS^{hi}CTLA-4^{hi}PD-1^{hi}CCR6^{hi}IL-17^{hi}TGF- β 1^{hi}IL-10^{lo}TNF^{lo}) by *L. murinus*.

I compared the expression of several cell-surface markers that distinguish their counterparts in the gut or lymph nodes; these markers are also partially shared by Th17 and conventional Treg from the lungs (Figure 44). Importantly, as in the gut, lung ROR γ ⁺ Treg expressed ICOS, which is considered as a marker of activated T cells, and as a mucosal Treg marker that is essential for their development and production of IL-10 (Brusse J Immunol 2012, RedpathEur J Immunol 2013). Lung ROR γ ⁺ Treg highly expressed CTLA-4, indicating a potential immuno-suppressive capacity (Ohnmacht *et al.*, 2015). In support of this, they also expressed PD-1 (and at higher levels than conventional Treg), which has not been assessed at steady states in other organs, but was observed in the lymph node ROR γ ⁺ Treg upon different types of stimulation (Kim *et al.*, 2017). Importantly, Gollwitzer and colleagues report that the set-up of a tolerogenic lung environment during colonization by the microbiota was dependent on the generation of PD-1⁺ Helios⁻ Treg (Gollwitzer *et al.*, 2014). In our experiments, *L. murinus* further accentuates the ICOS and PD-1 expression in lung ROR γ ⁺ Treg. Comparison of our results with the available literature suggests that lung iTreg induced during microbiota acquisition are indeed the ROR γ ⁺ Treg, and this Treg subset likely possesses anti-inflammatory functions (Al Nabhani *et al.*, 2019).

Unlike in the spleen, I was not able to detect CD25 expression on lung ROR γ ⁺ Treg or conventional Treg (Data not shown and Figure 44C). Another study suggests that Foxp3⁺CD25⁻ CD4⁺ T cells are the dominant Treg population in the lung (and also in the gut). Yet, unlike our study, the authors found that 40% of lung Treg still expressed CD25 (Coleman FEMS Immunol Med Microbiol 2012). In addition, Lochner and colleagues detect CD25 expression on lung Treg (independently of their expression of ROR γ) (Lochner *et al.*, 2008). Determining expression of CD25 is important because it acts as a “sink” for IL-2 consumption on Treg to deprive and inhibit effector T cell functions; such mechanism has not yet been described for ROR γ ⁺ Treg. We also observed that, as in the colon, lung ROR γ ⁺ Treg express

CCR6 at higher levels compared to conventional Treg, indicating these cells have the capacity to migrate in CCL20-dependent manner at sites of inflammation (Jetten *et al.*, 2008; Ohnmacht *et al.*, 2015; Yang *et al.*, 2016). Of note, Lochner and colleagues show that lung ROR γ ⁺ Treg also secrete CCL20, and propose that it may help them to track Th17 (highly expressing CCR6) migration towards inflammatory sites, where they can later inhibit the production of IL-17 to decrease inflammation (Lochner JEM 2008). While we confirm that lung Th17 also express CCR6, we did not stain for CCL20, and thus we cannot determine whether this hypothesis is valid in the tuberculosis context.

Regarding the cytokine profile, lung ROR γ ⁺ Treg produce IL-17A accompanied by noticeable high levels of the anti-inflammatory TGF- β propeptide and low levels of the pro-inflammatory TNF (Figure 45), which becomes strongly accentuated in mice treated with *L. murinus* compared to controls. IL-17 production by these cells was described to be pathologic in other contexts, and linked to aggravated inflammation (Esposito *et al.*, 2010; Kryczek *et al.*, 2011; Blatner *et al.*, 2012; Chellappa *et al.*, 2016; Kluger *et al.*, 2016, 2017). However, I propose that the ROR γ ⁺ Treg-driven production of IL-17A, in combination with a high ratio of anti-inflammatory to pro-inflammatory cytokines, is likely to have a homeostatic role that contributes to tissue integrity. This is also reflected in the simultaneous induction by *L. murinus* of Th17 cells expressing immunosuppressive markers, such as TGF- β , CTLA-4 and PD-1 (an issue discussed later on).

Importantly, we recently obtained Foxp3^{RFP}ROR γ ^{GFP} reporter mice from the lab of Dr. Gérard Eberl from the Pasteur Institut, Paris, France. The Foxp3^{RFP}ROR γ ^{GFP} reporter mice will help us to dissect many of these issues. For example, it will allow the simultaneous FACS sorting of these populations after *L. murinus* treatment to better characterize their phenotype using qPCR analysis, test their capacity to respond to stimuli *ex vivo* and define their cytokine/chemokine secretion content, and perform adoptive transfers into different mouse models, as detailed in the following section.

A role of lung ROR γ ⁺ Treg in Type 2 immunity

It is described that gut ROR γ ⁺ Treg inhibit type 2 immune response defined by the hallmark Th2 subset, expressing the GATA3 transcription factor. This is an aspect that was not explored in this project because, unlike Th1, Th17 or Tregs, Th2 cells are not critical for the etiology of tuberculosis. Yet, the generation of lung ROR γ ⁺ Treg may be one mechanism by which a microbiota at eubiosis reduces susceptibility to asthma (in particular during development). This hypothesis is strongly supported by the study of Gollwitzer and colleagues showing the reduction of type 2 cytokines in response to allergic challenge upon commensal-mediated generation of iTreg in the lungs or upon adoptive transfer of spleen Treg (Gollwitzer *et al.*, 2014). Our collaborators observed decreased susceptibility to asthma in neonates receiving nasal delivery of the lung commensal *Enterococcus faecalis* that was also associated to decreased type 2 immunity (Remot *et al.*, 2017). It would be interesting to show if this protection against asthma/allergy is associated to an increase of lung ROR γ ⁺ Treg. If it is the case, then we could adopt a “gain-of-function” approach by performing intranasal adoptive transfer of lung ROR γ ⁺ Treg isolated from Foxp3^{RFP}ROR γ ^{GFP} reporter mice treated with *Enterococcus faecalis* or with *L. murinus*. Of note, a mouse model exist to deplete ROR γ ⁺ Treg, the Foxp3-cre \times Rorc^{fl/fl} mice, which have been successfully used to demonstrate importance of ROR γ ⁺ Treg in protection against colitis (Sefik *et al.*, 2015). Such “loss-of-function” approach may be used to complement the adoptive transfer strategy to unequivocally demonstrate a role for lung ROR γ ⁺ Treg in diseases related to type 2 immunity. If type 2 immune response inhibition is mediated by ROR γ ⁺ Treg, it could be studied *in vitro* to investigate molecular mechanisms involved.

Suppressive functions of lung ROR γ t⁺ Treg may be assessed using co-culture assays with CFSE stained T cells from OTII transgenic mice and OVA peptide loaded APC. In particular, naïve CD4⁺ T cells from OTII mice can be differentiated *ex-vivo* toward distinct CD4⁺ T cell subsets using anti-CD3, anti-CD28, IL-2 and specific polarization cytokines. They can be driven toward a Th2 phenotype using IL-4 and IL-5/IL-13, combined with anti-IFN γ and anti-IL-12; toward a Th1 phenotype in presence of, IL-12 and anti-IL-4; and toward a Th17 phenotype by the presence of anti-IL-4 and anti-IFN γ . Their use as responder T cells in the suppressive assay may allow us to determine if the *L. murinus*-induced lung ROR γ t⁺ Treg suppress Th2 proliferation or modulate the Th17 subset, along with factors involved in either process. Inhibition of Th2 by ROR γ t⁺ Treg has been shown to be dependent on CTLA-4 (Ohnmacht *et al.*, 2015), but additional suppressive functions of Treg could be involved, as previously discussed and could be investigated in this suppressive assay.

Characterization of resident and *L. murinus*-induced lung Th17 cells

Lung resident Th17 cells are scarcely present in the lung of C57BL/6 mice at steady state, and they displayed the ICOS^{int}CTLA-4^{lo}PD-1^{lo}CCR6^{int}IL17A^{int}TGF- β 1^{int}TNF^{int} phenotype. Of note, to the best of my knowledge, the expression of CTLA-4, PD-1 or TGF- β has not been previously described in these homeostatic lung Th17. In my thesis work, I now report that the *L. murinus* administration increases significantly the total number of lung Th17, and strongly establishes the ICOS^{hi}CTLA-4^{hi}PD-1^{hi}CCR6^{hi}IL17A^{hi}TGF- β 1^{hi}TNF^{lo} phenotype, which I propose, may have homeostatic and anti-inflammatory functions similarly to lung ROR γ t⁺ Treg.

IL-17 production by Th17 is usually associated with autoimmunity, but this cytokine has other multiple roles. In particular, while its over-production leads to chronic inflammation and fibrosis (through TGF- β promotion) in the lungs, IL-17 also participates to resistance to infection by pulmonary pathogens and can improve epithelial damage repair through IL-22 induction (Gurczynski and Moore, 2018). In particular, it was described that Th17 induced by commensals (e.g., SFB) have an anti-inflammatory phenotype when compared to those induced by pathogens (Omenetti *et al.*, 2019). In this study, for example, Th17 induced by SFB were characterized by IL-22 and IL-17 production, along with an oxidative phosphorylation metabolic state, which is associated with immune cell anti-inflammatory properties. By contrast, pathogen-elicited Th17 cells show high plasticity towards pro-inflammatory cytokines (e.g., IFN- γ), dependency on aerobic glycolysis metabolic state (associated with pro-inflammatory functions), and a better engagement in *C. rodentium* clearance. Importantly, SFB-induced Th17 were shown to not be involved in models of systemic pathological inflammation, such as EAE (Experimental autoimmune encephalomyelitis). In fact, they do not participate in *C. rodentium* clearance, but instead decrease the barrier permeability and favor epithelium damage repair occurring during pathogen infections, and thus reducing pathogen invasion (Edelblum *et al.*, 2017). *L. plantarum* is also able to induce Th17 producing IL-17 and IL-22 that are essential for the maintenance of the intestinal barrier, which was dependent on the capacity of DC to recognize *L. plantarum* via Mincle (Martínez-López *et al.*, 2019).

These studies may be in accordance with the expression of suppressive markers observed in our model on *L. murinus*-induced Th17. Unfortunately, the key cytokines to assess this phenomenon in our model were not assessed in lung Th17; IL-22 was poorly detected by FACS and IFN- γ staining was not included in the antibody mix containing ROR γ t. Detection of these critical factors either by optimization of this flow cytometry staining strategy or by other methods (qPCR or ELISA on sorted cells in culture), may allow the corroboration of an anti-inflammatory profile of Th17 cells generated by *L. murinus*. Beyond

these cytokine issue, I was able to measure other factors that may support the homeostatic role for lung Th17. First, *L. murinus* significantly decreases the capacity of these cells to produce the pro-inflammatory TNF. And last, I demonstrate the induced lung Th17 cells failed to co-express T-bet along with ROR γ t (Figure 5); T-bet expression in Th17 cells has been associated to a pathogenic role affecting the ration of Th1/Th17 (Lyadova and Panteleev, 2015). These observations are crucial because high presence of *Lactobacillus species* observed in mouse model of COPD was associated with the pathological increase of IL-17 that aggravate the disease (Yadava *et al.*, 2016). Therefore, more experiments are required to assess the role of lung ROR γ t⁺ Treg and Th17 induced in our model. The use of the previously discussed reporter mice may allow us to assess other parameters characterizing lung ROR γ t⁺ Treg and Th17 induced, such as metabolism as assessed for Th17 in the study with SFB (Omenetti *et al.*, 2019). This could be achieved using the Seahorse technology that can measure the live oxygen consumption rates (indicator of oxidative phosphorylation), extracellular acidification rates (indicator of glycolysis), mitochondrial respiration or energetic phenotypes (measuring glycolysis potential). If both cells harbor anti-inflammatory profiles, I predict that the *L. murinus*-induced Th17 should be characterized by oxidative metabolism. This aspect could reinforce their characterization as homeostatic rather than pathologic cells. Whatever the case may be, I consider that the *L. murinus*-induced IL-17 production in these cells is likely to be beneficial for *M. tuberculosis*-infected mice because the bacterial burden is unchanged even though the leukocyte infiltration is significantly reduced (Gurczynski and Moore, 2018).

A role for ROR γ t⁺ Treg and Th17 induced by commensals in tuberculosis?

In our model of *M. tuberculosis* infection, both lung Th17 and ROR γ t⁺ Treg are significantly increased in mice having received *L. murinus* compared to controls. This is interesting because it suggests that the strong immunomodulatory properties of *M. tuberculosis* do not invalidate those previously established by the *L. murinus* administration. This was previously described in the gut, as the prior generation of Th17 by SFB was conserved during *Listeria monocytogenes* infection (Yang *et al.*, 2014). Our data thus suggest that this effect may be common to all commensal-induced T cells.

The role of Th17 in tuberculosis is starting to be comprehended and has both protective and pathological consequences. It is possible that this dual effect is due to effect of different Th17, as previously proposed in the gut (Omenetti *et al.*, 2019). Th17 participate in control of pathogen load and recruitment of neutrophils during tuberculosis (Torrado and Cooper, 2010; Gallegos *et al.*, 2011). However, while I observed the increased of lung Th17 and IL-17-producing CD4⁺ T cells, there was no significant reduction of the pathogen growth or increased neutrophils recruitment in mice infected by *M. tuberculosis* and having received *L. murinus* administration (Figure 38, 39, 40 and 42). Therefore, I predict that induction of Th17 cells in our model is a specific response to commensals and not to pathogen, and these may not be involved in mechanisms of resistance toward *M. tuberculosis*, lacking a pro-inflammatory potential. Alternatively, IL-17 has other functions such as granuloma development to contain the pathogen (Okamoto Yoshida *et al.*, 2010). Granuloma formation could be considered as a result of activation of disease tolerance mechanisms when the microbicidal mechanisms were not sufficient to rapidly eliminate the pathogen and potentially lead to tissue damage. In this hypothesis, induction of granuloma may be mediated partially by commensal-specific Th17 cells participating in pathogen containment and preservation of host tissue. While the C57BL/6 mouse model used for my thesis work is not optimal to study granuloma formation in response to *M. tuberculosis*, I predict that *L. murinus*-induced Th17 cells could have an impact on the incidence of necrotic granuloma and the

bacillus extra-pulmonary dissemination in the C3HeB/FeJ mouse model (as discussed in the following subsection).

The role of Treg in tuberculosis is also controversial as their early presence in the lung is thought to allow bacterial escape from immune control. By contrast, Treg presence is known to be critical to avoid over-inflammation leading to necrosis and tissue damage at later stages of infection (Windish *et al.*, 2009). It has been shown that *M. tuberculosis*-specific Treg are generated in the lymph node during the infection, and participate in the delay of the Th1 response observed in tuberculosis (Shafiani *et al.*, 2013). However, it is unknown if these cells belong to the ROR γ t⁺ Treg subtype, and whether the ROR γ t⁺ Treg induced by commensals could alter the specific response to the pathogen. Therefore, the role of ROR γ t⁺ Treg in tuberculosis remains to be determined. If ROR γ t⁺ Treg are able to inhibit Th2 during *M. tuberculosis* infection, it is possible that they may contribute to the development of the Th1 response. Yet, this is unlikely the case in our model because I did not observe differences in the number of Th1 cells at day 42 post-infection. Another aspect they could regulate is neutrophil-mediated tissue damage in active tuberculosis. Neutrophils may be protective at the beginning of the infection to avoid bacterial spread, but they often tend to be detrimental if their abundance becomes too high at later stages of infection due to their toxic inflammatory properties. However, I did not see that the neutrophil levels changed in the lungs at 42 post-infection in mice infected with *M. tuberculosis* depending on *L. murinus* administration. Therefore, it is unlikely that lung ROR γ t⁺ Treg regulate neutrophil activity in our model. Finally, I favor the idea that lung ROR γ t⁺ Treg may regulate the induction of *M. tuberculosis*-specific Th17, to limit the pathogenesis of tuberculosis. As detailed in the Introduction (Section 1.II.3), conventional Treg can induce the expression of IDO in DC *via* CTLA-4. This results in the production of AhR ligands, whose activation favor the generation of Treg over Th17 cells. In the tuberculosis context, one study demonstrated that IDO expression by lung epithelial and endothelial cells reduced *M. tuberculosis*-specific Th17 associated with neutrophilic inflammation and mortality (Desvignes and Ernst, 2009). As the lung ROR γ t⁺ Treg induced by *L. murinus* express high levels of CTLA-4, they may similarly down-modulate the generation of *M. tuberculosis*-specific Th17 cells, ultimately reducing lung immunopathology.

At the moment, I have not assessed the specificity of Th17 and ROR γ t⁺ Treg induced by *L. murinus* at steady state or during tuberculosis infection. If my hypothesis above is correct, I predict these cell populations are specific for *L. murinus* rather than for *M. tuberculosis*. To demonstrate T cell specificity, conventional Treg, ROR γ t⁺ Treg and Th17 could be sorted from the lungs of infected mice having received *L. murinus* administration. These T cells could then be labeled with CFSE and co-cultured with lung APC that would be pre-loaded with materials either from heat-killed *L. murinus* or *M. tuberculosis*. I would then assess the T cell proliferation after 3-5 days of co-culture and their capacity to produce key cytokines, which will reveal their activation if their cognate antigen is presented by APC (Yang *et al.*, 2014; Hepworth *et al.*, 2015). Since *ex-vivo Lactobacillus strains* can induce the generation of non-lactobacilli specific Treg, it is probable that this assay reveals that some of the ROR γ t⁺ Treg and Th17 induced by *L. murinus* are in fact *M. tuberculosis* specific. This would imply that our strain is able to modify the phenotype and functional profile involved in the response to this pathogen, favoring tolerance mechanisms at a late time point.

Determination of the role of ROR γ t⁺ Treg in tuberculosis could be evaluated using the Foxp3-cre \times Rorc^{fl/fl} mice to specifically delete this cell subset from the Treg compartment. Comparison of results obtained in this model with the CD4-Cre \times STAT3^{fl/fl} mice (Raz *et al.*, 1999; Wu *et al.*, 2015), where both Th17 and ROR γ t⁺ Treg should be depleted, would be even more interesting. In this manner, we might determine the relationship between these two cell populations and their role in the tuberculosis

disease outcome. Adoptive transfers may also be useful to assess involvement of *L. murinus*-specific Th17 and Treg during tuberculosis infection and associated lung inflammation.

Regulation of lung inflammation associated to tuberculosis by *L. murinus*

In many studies, leukocyte infiltration following *M. tuberculosis* infection, also described as granulomatous region formation, is associated to immunopathology leading to tissue damage. Antibiotherapy reduces bacterial load but does not prevent lung lesion formation if started at a late stage (Malherbe *et al.*, 2016). Therefore, host-directed therapies protecting or inducing reparation of lung tissue, without fomenting the bacteria growth, are highly needed to complement anti-tuberculosis antibiotics. In the context of my thesis work, it is difficult to definitely conclude about a protective role of *L. murinus* administration in this disease, in particular because the effect on leukocyte infiltration and pro-inflammatory cytokine secretion was minor (albeit significant) compared to controls (Figure 42). One factor influencing these results is the choice of our animal model. Unlike the guinea pig, for example, the mouse model is not ideal to study the immunopathology associated with tuberculosis. Yet, due to cost, space, and ethical reasons, the mouse model is still the most common one used in the investigation of tuberculosis. This is also the only animal model adapted to the BSL3 facility at our institute. Another influencing factor affecting our results is the choice of the C57BL/6 genetic mouse background. C57BL/6 mice were chosen because it is the best studied genetic background in tuberculosis research, and due to availability of many knockout and transgenic mouse models in this background. This is useful to study mechanisms involved in the observed phenotype as proposed previously. However, the C57BL/6 background does not mimic all stages of the tuberculosis disease. In fact, mice of this background are quite resistant to *M. tuberculosis* infection, granuloma formation does not recapitulate the classical features as in humans, and they develop few necrosis either at the beginning of the infection (macrophages efficiently contain the bacilli and few neutrophils are recruited) or at the late stages of the disease (they do not develop necrotic lung lesions) (Kramnik and Beamer, 2016). Accordingly, we observed *M. tuberculosis* growth and induction of type-1 immune response, but no hyper-activation or lung lesions in response to *M. tuberculosis*.

The 129/Sv mouse model was described in the literature as a susceptible genetic background to tuberculosis compared to C57BL/6 (Medina and North, 1998; Dorhoi *et al.*, 2014). One of the main reasons is that these mice succumb to uncontrolled inflammation driven by the type I IFN signaling pathway, which regulates neutrophil-mediated pro-inflammatory response in tuberculosis (Dorhoi *et al.*, 2014; Mayer-Barber *et al.*, 2014). This model therefore looked interesting to study the effect of *L. murinus* on immunopathology associated with tuberculosis infection. However, in this model we observed no changes in leukocyte infiltration according to *L. murinus* administration (Figure 43). This means that *L. murinus* administration has lower effect on immunopathology than we expected, or that the mechanism induced by our bacterial strain does not target the type I IFN signaling pathway. It should be noted that in the study by the team of Dorhoi, most of the 129/Sv mice died of *M. tuberculosis* infection at day 40 post infection (Dorhoi, Eur J Immunol 2014). In my hands, while the granulomatous region formation was increased in this model compared to the C57BL/6, no mice died from the infection; in fact, the mice did not even lose weight or exhibit temperature changes during the infection (data not shown), suggesting that I did not perform these experiments in the most optimal conditions as previously reported.

As mentioned earlier, the C3HeB/FeJ mice that develop necrotic granuloma lesions and may even develop cavities during tuberculosis infection as human, may also represent an attractive model for my thesis project (Kramnik and Beamer, 2016). In particular, it was shown in these mice that induction

of Treg can reduce immunopathology and improve survival of infected animals (Cardona *et al.*, 2016). In this study, the Treg were induced by administration of an environmental mycobacterium *Mycobacterium manresensis*, and were reactive to *M. tuberculosis* antigens. Heat-killed preparation of this bacteria is now commercialized under the name Nyaditum resae® and its administration in humans increases specific Treg (Montané *et al.*, 2017). While efficacy tests are ongoing, these studies suggest that the C3HeB/FeJ mouse model would be ideal to assess if commensal-induced RORγt⁺ Treg can protect against immunopathology and the potential of *L. murinus* as a probiotic strain for tuberculosis. At the end of my thesis, I tried one experiment with these mice background. However, a technical problem at the infection stage resulted in low infection load, and consequently provoked very low incidence of inflammation and leukocyte infiltration in control mice (data not shown). Due to time constraints, this experiment was not reproduced during the thesis.

A third factor influencing our results is the choice of the *M. tuberculosis* experimental strain: H37Rv. While IL-17 may be dispensable to control infection by the H37Rv, these cells are essential in response to the hypervirulent clinical isolate Beijing strain HN878, which causes exacerbated immunopathology in infected animals. The HN878 infection model demonstrated that IL-17 signaling is important for localization of T cells near infected macrophages in the granuloma, which is key for activation of their microbicidal functions (Gopal *et al.*, 2014). Therefore, *L. murinus* administration that increased IL-17 levels may be protective in response to this emerging *M. tuberculosis* strain.

Finally, susceptibility of the different mouse backgrounds to tuberculosis is usually associated to genetic variations, but it may also be correlated to microbiota divergence between mouse strains (Ericsson *et al.*, 2015). Probiotic administration may have a different effect depending on the composition of the microbiota. It is possible that the immune response induced by *L. murinus* in C57BL/6 mice results redundant to an already existing cooperation of the resident microbiota and host immune system. Indeed, *Lactobacillus* strains are already highly present in the lungs of SPF C57BL/6 mice, and thus the protective effect exerted by *L. murinus* (or of strains with similar effect) may be already happening in PBS treated mice. Microbiota divergence between different mouse backgrounds may also influence the capacity of *M. tuberculosis* to interact with the host. *M. tuberculosis* infection, which induces dysbiosis of the microbiota, may also impede the protective immunomodulatory effect established by the resident microbiota. This may be due to direct interaction between the pathogen and the resident microbiota (competition for nutrients, secretion of antimicrobial factors or modification of the environment), or by the capacity of the bacillus to turn the immune response against the commensal strains, such as AMP production that reach non-invasive bacteria and are not specific for the pathogen. For instance, Dectin-1 activation is involved in the induction of the signaling molecule S100A8/A9, which has an AMP activity shown to deplete the gut microbiota of *Lactobacillus* species, including *L. murinus* (Tang *et al.*, 2015). In this study, such loss of *Lactobacillus* species resulted in reduction of iTreg and consequently to susceptibility to colitis. Dectin-1 recognizes β-glucan of *M. tuberculosis* and is overexpressed on the surface of alveolar epithelial cells infected with mycobacteria (Lugo-Villarino *et al.*, 2011). S100A8/A9 are produced during tuberculosis and mediate neutrophil accumulation and inflammation associated with immunopathology in tuberculosis (Gopal *et al.*, 2013). These effects are usually attributed to a S100A8/A9-dependent role as a signaling molecule between immune cells. However, it probably mediates late inflammation via the targeted loss of *Lactobacillus* species that induce RORγt⁺ Treg. Similar to the gut, this would suggest that, while Dectin-1 has an important role to patrol against pathogen invasion, it also increases the risks to develop unnecessary inflammation in the lung (Tang *et al.*, 2015). Interestingly, loss of *Lactobacillus* species (among other commensals) after antibiotic treatment is associated with susceptibility to the pathogenic infection in mice that could be reduced by oral administration of *Lactobacillus plantarum*. However, immunopathology was not assessed in this study. Some dysbiosis conditions, such as infection by

Helicobacter hepaticus, lead to aggravated immunopathology, associated with altered CD4⁺ T cell response (Majlessi *et al.*, 2017). Testing nasal administration of *L. murinus* in models of dysbiosis caused by tuberculosis co-morbidity parameters (such as HIV infection, type 2 diabetes, or malnutrition among others), or due to antibiotic therapy against tuberculosis, may be the most obvious scenario to best define whether this pulmonary strain could be a probiotic candidate reducing immunopathology in tuberculosis and for which type of patients.

Applications to other respiratory diseases

L. murinus immunomodulatory effects on the lung were similar in absence of *M. tuberculosis* infection, suggesting that this strain may be useful for other respiratory infections associated with over-reaction of the immune system and dysbiosis of the microbiota. This could be the case of chronic infections such as COPD, in which disease severity correlates with high neutrophil-secreting proteases infiltration, high Th1 and Th17 levels and decrease in Treg in the lung (Rovina, Koutsoukou and Koulouris, 2013), or such as cystic fibrosis associated with high Th1, Th2 and Th17 response, high neutrophil and eosinophil infiltration, epithelium damages and mucus production (Yonker *et al.*, 2015). Of note, cystic fibrosis can be caused by another mycobacterium: *Mycobacterium abscessus*. One study suggests that the oral administration of *Lactobacillus animalis* (which is phylogenetically the closest species to *L. murinus*) modulates the immune response during *Mycobacterium avium subspecies tuberculosis*⁵⁸ infection in mice. It reduces the levels of IFN- γ and IL-6, while increases those of TNF- α and IL-17, which were associated to reduction of early pathogen load (Karunasena *et al.*, 2013). Based on these studies, I predict that *L. murinus* could mediate protection against over-inflammation caused by different mycobacteria infections and possibly caused by other pathogens. Beyond mycobacterial infections, there are several acute infections such as Influenza involving high immunopathology that may be ameliorated by *L. murinus* administration. In this disease, the resolution of immunopathology is associated with the balanced induction of Treg (to prevent reinfection) and establishment of tissue repair mechanisms (Wang *et al.*, 2013; Herold *et al.*, 2015). As previously proposed, induction of ROR γ t⁺ Treg by *L. murinus* may also be protective in type 2 inflammatory diseases, such as asthma.

Given its potential as probiotic, the use of *L. murinus* was patented as “Treatment of respiratory diseases using the bacteria *Lactobacillus animalis*⁵⁹.” **Patent FR1903364** (*Provisional application filed March 29, 2019*). Bernard L, Lugo-Villarino G, Neyrolles O, Thomas M, Remot A, Langella P. This patent (in French) is provided in the Appendix 2 and it contains results presented in this thesis main text.

Potential of commensal strains from lung origin to modulate the lung immune system

One interesting aspect of this PhD thesis was the focus given to strains from the lung microbiota. These commensals may have unique interactions with the lung immune system and represent valuable target to modulate respiratory diseases, but these aspects have been poorly described. The results obtained during my PhD strongly support this hypothesis. Indeed, we observe that nasal administration of other strains than *L. murinus* also isolated from the airway microbiota, and belonging to *Lactobacillus*,

⁵⁸ *Mycobacterium avium subspecies tuberculosis* is a pathogen causing Johne's Disease in ruminant animals, leading to chronic gastro-intestinal inflammation, and involved in human Crohn's disease.

⁵⁹ The strain patented was identified as *Lactobacillus murinus* after the patent provisional application.

Neisseria and *Staphylococcus* genera, modulate the lung CD4⁺ T cell compartment (Figure 35 and 47). To my knowledge, the study by our collaborators is the only other one showing that strains isolated from the lung microbiota possess immunomodulatory properties (Remot *et al.*, 2017). Of note, in the study by Remot and colleagues, the CD4⁺ T cell compartment was not assessed. In fact, the modulation of lung CD4⁺ T cells by the different microbiota-based approaches is poorly described in the literature. Given the role of these cells in orchestrating the immune response, understanding their modulation by the microbiota is key. In our study, depending on the species, total number of CD4⁺ T cells was increased or not, and the subsets induced were differentially modulated. All strains tested, were able to increase levels of ROR γ t⁺ Treg in the lungs, with the highest induction observed after administration of the *Neisseria mucosa* strain and the lowest by the *Lactobacillus rhamnosus* strain. Importantly, only one other study reported the presence of ROR γ t⁺ Treg in the lungs (Lochner *et al.*, 2008) and we are the first ones demonstrating that as in the gut, these cells are induced by the microbiota in the lungs (Sefik *et al.*, 2015; Yang *et al.*, 2016). Interestingly, oral administration of *Lactobacillus* or *Staphylococcus* species also leads to an induction of ROR γ t⁺ Treg in the gut, suggesting there are conserved immunomodulatory mechanisms of resident commensals at both mucosal sites. It is, however, possible that due to different environmental constrictions between these two organs, the natural major inducers of these cells may not be the same commensal bacterial species. Interestingly, some of these pulmonary strains also induced high levels of Th17 and Th1. This was particularly the case of *Neisseria mucosa*, which was the strongest inducer of all CD4⁺ T cell populations observed. This suggests that, similar to the gut, different commensals in the lungs will have different interactions with immune cells. This is in accordance with our collaborators' study showing that *Enterococcus faecalis* nasal administration, but not *Staphylococcus sciuri*, alters the immune response in the context of asthma.

Deciphering which strains would be beneficial or detrimental in a given context is a current challenge in probiotic development. Our study reveals the use of naïve and microbiota-competent mice, rarely studied in this context, could be a valuable intermediate/screening step between *in vitro* tests and disease models. Interestingly, the use of aerosolized or nasal antibiotics was recently shown to alter the lung microbiota and immune system (Barfod *et al.*, 2015; Noci *et al.*, 2018). This could be helpful to generate models of respiratory microbiota dysbiosis, which could be used as a screening step to select strains able to compensate locally for dysbiosis. The combination of lung microbiota depletion and nasal administration of commensals may greatly improve in the near future our understanding of the role of the lung microbiota. Comparison of different antibiotic or commensal delivery may also help to better understand the dynamics between the lung and gut microbiota. This fundamental knowledge will be crucial to improve the rationalized development of probiotic for respiratory diseases. It will also be key to improve administration protocols. In our study, administration of *L. murinus* intragastrically also induced ROR γ t⁺ Treg in the lungs. This induction was slightly lower than the one induced by intranasal administration, mirrored by equally low levels of lung Th17 cells (data not shown). However, it is difficult to assess if administration protocols are comparable in term of bacterial quantity that may reach the lung and gut mucosa. *L. murinus* strains are found in the gut mucosa, thus making it possible that this species has fitness for both the lung and gut environment. However, nasal administration may allow to benefit from colonization resistance mechanisms, which could be very interesting, in particular in preventive approaches. Some studies suggest that live bacteria administration in nasal spray to humans is safe (Cervin, 2018). While live strains may have more potent effects than heat-killed ones, there is a strong need to verify that the identified strain does not become a pathobiont under certain conditions, such as administration in immunocompromised individuals (which may be the case in the tuberculosis context). In this case, postbiotics or engineered *Lactobacillus* strains may be an interesting alternative. While the study of the lung

microbiota is still in its infancy, transfer of knowledge from the gut microbiota (or skin) may allow a rapid characterization of its interaction with the immune system. This should lead in a near future to microbiota-based therapies targeting this new player in respiratory diseases.

Conclusion

Altogether, my PhD work demonstrates that administration of commensals from the lung microbiota, such as *L. murinus* CNCM I-5314, induces lung ROR γ ⁺ Treg and Th17 populations expressing anti-inflammatory markers in naïve and *M. tuberculosis*-infected mice. Thus, these results contribute to the existing evidence for a major role of the lung microbiota in respiratory health and disease, and highlight the importance of ROR γ ⁺ Treg and Th17 cells as key mediators of its immunomodulatory role. I hypothesized that a better characterization of the mechanisms leading to the generation of these cell populations by commensals, and of their role in lung immune responses to pathogens, allergens or self-antigens, will deeply improve our understanding of the mechanisms involved in protective versus pathological immune responses. This knowledge will greatly improve the development of host-directed therapies targeting the gut and lung microbiota for the prevention and treatment of respiratory diseases. In particular, while more work is needed to assess if *L. murinus* CNCM I-5314 would be the most promising candidate to improve tuberculous patient health, our study is the second one suggesting a high potential for microbiota-based interventions in this disease, and the first one focused on the lung microbiota. The understanding of interactions between our body and its resident microbes is still today a complex and challenging issue. In particular, various immune mechanisms are involved in the interaction with both symbionts and pathobionts, and their role as tolerance or resistance mechanisms could be beneficial or pathological depending on genetic and environmental factors affecting the host. However, in the past, our gain of knowledge regarding pathogen interaction with our body led to changes in life habits improving infection prevention (e.g., hygiene, pasteurization) and to the development of antibiotics targeting microbes and successful vaccines to eradicate some diseases (e.g., poliomyelitis). Similarly, our current understanding of the mechanisms linking the microbiota to host health and disease is leading to new changes in life habits (e.g., diet adaptation) with the goal to prevent the global occurrence of infections, but also inflammatory diseases and cancers. Likewise, the ongoing development of host- and microbiota-targeted strategies may in parallel help specific prevention and treatment of multiple diseases, as suggested by the current success against *C. difficile* infection. Detection of factors from host and microbiota origin that are involved in susceptibility to develop diseases at the individual level may spearhead personalized applications of these microbiota-based approaches. Combined approaches targeting pathogens (e.g., antibiotics) and the host response (e.g., vaccines, probiotics) may enhance the prevention and cure of many diseases, including tuberculosis. Therefore, a better characterization of our interaction with the microbiota, including bacteria from gut and other sites as well as viruses and fungi, is interesting on a fundamental point of view and possesses high potential to improve human health in infection and many others disease contexts, which was the main theme of my PhD thesis.

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Appendixes

- [Appendix 1](#): Minireview: “The role of the lung microbiota and the gut–lung axis in respiratory infectious diseases”
- [Appendix 2](#): Patent FR1903364: “Traitement des maladies respiratoires avec la bactérie *Lactobacillus animalis*”

MICROREVIEW

The role of the lung microbiota and the gut–lung axis in respiratory infectious diseases

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Abstract

The pulmonary microbial community, described only a few years ago, forms a discreet part of the human host microbiota. The airway microbiota has been found to be substantially altered in the context of numerous respiratory disorders; nonetheless, its role in health and disease is as yet only poorly understood. Another important parameter to consider is the gut–lung axis, where distal (gut) immune modulation during respiratory disease is mediated by the gut microbiota. The use of specific microbiota strains, termed “probiotics,” with beneficial effects on the host immunity and/or against pathogens, has proven successful in the treatment of intestinal disorders and is also showing promise in the context of airway diseases. In this review, we highlight the beneficial role of the body's commensal bacteria during airway infectious diseases, including recent evidence highlighting their local (lung) or distal (gut) contribution in this process.

1 | INTRODUCTION

“Microbiota” refers to the community of microorganisms, including bacteria, viruses, fungi, and protozoans, that live in a host (Limon et al., 2017; Mirzaei et al., 2017). The human body is host to at least as many microbes as there are human cells (Sender et al., 2016), and these microbes are present in all mucosal sites. The beneficial role of our microbiota in shaping the immune system and in maintaining homeostasis has been known for more than a century from studies of the gut, the organ where the microbial population is the more abundant (Brestoff et al., 2013; Lozupone et al., 2012). Studies using germ-free (GF) mice or mice treated with broad-spectrum antibiotics have highlighted the protective role of the host microbiota in a variety of pathological settings, ranging from metabolic disorders (Turnbaugh et al., 2006) to inflammatory and infectious diseases in the gut and at distal body sites, such as the skin and the lungs (Khosravi et al., 2014; Samuelson et al., 2017; Zhang et al., 2015).

The host-microbiota symbiotic equilibrium is highly sensitive to a number of intrinsic and environmental factors, including the host genetic background, the use of antibiotics, diet, the presence of allergens or infectious agents, all of which can disturb microbiota composition, leading to a state of “dysbiosis” (Levy et al., 2017). Dysbiosis

can result in disease aggravation or increased susceptibility to new disorders, such as the growth of potentially pathogenic commensals or pathobionts. The development of metaomics approaches (including 16S ribosomal RNA profiling and the more accurate shotgun-sequencing technology) has allowed a more detailed and extensive definition of the microbiome of healthy subjects throughout life and its dependence on gender, diet, or geographical location, as well as on the body sites sampled (Eckburg et al., 2005; Peterson et al., 2009; Yatsunenko et al., 2012) and its alteration during disease (Ley et al., 2006; Nelson et al., 2012). Metagenomic approaches also revealed the presence, composition, and relative abundance of microbial commensals at body sites previously thought to be sterile, such as the lungs. Such sequencing-based approaches were complemented and confirmed by culture-based approaches (Remot et al., 2017). Since 2010, studies have described alterations of the lung microbiota in a number of disease conditions, such as chronic obstructive pulmonary disease, cystic fibrosis, and asthma (Hilty et al., 2010; Pragman et al., 2012; Willner et al., 2012), indicating that the lung microbiota influences both respiratory health and disease. In addition, the gut microbiota has been shown to influence pulmonary immunity through what is commonly referred to as the gut–lung axis (Budden et al., 2017; Trompette et al., 2014).

This review focuses on respiratory infectious diseases, caused by a broad range of airborne pathogens, causing acute or chronic infections. We highlight the beneficial role of the host commensal bacteria during these pathologies, notably in mouse models of dysbiosis, which involve the gut–lung axis (Table 1). Finally, we discuss strategies aimed at modifying the host microbiota (e.g., using probiotics), especially targeting the lungs as a strategy to combat respiratory infections (Figure 1).

2 | THE LUNG MICROBIOTA IN HEALTH AND DISEASE

The lungs are inhabited by a microbial population distinct from that of the gut (Man et al., 2017). Although the human core gut and lung microbiota are similar at the phylum structure level (e.g., Bacteroidetes and Firmicutes predominate in the gut, and Bacteroidetes, Firmicutes, and Proteobacteria are predominant in the lung), they differ in their bacterial species composition. For example, although *Faecalibacterium prausnitzii* and *Bacteroides thetaiotaomicron* are found in the intestinal tract and not in the lung (Lopez-Siles et al., 2012; Mahowald et al., 2009), *Haemophilus spp.*, *Pseudomonas spp.*, *Streptococcus spp.*, and *Veillonella spp.* are frequently found in the airways and not in the gut (Charlson et al., 2011; Morris et al., 2013). The characterisation of the lung microbiota in humans is complicated by the invasive methods required to sample the lower respiratory tract, making contamination with populations of the upper respiratory tract a serious problem (Dickson et al., 2017). However, in mice, it is possible to directly collect lung tissues aseptically in order to carefully assess their microbial composition. Studies in mice have confirmed that the lung microbiota is variable and different of those from other body sites, such as the oral cavity or the gut (Dickson et al., 2018; Kostric et al., 2018).

Alteration of the lung and gut microbiota has been observed in many respiratory diseases (Dickson et al., 2014; Taylor et al., 2016). Whether microbial dysbiosis at both sites is a cause or a consequence of disease remains to be determined, for example, using the mouse models. However, since the gut microbiota is the largest and most diverse community of the mammalian microbiome, with an important impact on immunity in both the gut and the lungs, the specific contribution of the lung microbiota to the host immunity remains difficult to assess. The intensive present focus on the gut–lung axis overshadows the potential role of local microbiota in health and airway diseases (Dickson et al., 2017).

3 | PROTECTIVE ROLE OF THE HOST MICROBIOTA DURING DISEASES OF THE AIRWAYS: INVOLVEMENT OF LOCAL AND DISTAL COMMUNITIES

3.1 | Acute bacterial infections

The composition of the airway microbiota has been studied extensively in patients with chronic airway diseases of both infectious and non-infectious origins (Marsland et al., 2014). By contrast, analysis of

the composition of this microbial community has been poorly explored in patients suffering from acute lung infections, such as pneumonia, probably due to the short duration of such diseases. However, the beneficial role of the host microbiota during acute bacterial infections of the lungs has been demonstrated in numerous settings using GF mice. For example, GF mice are more sensitive to lung infection by *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae* (Brown et al., 2017; Fagundes et al., 2012; Fox et al., 2012). Dissection of the molecular mechanisms involved in host protection against lung infections by the microbiota has been limited because of the complication that, in addition to profound anatomical and developmental differences compared with specific pathogen-free mice, GF mice display an altered immune system (Al-Asmakh et al., 2015). To avoid this problem, recent studies have used combinations of broad-spectrum antibiotics to provoke dysbiosis and assess the role of the bacterial microbiota in resistance to pulmonary infections in mice (Table 1).

In general, studies using antibiotic-treated mice have not addressed whether oral antibiotic administration also eliminated all or part of the lung microbiota, in addition to gut microbial communities; however, it seems likely that this is the case, because such treatments are known to affect the upper airways community (Cheng et al., 2017). Antibiotic-treated mice have been reported to be more susceptible to respiratory pathogens such as *S. pneumoniae* and *K. pneumoniae*. In particular, antibiotic-treated mice infected with *S. pneumoniae* suffered a defect in lung cytokine production. Faecal transplantation by oral gavage with a normal gut microbiota restored both control of the infection in the mice and cytokines' levels in the lungs, illustrating the contribution the gut microbiota makes to lung immunity (Schuijt et al., 2016). Nevertheless, the potential role of the local, pulmonary microbiota in host defence against *S. pneumoniae* remains to be evaluated (Lankelma et al., 2017). In another study, susceptibility to *S. pneumoniae* and *K. pneumoniae* infection in antibiotic-treated mice correlated with reduced production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-17A in the lungs (Brown et al., 2017). Control of infection was restored after the transfer of a normal microbiota from the upper respiratory tract (via the intranasal route) or after faecal transplantation (via the oral route), demonstrating a beneficial role of both local and distal microbiota on lung immunity. This beneficial effect was abolished when a GM-CSF-neutralising antibody was delivered intranasally. GM-CSF levels were restored following intranasal administration of IL-17A in antibiotic-treated mice, indicating that the microbiota protects the host from respiratory pathogens via IL-17A, which in turn regulates local GM-CSF production (Brown et al., 2017). Finally, in the context of infection with *K. pneumoniae*, microbiota depletion by oral antibiotic administration prevented the innate immune clearance of this pathogen (Clarke, 2014). Although the oral administration of bacterial nucleotide-binding oligomerization domain (NOD)-like receptor ligands rescued the host's ability to control the infection, intranasal administration of these ligands failed to do so. This study highlights the complexity of the mechanisms employed by the microbiota in the gut–lung axis for sustaining host immunity in the lungs.

In the context of acute infections, it has also been shown that specific commensals have the capacity to regulate host immunity.

TABLE 1 Studies of the impact of the microbiota or probiotics administration on host resistance to pulmonary pathogens

Mouse model	Pathogen studied	Main findings	Phenotype rescue (route of administration)	References
Germ free	<i>Klebsiella pneumoniae</i>	Survival ↓; CFUs ↑ in blood and lungs; neutrophils, CXCL1, TNFα ↓, and IL-10 ↑ in lungs	IL-10 blocking antibody (s.c.); LPS (i.p.); faecal transplant (i.g.)	Fagundes et al., 2012
	<i>Streptococcus pneumoniae</i>	CFUs ↑ in lungs	High NOD2-stimulating bacteria group (i.g.)	Brown et al., 2017
	<i>Pseudomonas aeruginosa</i>	Survival ↓; TNFα and IL-1β ↓ in BALF; intestinal epithelial apoptosis ↓		Fox et al., 2012
	<i>P. aeruginosa</i>	CFUs ↑ in lungs and blood		Robak et al., 2018
Antibiotics treated	<i>Escherichia coli</i> (pneumonia)	Survival ↓; CFUs ↑ in blood and lungs; NF-κB binding activity, TNFα ↓, and IL-6, IL-1β ↑ in lungs; MPO activity in lungs and killing activity of AM ↓	LPS (oral, drinking water)	Chen et al., 2011
	<i>K. pneumoniae</i>	CFUs ↑ in lungs; TNFα and IL-6 ↓ in lungs; ROS-mediated killing by AM ↓	NLR ligands (i.g.); AM (i.n.)	Clarke, 2014
	<i>K. pneumoniae</i> <i>S. pneumoniae</i>	Survival ↓; CFUs ↑ in lungs; GM-CSF, IL-17A, CXCL1, and CXCL2 ↓ in lungs	Faecal transplant (i.g.) and lavage fluid (i.n.); NLR-stimulating bacteria (i.n.); GM-CSF, IL-17A-neutralising antibodies, and GM-CSF, IL-17A recombinant cytokines (i.n.)	Brown et al., 2017
	<i>S. pneumoniae</i>	Survival ↓; CFUs ↑ in blood and lungs; inflammatory and tissues damages ↑ in lungs and gut; IL-10, TNFα ↓, and IL-1, IL-6, CXCL1 ↑ in lungs; phagocytosis and cytokine production by AM ↓	Faecal transplant (i.g.)	Schuijt et al., 2016
	<i>P. aeruginosa</i>	Survival ↓; CFUs ↑ in lungs, BALF, and blood; IL-6, CXCL2, and neutrophils ↑ in BALF; IgA ↓ in BALF and blood	IgA purified (i.n.)	Robak et al., 2018
	<i>Mycobacterium tuberculosis</i>	Lungs damages and CFUs ↑ in lungs, spleen, and liver; Treg ↑ and Th1 ↓ in spleen	Faecal transplant (i.g.)	Khan et al., 2016
	Conventional	Influenza virus	Body weight loss, virus titre, symptoms, and histopathology scores ↓; myeloid cells, NK, IL-1-α/β, IL-33 ↑, and eotaxin, MIP-1, MCP-1, IFNγ ↓ in lungs; IL-10 in lungs ↓ (early response) and ↑ (late response)	<i>Lactobacillus paracasei</i> CNCM I-1518 (i.g.)
Influenza virus		Body weight loss, histopathology, and viral titre ↓; TNFα, IL-6 ↓, and IL-12, IFNγ ↑ in BALF	<i>Lactobacillus plantarum</i> DK119 (i.g. or i.n.)	Park et al., 2013
Influenza virus		Survival ↑ (highest protection with <i>Lactobacillus fermentum</i> -1); virus titre and histopathology ↓; IgA ↑ and TNFα and IL-6 ↓ in lungs	eight different lactobacillus strains (i.g. or i.n.)	Youn et al., 2012
<i>P. aeruginosa</i>		Survival ↑; CFUs and histopathology scores ↓; IL-6 ↓ and IL-10 ↑ in serum and lungs; neutrophils ↓ and Treg ↑ in lungs	<i>Lactobacillus rhamnosus</i> GG (i.g.)	Khailova et al., 2013
<i>S. pneumoniae</i>		CFUs and histopathology scores ↓; specific IgG and IgA ↑ in BALF; neutrophils, TNFα ↑ (early response),	<i>Lactobacillus casei</i> (i.g.)	Racedo et al., 2006

(Continues)

TABLE 1 (Continued)

Mouse model	Pathogen studied	Main findings	Phenotype rescue (route of administration)	References
		and \downarrow (late response) in serum and lungs; IL-10 \uparrow in serum and lungs		
	<i>K. pneumoniae</i>	Survival \uparrow ; body weight loss, CFUs and histopathology score \downarrow ; TNF α and IL-6 in BALF \downarrow ; IL-10 \uparrow in lungs; ROS and bacterial killing by AM \uparrow	<i>Bifidobacterium longum</i> 5 ^{1A} , live, or heat-killed bacteria (i.g.)	Vieira et al., 2016
	RSV and <i>S. pneumoniae</i>	Body weight loss, viral titre, CFUs, and histopathology score \downarrow ; IFN β , IFN γ , TNF α , IL-6, IL-10 \uparrow in BALF; DC, AM, CD4 T cells \uparrow in lungs	<i>Corynebacterium pseudodiphthericum</i> 090104 (i.n.)	Kanmani et al., 2017

Note. AM: alveolar macrophages; BALF: broncho alveolar lavage fluid; CFUs: colony forming units; CXCL: chemokine (C-X-C motif) ligand; DC: dendritic cells; GM-CSF: granulocyte colony stimulating factor; IFN: interferon; Ig: immunoglobulin; IL: interleukin; i.g.: intragastric administration (gavage); i.n.: intranasal administration; i.p.: intraperitoneal administration; LPS: lipopolysaccharide; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; MPO: myeloperoxidase; NF- κ B: nuclear factor kappa-light chain enhancer of activated B cells; NK: natural killer lymphocytes; NLR: nucleotide-binding domain leucine-rich repeat containing; NOD: NLR, nucleotide oligomerization domain-like receptor ligands; ROS: reactive oxygen species; RSV: respiratory syncytial virus; s.c.: subcutaneous administration; Th: T-helper lymphocyte; TLR: toll like receptor; TNF: tumour necrosis factor; Treg: regulatory T cells.

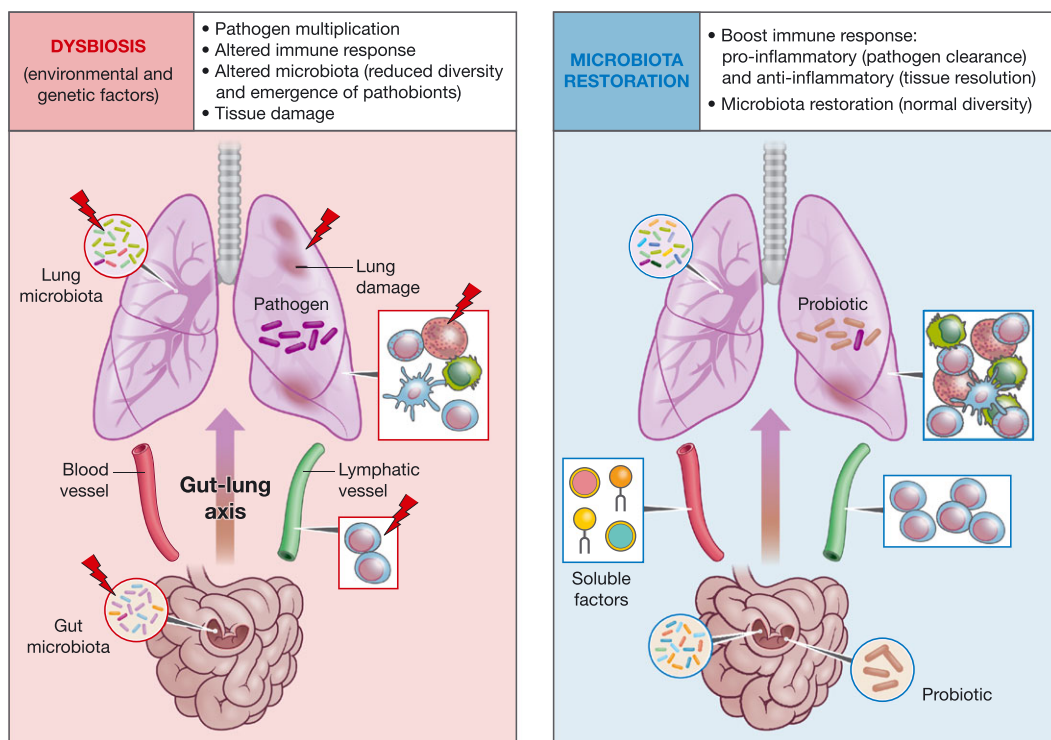


FIGURE 1 Model of the host-microbiota interaction during dysbiosis (and microbiota restoration) within the context of pulmonary infectious disease: the gut-lung axis. The dysbiotic state (i.e., the alteration of the function and composition of the microbiota) can be caused by a variety of environmental (e.g., pathogenic and allergenic contexts and diet) or genetic-induced (e.g., autoimmunity) factors and/or disorders. In this model, a respiratory pathogen causes dysbiosis where the commensal bacterial diversity is perturbed, and pathobionts (i.e., any potentially disease-causing microorganism) can subsequently emerge in the gut and/or the lungs. As a consequence of dysbiosis, there is a disturbance of the level and activation of leucocytes, potentially leading to lung damage. Reintroduction of beneficial microbial strains (i.e., probiotics) may help to recover a healthy status (e.g., microbiota function and composition, leucocyte homeostasis, and/or activation to control infection and immunopathology) through microbiota-derived (e.g., short chain fatty acids) or host-derived products (e.g., cytokines and chemokines) at the local (lung) or distal (gut) level. The “gut-lung axis” refers to the crosstalk between these two mucosal sites of the body

One of the best-known cases is that of segmented filamentous bacteria (SFB), a bacterial lineage of the gut microbiota, which is sufficient to induce the appearance of T-helper (Th)17 cells in the lamina propria in mice. SFB-induced Th17 immunity protects the

host from infection by either intestinal or pulmonary pathogens (Gauguet et al., 2015; Ivanov et al., 2009). Because colonisation of the gut by defined microbial species seems to be important in the context of infectious diseases, it would be interesting to

colonise the lungs with one or several well-defined microbiota species in order to better characterise their local and/or distal role in host immunity development and homeostasis.

3.2 | Chronic bacterial infections: The case of *Mycobacterium tuberculosis*

Chronic infections by the airborne bacterial pathogen *M. tuberculosis*, which causes tuberculosis (TB), can remain latent for years or decades before becoming reactivated and leading to chronic immunopathological lung tissue destruction. Several studies have compared the lung microbiota composition in sputum or bronchoalveolar lavages' fluids, which are more representative of the lower respiratory tract, in TB patients and healthy controls. Although these studies have revealed differences in the airway microbiota composition between patients and controls, the results are not always consistent, likely because of poorly standardised experimental protocols, from initial sampling to sequencing and to the absence of harmonised experimental controls. Six such studies have already been reviewed and summarised by Hong et al (Hong et al., 2016). The same group recently published a meta-analysis study, using the same methods, to reanalyse four of these studies and an additional cohort of healthy individuals (Hong et al., 2018). The authors did not find any difference in the overall global lung microbiota diversity between TB patients and healthy controls. However, they did find that TB patients differed from healthy controls in the abundance of a limited number of bacterial species, with some species being specifically associated with the presence of *M. tuberculosis*.

Gut microbiota has been analysed during the course of TB infections and also during anti-TB treatment in humans. These studies revealed that bacterial diversity in the gut of TB patients is altered and this may correlate with the progress of the disease (Luo et al., 2017; Maji et al., 2018). Anti-TB therapy includes antibiotics, such as rifampicin, that target bacteria other than mycobacteria, and one study showed that prolonged anti-TB treatment broadly alters the gut microbiota of TB patients and that the resulting dysbiotic state persists following cessation of therapy (Wipperman et al., 2017). This suggests that the long anti-TB treatment, which lasts at least 6 months, may render the patients more susceptible to other disorders and infections.

How the gut microbiota influences anti-TB immunity in the lungs is not yet fully understood. Two recent studies have shown that the short chain fatty acid (SCFA) butyrate modulates the production of *M. tuberculosis*-induced pro- and anti-inflammatory cytokines in the lungs, associated with an increased susceptibility to *M. tuberculosis* (Lachmandas et al., 2016; Segal et al., 2017). Because SCFAs, which are produced and released by microbial species of the gut, are mediators of immunity and play a crucial role in gut homeostasis, it remains to be determined whether and how these metabolites could affect the *M. tuberculosis* growth and resilience in the infected host.

The first study addressing the possible functional role of the host microbiota in TB immunity in vivo found that multiplication and dissemination of *M. tuberculosis* were elevated in mice treated with a cocktail of broad-spectrum antibiotics during the course of infection,

and this treatment was associated with a higher number of TB-associated lung lesions (Khan et al., 2016). This correlated with decreased production of tumour necrosis factor (TNF) α -positive and interferon- γ -positive CD4 T cells and an increase of FoxP3-positive regulatory T cells (Treg) in the spleen, suggesting that microbiota dysbiosis following antibiotic treatment adversely altered the immune response to *M. tuberculosis* infection. However, this study did not evaluate the status of local immunity in the lungs of antibiotic-treated animals. More analyses are required to determine the involvement of the host microbiota in the immune response to TB.

4 | TARGETING THE LUNG MICROBIOTA DURING RESPIRATORY DISEASES

In light of our increasing appreciation of the protective role that bacterial commensals play in lung homeostasis, a number of approaches have been developed that target the microbiota-host immune system interaction, with the goal of improving both prevention and treatment of respiratory diseases. Administration of microbes (using probiotics or faecal transfer), microbe components, or products favouring their growth (e.g., prebiotics) has been suggested to confer host protection through direct competition with the disease-causing microbes, enhancement of epithelial barrier functions, or immune modulation during respiratory diseases (Alexandre et al., 2014; Trompette et al., 2018). In this section, we highlight reports of the improved immune responses to lung infections seen upon treatment with probiotics.

Until now, most studies in mice have focused on two infection models—influenza and pneumonia—in which a beneficial effect of oral or nasal probiotic administration has been characterised by improved survival, decreased weight loss, decreased viral titre or bacterial load in the lung, and decreased bronchial epithelium damage (summarised in Table 1). These studies report that the protective effect was mediated by specific immune modulation, distinguished by an early recruitment in the lung of innate leucocytes displaying potent killing properties, such as alveolar macrophages (Park et al., 2013; Vieira et al., 2016), neutrophils (Racedo et al., 2006), or natural killer lymphocytes (Belkacem et al., 2017; Kawahara et al., 2015), and elevated levels of pro-inflammatory cytokines (e.g., TNF- α , IL-6). This inflammatory boost then rapidly diminished, likely due the subsequent increase of anti-inflammatory factors, such as Treg cells and IL-10 in the lungs, reducing lung injuries observed in nontreated mice (Khailova et al., 2013). However, in most studies, causality between immune modulation and host protection has not been demonstrated, and global analysis of the data is complicated by differences in the protocols used for probiotic administration (e.g., oute, dose, and duration).

A number of more experimentally sophisticated studies have recently been published that corroborate the potential of probiotics. Oral administration of *Bifidobacterium longum* has been shown to improve the immune response of *K. pneumoniae*-infected mice, enhancing their survival. It was also shown that the administration in GF mice was able to induce the same beneficial effects as that provided by faecal transfer of whole microbiota (Fagundes et al., 2012; Vieira et al., 2016). This effect may be enacted by acetate production

by *B. longum*, which is consistent with a recent report demonstrating significant protection against *K. pneumoniae* in acetate-treated mice (Galvao et al., 2018).

Concerning administration protocols, comparative studies have suggested that intranasal administration and the use of viable bacteria are the most effective methods for reducing disease features in mice (Youn et al., 2012). It seems that short pretreatment induces a more protective immune response than the classical longer treatments (Racedo et al., 2006). Also, it seems that protection granted against a given pathogen is not only different when using different microbiota species but also between different strains from the same species (Youn et al., 2012). Such results may suggest why, until now, clinical studies using probiotics have shown a promising yet limited potential for the reduction of respiratory disease incidence (Alexandre et al., 2014; Lehtoranta et al., 2014). Therefore, further experimentations helping to identify the most effective strains as well as optimal administration protocols are needed to improve clinical trials.

Lastly, although most commensal bacteria being used as probiotics are of gastrointestinal origin, an alternative strategy is to use natural resident commensal bacteria from the respiratory system. A pioneering study reported the isolation of 20 lower respiratory commensal bacteria species in mice, including *Enterococcus faecalis* that modulates asthma susceptibility in mice (Remot et al., 2017). In the context of infectious diseases, it has been demonstrated that intranasal administration in infant mice of *Corynebacterium pseudodiphtheriticum*, which is present in the respiratory tract, was able to reduce features of both respiratory syncytial virus primary infection and of secondary *S. pneumoniae* superinfection, decreasing both pathogen burden and lung damage (Kanmani et al., 2017).

There has been tremendous progress in leveraging the potential of probiotics in the prevention and treatment of respiratory infections. In spite of this, there is still much more that needs to be done. One key area is extending the successes seen in the various mouse models to the so far more limited successes seen in clinical trials, as already reported for the use of probiotics in intestinal diseases (Suez et al., 2018). Likewise, more detailed studies are needed to address how probiotics (a) modulate the resident lung microbiota community, (b) persist and are localised in airways, (c) interact with resident cells and leucocytes, and (d) influence the homeostasis of lung immunity during and after infection with different pathogens. Finally, we envision the emergence of the characterisation of novel lung microbiota strain interaction with both the local and distal immune systems that could become the next generation of probiotics for respiratory diseases.

5 | PERSPECTIVE

We are only just beginning to understand the functional implications of the lung microbiota in health and disease. It is clear that a much greater research effort needs to be dedicated to uncovering how the lung microbiota interacts and collaborates (or antagonises) with the now increasingly well-studied gut microbiota. Although the existence of a gut–lung axis has been clearly established (Figure 1), the contribution of the airway resident microbiota to this axis remains to be

elucidated. Even if it presently appears difficult to specifically target the lung microbiota without distal effect on other communities, its role needs to be carefully evaluated, possibly by using intranasally delivered antibiotics in order to modify the lung communities (Barfod et al., 2015). Another possible approach involves the introduction, via the oropharyngeal route, of probiotics, which seem to modulate lung immunity, increase protection against respiratory pathogens, and reduce lung damage. Application of these findings in the clinic is moving ever closer, but safety issues concerning the oropharyngeal delivery of living gut or newly identified lung strains remain to be clearly delineated, along with the search for specific active compounds. Similarly, faecal microbial transplantation in humans showed systemic effects (Li et al., 2014) that may prove beneficial in the context of respiratory diseases.

Another outstanding question is whether specific interactions exist between different airways commensals (i.e., trophical niches), as is the case with gut commensals. In addition, even if the great majority of the host microbiota is composed of bacteria, recent evidence has demonstrated that fungal or viral agents can also have beneficial functions (Jiang et al., 2017; Kernbauer et al., 2014) and are a natural part of the lung microbiota (Nguyen et al., 2015; Wylie, 2017), indicating that there is a clear need to better study this (somewhat neglected) aspect of the microbiota during health and disease. In this regard, the use of antifungal and/or antiviral treatments, along with the traditional antibiotic treatments used in mouse models of infections, would represent a highly valuable approach. A potential complication in many studies is the use of laboratory mice confined to highly sterile environments, which is not a representative of the complexity of microbiota present in wild mice and results in altered immune development (Rosshart et al., 2017). Even further complicating this situation, it is known that mice and humans share only 15% microbiota species in the gut (Ley et al., 2005). This is likely similar or even more divergent at other body sites. This will have to be taken into account in future studies.

We envision the application of “omics” studies, such as metagenomics, metabolomics, metatranscriptomics, and metaproteomics, to reduce the gap in knowledge of how the lung microbiome communities affect health and disease. We also encourage functional and mechanistic experimental studies that aim to better understand lung microbial community functions and causality, microbe–microbe and microbe–host cell interactions, as well as inflammatory and resolution pathways/circuits.

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Numéro d'enregistrement : FR1903364

Lieu de dépôt : 92 INPI - Dépôt électronique

Date de la demande : 29/03/2019

Référence client : B251060FR D39013

Type de brevet : Brevet français

Rubrique 1 : NOM ET ADRESSE DU DEMANDEUR OU DU MANDATAIRE À QUI LA CORRESPONDANCE DOIT ÊTRE ADRESSÉE

Société/Cabinet : REGIMBEAU

Nom/Prénom : Monsieur BOUQUIN Nicolas

Adresse :

20, rue de Chazelles
75847 PARIS CEDEX 17
France

Rubrique 2 : TITRE DE L'INVENTION

Titre : TRAITEMENT DES MALADIES RESPIRATOIRES AVEC LA BACTERIE LACTOBACILLUS ANIMALIS

Rubrique 3 : PRIORITÉS

Néant

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Rubrique 5 : MANDATAIRE

Société/Cabinet : REGIMBEAU

Nom/Prénom : Monsieur BOUQUIN Nicolas

Qualité : CPI brevets

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Renvoi à une demande de brevet déjà déposée : Non

Rubrique 7 : RAPPORT DE RECHERCHE EQUIVALENT

Rapport de recherche équivalent : Non

Rubrique 8 : RÉDUCTION DES REDEVANCES

Bénéficiaire de la réduction des redevances : Oui

En qualité de : Organisme à but non lucratif dans le domaine de l'enseignement ou de la recherche (attestation à fournir dans le mois du dépôt)

Rubrique 9 : DÉPÔT DE MATIÈRE BIOLOGIQUE

Dépôt N° 1

Nom de Souche : I-5314

Autorité de dépôt : Collection nationale de cultures de micro-organismes (CNCM)

Référence de dépôt : I-5314

Numéro de dépôt : I-5314

Accessibilité limitée aux experts : Oui

Dépôt N° 2

Nom de Souche : I-4967

Autorité de dépôt : Collection nationale de cultures de micro-organismes (CNCM)

Référence de dépôt : I-4967

Numéro de dépôt : I-4967

Accessibilité limitée aux experts : Oui

Dépôt N° 3

Nom de Souche : I-4968

Autorité de dépôt : Collection nationale de cultures de micro-organismes (CNCM)

Référence de dépôt : I-4968

Numéro de dépôt : I-4968

Accessibilité limitée aux experts : Oui

Rubrique 10 : SÉQUENCES DE NUCLÉOTIDES ET/OU D'ACIDES AMINÉS

La demande contient une liste de séquences : Non

Rubrique 11 : DOCUMENTS ET FICHIERS JOINTS

N°	Type de document	Fichier électronique	Détails
1	Contenu de la demande DOCX	B251060FR_TEXTE_DEPOT_ePROCEDURE.docx	
2	Contenu de la demande PDF	B251060FR_TEXTE_DEPOT_ePROCEDURE.docx.pdf	
3	Contenu de la demande ZIP	B251060FR_TEXTE_DEPOT_ePROCEDURE.docx.zip	
4	Contenu de la demande XML	application-body.xml	
5	Récépissé officiel de l'autorité de dépôt internationale	I5314.pdf	
6	Récépissé officiel de l'autorité de dépôt internationale	I4967.pdf	
7	Récépissé officiel de l'autorité de dépôt internationale	I4968.pdf	

Rubrique 12 : DEMANDE DE DELIVRANCE ACCELEREE

Demande de délivrance accélérée : Non

Rubrique 13 : EXTENSION DE LA PROTECTION

Extension de la protection à la Polynésie Française : Non

Rubrique 14 : PAIEMENT DES REDEVANCES

Mode de paiement : Compte Client

Prestation	Tarif	Quantité	Total
Dépôt d'une demande de brevet / certificat d'utilité sous forme électronique (tarif réduit)	13	1	13
Rapport de recherche (tarif réduit)	260	1	260
Revendication, à partir de la 11ème (tarif réduit)	21	2	42
Total (EURO) :			315

Rubrique 15 : DATE ET SIGNATURE

Nom/Prénom : Monsieur BOUQUIN Nicolas

Qualité : CPI

N°CPI : 13-0202

Date de signature : 29/03/2019

Ce document récapitule les données du dépôt déclarées conformes par le signataire.



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Rubrique - 3 : INVENTEUR

Nom/Prénom : Monsieur NEYROLLES Olivier

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Nom/Prénom : Monsieur LANGELLA Philippe

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Description

Titre de l'invention : TRAITEMENT DES MALADIES RESPIRATOIRES AVEC LA BACTERIE LACTOBACILLUS ANIMALIS

- [0001] Le microbiote, composé de bactéries mais aussi de virus, parasites et champignons présents au-niveau de toutes les muqueuses, telles que l'intestin ou les poumons, est une composante majeure des interactions hôte-pathogène. Le microbiote intestinal, par exemple, exerce un effet protecteur, tant pour le maintien de l'homéostasie (mécanisme de tolérance), que pour la protection contre des pathogènes [1]. Notamment, l'orientation du système immunitaire vers un profil anti-inflammatoire, permettant de limiter l'inflammation, dépend largement du microbiote [2]. Son étude a ainsi permis l'identification parmi les bactéries du microbiote intestinal, de souches dites probiotiques, possédant des propriétés spécifiques permettant la prévention ou le traitement de différentes maladies (notamment les infections pulmonaires) [3, 4].
- [0002] Bien que longtemps considérés comme stériles, les poumons possèdent eux aussi un microbiote. Les données disponibles dans la littérature proviennent majoritairement d'étude de métagénomique [5,6, 7]. En effet, la charge bactérienne dans les poumons en bonne santé est au moins d'un ordre de grandeur plus faible que celle de l'intestin. Il a pu être montré que le microbiote du poumon se compose d'une relativement grande diversité d'espèces bactériennes.
- [0003] La colonisation par le microbiote a un impact très important sur l'immunité et la santé. Les présents inventeurs ont ainsi mis en évidence l'effet protecteur d'une souche d'*Enterococcus faecalis* contre l'asthme allergique [8]. En outre, un candidat probiotique issu du microbiote pulmonaire, *Corynebacterium pseudodiphtheriticum*, améliore la réponse immunitaire pulmonaire contre l'infection par le virus respiratoire syncytial (VRS) et la pneumonie résultant de l'infection secondaire par *Streptococcus pneumoniae* [9]. Ces questions sont plus largement développées dans [10, 11].
- [0004] La tuberculose est l'une des 10 premières causes de mortalité dans le monde. Grâce aux traitements actuels (vaccin BCG (Bacillus Calmette–Guérin) et quadrithérapie antibiotique), l'incidence de la maladie diminue en moyenne de 1,5 % par an. Cependant, l'apparition de formes de tuberculose résistante aux antibiotiques souligne le besoin d'identifier de nouvelles stratégies thérapeutiques [12].
- [0005] La tuberculose est une maladie infectieuse causée par une bactérie (*Mycobacterium tuberculosis*) et touchant le plus souvent les poumons [13]. La multiplication du pathogène et l'expression de certains composés moléculaires induisent une hypersensibilité immunologique conduisant à une inflammation incontrôlée [14].

- [0006] Une fois que le bacille tuberculeux inhalé a atteint les alvéoles, il est phagocyté par différentes cellules immunitaires, dont notamment des macrophages alvéolaires. Cette défense cellulaire est complétée par une défense immune, impliquant les lymphocytes T par l'intermédiaire de leurs récepteurs avec les antigènes de *M. tuberculosis*. Ces cellules après s'être multipliées localement vont migrer dans l'organisme et gagner le foyer infectieux primaire où elles vont déclencher une réaction inflammatoire.
- [0007] Les facteurs toxiques sécrétés par les cellules immunitaires, délétères pour les bactéries, mais aussi pour les cellules de l'hôte, conduisent à des dommages tissulaires importants au niveau des poumons [15]. Ainsi, pour lutter efficacement contre la tuberculose, il est nécessaire de développer de nouveaux antibiotiques, mais aussi d'identifier des outils permettant de maintenir une inflammation suffisante pour contrôler la bactérie mais sans être délétère pour l'hôte. Ce type de thérapeutique est important également en dehors de la tuberculose puisque de nombreuses maladies respiratoires sont liées à une inflammation trop importante, comme par exemple les infections par *Francisella tularensis* ou *Pseudomonas aeruginosa*.
- [0008] La présente invention a pour objet de nouveaux traitements de l'inflammation liée à une maladie respiratoire, notamment la tuberculose. La présente invention a notamment pour objet leur prévention.
- [0009] Plus particulièrement, les présents inventeurs ont montré que la bactérie *Lactobacillus animalis* possède des propriétés tout à fait avantageuses dans le traitement et/ou la prévention de maladies respiratoires liées à l'inflammation telles que la tuberculose.
- [0010] En effet, l'administration de cette bactérie confère une forte protection contre l'infiltration leucocytaire des poumons, un marqueur clinique important de l'inflammation. En outre, elle entraîne une forte diminution de la population de leucocytes produisant des cytokines pro-inflammatoires dans les poumons. Dans le même temps, les lymphocytes T régulateurs produisant des cytokines anti-inflammatoires sont fortement stimulés. En particulier, les lymphocytes iTregs sont induits. Encore plus particulièrement, les lymphocytes iTregs qui sont induits sont des lymphocytes T régulateurs bifonctionnels.
- [0011] Dans un premier aspect, l'invention a pour objet la bactérie *L. animalis* pour son utilisation dans le traitement et/ou la prévention de l'inflammation liée à une maladie respiratoire, notamment la tuberculose. Elle a aussi pour objet l'utilisation de la bactérie *L. animalis* pour la préparation d'un médicament pour traiter et/ou prévenir l'inflammation liée à une maladie respiratoire, notamment la tuberculose. Préférentiellement, le sujet affecté par ladite maladie respiratoire est un mammifère, y compris l'homme, le chien, le chat, les équidés, les bovins, les caprins, les porcs, les ovins et les

primates non-humains. De façon plus préférée, ledit sujet est un sujet humain. Alternativement, le sujet peut être un mammifère non-humain, tel qu'un chien, un chat ou un équidé.

- [0012] L'invention a notamment pour objet une souche particulière de *L. animalis* pour son utilisation dans le traitement et/ou la prévention de l'inflammation liée à une maladie respiratoire, notamment la tuberculose. Plus spécifiquement, l'invention a pour objet la souche de *L. animalis* déposée sous le numéro I-5314 le 16 avril 2018 à la Collection Nationale des Cultures de Microorganismes (CNCM), 25 rue du Docteur Roux, 75724 Paris Cedex 15, France, pour son utilisation dans le traitement et/ou la prévention de l'inflammation liée à une maladie respiratoire, notamment la tuberculose.
- [0013] L'invention a aussi pour objet une souche particulière de *L. animalis* possédant des propriétés de prévention et/ou de traitement des maladies respiratoires liées à l'inflammation. Plus spécifiquement, l'invention a pour objet la souche de *L. animalis* déposée sous le numéro I-5314 le 16 avril 2018 à la Collection Nationale des Cultures de Microorganismes (CNCM), 25 rue du Docteur Roux, 75724 Paris Cedex 15, France.
- [0014] La souche I-5314 est produite par culture, par exemple, dans un milieu de croissance connu de l'homme du métier (par exemple, un milieu liquide MRS : « Man, Rogosa et Sharpe ») pendant 1 à 2 jours en conditions aérobies, à une température de 30-37°C, avec ou sans ajustement du pH. Le bouillon de fermentation contenant les cellules bactériennes est recueilli. Le bouillon peut être utilisé tel quel, concentré ou lyophilisé. Avantagusement, les bactéries seront recueillies, par exemple par centrifugation puis remises en suspension dans un tampon approprié, par exemple du PBS (« phosphate-buffered saline »). La concentration bactérienne peut être établie en utilisant un cytomètre de flux ou un autre procédé équivalent.
- [0015] La souche de l'invention est particulièrement avantageuse en ce qu'elle entraîne une forte augmentation des populations aussi bien de lymphocytes Th17 que de lymphocytes Tregs. L'induction des lymphocytes Treg est notamment importante car ce sont avant tout des Tregs bifonctionnels qui ont à la fois des propriétés pro- et anti-inflammatoires. Ainsi, en fonction du contexte, les biTregs peuvent réguler de manière positive ou négative la réponse inflammatoire intervenant au cours de la maladie infectieuse [16, 17, 18].
- [0016] Selon un mode préféré de réalisation, l'invention a donc pour objet la bactérie *L. animalis* pour son utilisation dans le traitement et/ou la prévention de l'inflammation liée à une maladie respiratoire, notamment la tuberculose, ledit traitement et/ou prévention comprenant une diminution de l'infiltration leucocytaire et une augmentation des populations pulmonaires lymphocytes Th17 que de lymphocytes Tregs.
- [0017] Selon un autre mode préféré de réalisation, l'invention a pour objet l'utilisation de la bactérie *L. animalis* pour la préparation d'un médicament pour traiter et/ou prévenir

l'inflammation liée à une maladie respiratoire, notamment la tuberculose, ledit traitement et/ou prévention comprenant une diminution de l'infiltration leucocytaire et une augmentation des populations pulmonaires lymphocytes Th17 que de lymphocytes Tregs.

- [0018] Selon un mode de réalisation encore plus préféré, les lymphocytes Tregs sont des lymphocytes iTregs. Encore plus préférentiellement, les lymphocytes iTregs sont des lymphocytes iTregs bifonctionnels.
- [0019] Les « lymphocytes T » ou « cellules T » sont un type de lymphocytes (globules blancs) jouant un rôle central dans l'immunité à médiation cellulaire. Ils peuvent être distingués d'autres lymphocytes, tels que les cellules B et les cellules tueuses naturelles (cellules NK), par la présence d'un récepteur de cellules T (TCR) à la surface de la cellule. Tel qu'utilisé ici, le terme « récepteur de cellules T » ou « TCR » représente un récepteur présent à la surface des cellules T qui est responsable de la reconnaissance des antigènes liés aux molécules du complexe majeur d'histocompatibilité (MHC). Les cellules T ne présentent pas d'antigènes et dépendent d'autres lymphocytes (cellules tueuses naturelles, cellules B, macrophages, cellules dendritiques) pour faciliter la présentation de l'antigène. Les types de cellules T incluent notamment les cellules T auxiliaires (cellules Th), les cellules T mémoire (Tcm, Tern ou Temra), les cellules T régulatrices (Treg), les cellules T cytotoxiques (CTL), les cellules T tueuses naturelles (cellules NKT), les cellules T gamma delta et les cellules T invariantes associées à la muqueuse (MAIT).
- [0020] Parmi les lymphocytes T, les « lymphocytes T CD4⁺ », appelés également « T auxiliaires » et aussi « T helper (Th) », ont pour fonction principale de réguler positivement ou négativement d'autres cellules immunitaires. Ces cellules expriment la glycoprotéine CD4 à leur surface. Le terme « CD4 », tel qu'utilisé ici, désigne une glycoprotéine membranaire de lymphocytes T qui interagit avec les antigènes du complexe majeur d'histocompatibilité (CMH) de classe II et est également un récepteur du virus de l'immunodéficience humaine. La protéine fonctionne pour initier ou augmenter la phase précoce d'activation des cellules T. De préférence, la molécule CD4 de l'invention est un polypeptide ayant la séquence d'acides aminés représentée par NP_038516.
- [0021] Les lymphocytes T CD4⁺ peuvent être classifiés selon le type de cytokines qu'ils produisent. On peut ainsi notamment identifier des lymphocytes T CD4⁺ Th1, des lymphocytes T CD4⁺ Th2, des lymphocytes T CD4⁺ Th17 ou encore des lymphocytes T CD4⁺ régulateurs.
- [0022] On appelle « lymphocytes T CD4⁺ Th1 » ou « lymphocytes Th1 » ou « Th1 » une population de lymphocytes T CD4⁺ activés qui orientent la réponse immunitaire vers la réponse cellulaire et la cytotoxicité. Les Th1 produisent principalement les cytokines

IL-2, TNF α et IFN γ et expriment le facteur de transcription T-bet. Le terme « T-bet » ou « TBX21 », tel qu'utilisé ici, représente un facteur de transcription de la famille des facteurs de transcription à T-box, qui est nécessaire à la différenciation des lymphocytes T Th1 et des lymphocytes T cytotoxiques Tc1 (c'est-à-dire un lymphocyte T cytotoxique présentant à sa surface des récepteurs pouvant se lier à des complexes formés par un peptide présenté par une molécule CMH de classe I), deux populations lymphocytaires capables de sécréter l'IFN γ . Dans un mode préféré de réalisation, la protéine T-bet possède la séquence d'acides aminés représentée par NP_037483.1.

- [0023] Les lymphocytes Th1 sont induits par la cytokine IL-12 en réponse aux infections par des pathogènes viraux ou bactériens (comme *M. tuberculosis* par exemple). Les cytokines alors produites par les Th1 activent les macrophages qui détruisent les pathogènes. Cependant, cette réponse anti-infectieuse Th1 peut aussi être à l'origine des lésions immunopathologiques tissulaires, notamment en présence d'une infection chronique.
- [0024] Par « lymphocytes T CD4⁺ Th17 » ou « lymphocyte Th17 » ou « cellules Th17 » ou « Th17 », on entend ici une population de lymphocytes T CD4⁺ auxiliaires exprimant le facteur de transcription RAR-related orphan receptor- γ t (ROR- γ t) et produisant la cytokine IL-17A, une cytokine fortement pro-inflammatoire. Avantagusement, les cellules Th17 sont également caractérisées par la libération d'IL-17F, d'IL-21 et d'IL-22 et la co-expression des marqueurs membranaires CCR6, ICOS et CCR4. Les cellules Th17 sont impliquées dans le contrôle des infections bactériennes extracellulaires et fongiques.
- [0025] Tel qu'utilisé ici, le terme « cellules T régulatrices » ou « cellules Tregs » ou « Tregs » ou « cellules T suppresseurs » désigne une population de cellules T qui expriment le facteur de transcription FOXP3 et qui maintiennent la tolérance immunologique. Les cellules Tregs sont importantes pour maintenir l'homéostasie, contrôler l'ampleur et la durée de la réponse inflammatoire et prévenir les réponses auto-immunes et allergiques. Au cours d'une réponse immunitaire, les Tregs suppriment ainsi les réactions immunitaires médiées par les cellules T effectrices, telles que les cellules T effectrices CD4⁺ ou CD8⁺.
- [0026] Les cellules T régulatrices peuvent être des cellules Tregs naturelles ou des cellules Tregs induites. Par « cellules Tregs naturelles » ou « Tregs naturels », on entend ici des cellules T d'origine thymique qui expriment des marqueurs particuliers de la surface des cellules, à savoir les marqueurs CD4 et CD25. Lesdites cellules sont donc de préférence de phénotype CD4⁺CD25^{high}FOXP3^{high}. En outre, les Tregs naturels expriment le facteur de transcription Helios.
- [0027] Les « lymphocytes Tregs induits » ou « cellules Tregs induites » ou « cellules iTregs », tel qu'on les entend ici, sont des cellules T d'origine périphérique dont la dif-

férenciation est induite à la suite d'une interaction antigénique en présence de cytokines telles que TGF- β et IL-2. Les iTregs sont caractérisées par la présence de la chaîne α du récepteur de l'IL-2 (CD25) et de CCR4 à leur surface et la production de cytokines suppressives comme par exemple IL-10, en sus de l'expression de FOXP3. En outre, les cellules iTregs n'expriment pas le facteur de transcription Helios.

[0028] Le terme « CD25 », tel qu'utilisé ici, désigne la chaîne alpha du récepteur de l'IL-2. Cette protéine est une protéine transmembranaire de type I présente sur les cellules T activées, les cellules B activées, certains thymocytes, les précurseurs myéloïdes et les oligodendrocytes qui s'associent à CD122 pour former un hétérodimère pouvant servir de récepteur à haute affinité pour IL-2. Les Tregs en particulier expriment CD25 en plus des CD4 et FOXP3. De préférence, la molécule CD25 de l'invention est un polypeptide ayant la séquence d'acides aminés représentée par NP_032393.

[0029] Par « Helios », on entend ici un facteur de transcription à doigt de zinc codé par le gène IKZF2. Le facteur de transcription Helios forme des homodimères, voire des hétérodimères avec les facteurs de transcription Iskaros et Aiolos. Helios est exprimé notamment dans les cellules Tregs. Plus spécifiquement, Helios est exprimé exclusivement dans les Tregs naturels, mais pas dans les Tregs induits. Préférentiellement, la protéine Hélios telle qu'on l'entend ici correspond à deux isoformes, dont les séquences en acides aminés sont représentées par NP_057344.2 et NP_001072994.1, respectivement.

[0030] Dans un mode de réalisation préférentiel, les cellules iTregs sont des cellules possédant une double fonctionnalité et sont appelées « lymphocytes iTregs bifonctionnels » ou « lymphocytes bi-Tregs » ou « cellules iTregs bifonctionnelles » ou « cellules bi-Tregs » ou « biTregs ». Selon ce mode de réalisation, les cellules biTregs sont des cellules qui expriment à la fois ROR- γ t et FOXP3. Préférentiellement, les cellules biTregs produisent à la fois la cytokine pro-inflammatoire IL-17 et la cytokine anti-inflammatoire IL-10. De façon encore plus préférée, les cellules biTregs produisent en outre les cytokines TGF- β et IL-35.

[0031] Par « RAR-related orphan receptor- γ t » ou « ROR- γ t », on entend ici un facteur de transcription de la famille des récepteurs nucléaires des hormones stéroïdiennes, exclusivement exprimé dans les cellules du système immunitaire. Le facteur de transcription ROR- γ t joue ainsi un rôle clé dans la régulation de la différenciation des cellules Th17. De préférence, le facteur de transcription ROR- γ t est un polypeptide ayant la séquence d'acides aminés représentée par NP_001001523.1.

[0032] Tel qu'utilisé ici, le terme « FOXP3 » désigne un facteur de transcription appartenant à la famille des régulateurs de transcription « forkhead / winged helix ». Le facteur de transcription FOXP3 est le régulateur principal du développement et de la fonction des lymphocytes Tregs. En outre, FOXP3 est un marqueur des lymphocytes Tregs,

l'expression de ce facteur de transcription dans un lymphocyte T CD4⁺ suffisant à caractériser un lymphocyte Treg. De préférence, le facteur de transcription FOXP3 est un polypeptide ayant la séquence d'acides aminés représentée par NP_001186276.

- [0033] Les présents inventeurs ont ainsi montré que l'administration de la bactérie *L. animalis* conduit à une augmentation de la population pulmonaire de cellules iTregs. Cette augmentation n'est pas causée par un accroissement de la prolifération cellulaire, mais par une induction de la différenciation de ces cellules. De préférence, l'administration de *L. animalis* conduit à une induction dans le poumon de lymphocytes iTregs sécrétant aussi bien des cytokines pro-inflammatoires (par exemple, IL-17A) que des cytokines anti-inflammatoires (par exemple IL-10).
- [0034] Le terme « cytokine », tel qu'on l'entend ici, se rapporte à une famille de petites protéines sécrétées régulatrices ayant un rôle crucial dans les réponses immunitaires. Les cytokines sont impliquées dans la communication entre cellules et régulent de nombreuses fonctions cellulaires, comme par exemple la survie et la croissance des cellules, ainsi que l'induction de l'expression de nombreux gènes. Les cytokines peuvent être produites par de nombreux types cellulaires. Comme expliqué plus haut, le type cellulaire d'un lymphocyte donné est notamment déterminé par son profil cytokinique. Ainsi, les « cytokines Th1 », telles qu'on les entend ici, sont les cytokines produites par les lymphocytes T CD4 Th1 (notamment IL-2, IFN γ et TFN α).
- [0035] Par « cytokine pro-inflammatoire », on entend ici les cytokines qui conduisent à une augmentation de l'inflammation. Elles comprennent notamment des cytokines telles que, par exemple, IL-1 β , TNF α , IL-6, IL-15, IL-17, IFN- γ et IL-18. Selon un mode de réalisation préféré, les cytokines pro-inflammatoires sont TNF α , IL-6, IFN- γ et IL-17, plus préférentiellement IL-17. Les « cytokines anti-inflammatoires » sont celles qui contrôlent la réponse des cytokines pro-inflammatoires. Les cytokines anti-inflammatoires agissent de concert avec des inhibiteurs de cytokines spécifiques et des récepteurs de cytokines solubles pour réguler la réponse immunitaire humaine. Les cytokines anti-inflammatoires majeures comprennent l'antagoniste au récepteur d'IL-1, IL-10 et TGF- β . Préférentiellement, les cytokines anti-inflammatoires sont IL-10 et TGF- β .
- [0036] Selon un mode de réalisation plus particulièrement préféré, l'administration de *L. animalis* entraîne l'induction dans le poumon de lymphocytes iTreg produisant de l'IL-10, du TGF- β et de l'IL-17. Les inventeurs ont d'ailleurs montré que le nombre de cellules produisant ces cytokines est augmenté après administration de *L. animalis*. En revanche, la concentration de cytokines pro-inflammatoires telles que TNF α , IL-6 et IFN- γ n'est pas affectée.
- [0037] Le terme « interleukine 17 » ou « IL-17 » ou « IL-17A », tel qu'on l'entend ici, représente une glycoprotéine homodimérique de 20-30 kDa. Le gène de l'IL-17 humaine

code une protéine constituée de 155 acides aminés, dont une séquence signal de 19 acides aminés et un segment mature de 136 acides aminés. Il-17 est une cytokine pro-inflammatoire qui participe aux défenses contre les infections bactériennes extra-cellulaires et fongiques. Une fois sécrétée, cette cytokine agit sur les cellules épithéliales, les cellules endothéliales, les fibroblastes ainsi que sur d'autres cellules du système immunitaire, en les activant pour qu'elles produisent des cytokines pro-inflammatoires comme l'IL-1, l'IL-6, le TNF- α , des chimiokines, du GM-CSF, etc.

[0038] Par « interleukine 10 » ou « IL-10 », on entend ici une protéine homodimérique composée de deux sous-unités en hélice α liées par des interactions non covalentes. De façon préférée, chaque monomère d'IL-10 est exprimé sous la forme d'un précurseur dont la séquence en acides aminés est représentée par NP_000563.1. L'IL-10 est une cytokine anti-inflammatoire clé produite par des cellules immunitaires activées qui joue un rôle critique dans le contrôle des réponses immunitaires. En particulier, elle réduit l'expression des cytokines Th1, des antigènes du CMH de classe II et des molécules co-stimulatrices sur les macrophages. Elle améliore également la survie, la prolifération et la production d'anticorps des cellules B. L'IL-10 peut bloquer l'activité de NF-KB et est impliquée dans la régulation de la voie de signalisation JAK-STAT.

[0039] On entend ici par « TGF- β » ou « transforming growth factor- β » ou « facteur de croissance transformant- β » une cytokine multifonctionnelle appartenant à la superfamille des facteurs de croissance transformants et comprenant quatre isoformes différentes (TGF- β 1 à 4). De façon préféré, TGF- β 1, 2, 3 et 4 ont des séquences en acides aminés représentées par NP_000651, NP_001129071 ou NP_003229, NP_001316867 ou NP_001316868 ou NP_003230, et Q64280.1. Le TGF- β est impliqué dans de multiples processus. Notamment, il joue un rôle immunosuppresseur et anti-inflammatoire en favorisant la résolution de l'inflammation et le retour à l'homéostasie. Il supprime ainsi la production de cytokines en inhibant l'activité des macrophages et des cellules Th1. En particulier, il neutralise l'IL-1, l'IL-2, l'IL-6 et le TNF α , et induit IL-1RA.

[0040] Le terme « augmenté », tel qu'il est utilisé ici dans certains modes de réalisation, signifie une plus grande quantité, par exemple, une quantité légèrement supérieure à la quantité d'origine ou par exemple une quantité en grand excès par rapport à la quantité d'origine, et notamment toutes les quantités dans l'intervalle. En variante, « augmentation » peut faire référence à une quantité ou une activité qui est au moins 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% ou 20% de plus que la quantité ou de l'activité pour laquelle la quantité ou de l'activité accrue est comparé. Les termes « augmenté », « plus grand que », et « accru » sont utilisés ici de manière interchangeable. Une « population de lym-

phocytes augmentée » signifie ainsi une population desdits lymphocytes, par exemple des lymphocytes Th17 ou iTregs, notamment des biTregs, accrue par rapport à un contrôle de référence, tel que, par exemple, un contrôle n'ayant pas été traité avec la présente bactérie *L. animalis*. En d'autres termes, une « population de lymphocytes augmentée » dans les poumons, par exemple des lymphocytes Th17 ou iTregs, notamment des biTregs, signifie que le nombre desdits lymphocytes dans les poumons est augmenté par rapport à un contrôle de référence, tel que, par exemple, un contrôle n'ayant pas été traité avec la présente bactérie *L. animalis*. Cette augmentation peut résulter notamment d'une augmentation de la différenciation des lymphocytes T dans le type de lymphocytes d'intérêt (par exemple des lymphocytes Th17 ou iTregs, notamment des biTregs) et/ou d'une augmentation de la prolifération cellulaire. Préférentiellement, cette augmentation de la population de lymphocytes d'intérêt (par exemple des lymphocytes Th17 ou iTregs, notamment des biTregs) ne résulte pas d'une augmentation de la prolifération cellulaire.

- [0041] Le terme « diminution », tel qu'il est utilisé ici dans certains modes de réalisation, signifie une plus petite quantité, par exemple, une quantité légèrement inférieure à la quantité d'origine, ou par exemple une quantité beaucoup plus petite que la quantité d'origine, et notamment toutes les quantités dans l'intervalle. En variante, « diminution » peut faire référence à une quantité ou une activité qui est au moins 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% ou 20% de moins que la quantité ou de l'activité pour laquelle la quantité ou de l'activité diminuée est comparé. Les termes « diminué », « plus petit que », « moindre » et « décru » sont utilisés ici de manière interchangeable. Une « population de lymphocytes diminuée » signifie ainsi une population desdits lymphocyte réduite par rapport à un contrôle de référence, tel que, par exemple, un contrôle n'ayant pas été traité avec la présente bactérie *L. animalis*. En d'autres termes, une « population de lymphocytes diminuée » dans les poumons signifie que le nombre desdits lymphocytes dans les poumons est réduit par rapport à un contrôle de référence, tel que, par exemple, un contrôle n'ayant pas été traité avec la présente bactérie *L. animalis*.
- [0042] Ledit « contrôle » tel qu'utilisé ici peut être un patient, un modèle animal ou encore un modèle cellulaire in vitro. De préférence, le « contrôle » est un patient. Par « patient », on entend ici un sujet humain souffrant d'une inflammation liée à une maladie respiratoire, notamment la tuberculose. Selon un autre mode de réalisation préféré, le sujet est un animal, notamment un chien, un chat ou un cheval.
- [0043] Dans un autre aspect, la présente invention a aussi pour objet une composition pharmaceutique comprenant la souche I-5314 et au moins un excipient pharmaceutiquement acceptable.
- [0044] La bactérie inactivée induit les mêmes effets que la souche vivante et possède donc

- elle aussi des propriétés de prévention et/ou de traitement des maladies respiratoires.
- [0045] Selon un mode de réalisation particulier de l'invention, la souche I-5314 présente dans la composition pharmaceutique est une souche inactivée. Par « souche inactivée », on entend ici une souche bactérienne qui est incapable de croître et/ou de se diviser. Préférentiellement, une souche inactivée n'a plus d'activité métabolique. Toutefois, les bactéries inactivées selon l'invention sont toujours capables de modérer l'inflammation, c'est-à-dire que l'administration de la bactérie inactivée entraîne une diminution de l'infiltration leucocytaire et une augmentation des populations pulmonaires de lymphocytes Tregs.
- [0046] Les techniques d'inactivation des bactéries sont bien connues de l'homme du métier. On citera par exemple l'inactivation par la chaleur, l'irradiation par les UV ou les rayons gamma, le traitement par les acides, le traitement par le peroxyde d'hydrogène, etc. Les bactéries *L. animalis* seront préférentiellement inactivées par traitement à la chaleur.
- [0047] Il est particulièrement avantageux d'utiliser des extraits de la souche I-5314 dans les présentes compositions pharmaceutiques. Un « extrait », tel qu'on l'entend ici, désigne tout matériel cellulaire obtenu suite à la lyse d'une ou plusieurs souches bactériennes. Avantagusement, un extrait a subi une ou plusieurs étapes supplémentaires d'extraction et/ou de purification. Préférentiellement, l'extrait est obtenu à partir d'une seule souche ; plus préférentiellement, ladite souche est la présente souche I-5314.
- [0048] La lyse peut être réalisée par tous les moyens connus de l'homme du métier : lyse alcaline, lyse par sonication, lyse par haute pression (presse de French), etc. L'extrait obtenu par la lyse cellulaire peut ensuite être soumis à des étapes d'extraction et/ou de purification supplémentaires. Celles-ci peuvent comprendre n'importe quel traitement usuel de tels extraits et connu de l'homme du métier : on mentionnera entre autres les centrifugations (par exemple pour séparer la membrane plasmique du cytoplasme), les filtrations, les précipitations et les séparations des différents constituants cellulaires (par exemple en utilisant l'un des nombreux types de chromatographie), etc. Chacun des différents extraits obtenus à chacune de ces étapes peut être utilisé dans la méthode de l'invention pour autant qu'il soit toujours capable de modérer l'inflammation, c'est-à-dire que l'administration dudit extrait entraîne une diminution de l'infiltration leucocytaire et une augmentation des populations pulmonaires de lymphocytes Th17 et de lymphocytes Tregs.
- [0049] Les présentes compositions sont utiles pour le traitement de l'inflammation liée aux maladies respiratoires.
- [0050] Par « maladie respiratoire », on entend ici les maladies de l'appareil respiratoire, notamment des poumons ou des bronches, ou provoquant des troubles de la respiration. Parmi ces maladies respiratoires, un grand nombre sont liées à une inflammation de

l'appareil respiratoire, notamment des poumons ou des bronches. On citera ainsi, et de manière non exhaustive, l'asthme (légère, modérée ou sévère), par exemple, bronchique, allergique, intrinsèque, extrinsèque, induite par l'exercice, d'origine médicamenteuse (y compris l'aspirine et aux AINS) et l'asthme induit par la poussière, l'asthme résistant aux stéroïdes, la bronchite, y compris la bronchite infectieuse et à éosinophiles, d'une maladie pulmonaire obstructive chronique (COPD), comme la BPCO (broncho-pneumopathie chronique obstructive), la fibrose cystique, la fibrose pulmonaire, y compris cryptogénique alvéolite fibrosante, la fibrose pulmonaire idiopathique, pneumonias interstitielles idiopathiques, la fibrose compliquant une thérapie anti-néoplasique et chronique, l'infection, y compris la tuberculose, la tularémie (causée par *F. tularensis*), l'aspergillose et d'autres infections bactériennes (par exemple par *Francisella Novicida* ou par *P. aeruginosa*) ou fongiques (par exemple par *Candida albicans* ou par *Aspergillus fumigatus*) ; complications de la transplantation pulmonaire; vascularite et troubles thrombotiques du système vasculaire pulmonaire et hypertension artérielle pulmonaire (y compris l'hypertension artérielle pulmonaire); activité antitussive comprenant le traitement de la toux chronique associée à des affections inflammatoires et sécrétoires des voies respiratoires et la toux iatrogène; rhinite aiguë et chronique, y compris la rhinite médicamenteuse, et la rhinite vasomotrice; perannuelle et saisonnière rhinite allergique y compris la rhinite nerveuse (rhume des foins); polypose nasale; infection aiguë virale, y compris le rhume, et l'infection due au virus respiratoire syncytial, la grippe, coronavirus (SRAS notamment) et l'adénovirus, un œdème pulmonaire, embolie pulmonaire, pneumonie, sarcoïdose pulmonaire, la silicose, poumon de fermier et les maladies apparentées; pneumopathie d'hypersensibilité, insuffisance respiratoire, syndrome de détresse respiratoire aiguë, l'emphysème, la bronchite chronique, la tuberculose et le cancer du poumon, etc. Les maladies respiratoires telles qu'on l'entend ici comprennent aussi les maladies respiratoires affectant spécifiquement les animaux, notamment les chats, les chiens ou les chevaux. Elles comprennent notamment la toux du chenil, causée en particulier par les infections au virus *Parainfluenza* et la bactérie *Bordetella bronchiseptica*. Selon un mode de réalisation préféré, ladite maladie respiratoire est la tuberculose.

[0051] Par « tuberculose », on entend ici une maladie infectieuse causée par la bactérie *Mycobacterium tuberculosis*. Dans la grande majorité des cas, la tuberculose est une tuberculose pulmonaire, ce qui signifie que l'infection touche les poumons. Lorsqu'elle se déclare, la tuberculose pulmonaire se manifeste par une toux, parfois productive ou sanglante, des douleurs thoraciques, une asthénie, une perte de poids et des sueurs nocturnes. En outre, la tuberculose peut être responsable d'une inflammation d'évolution prolongée, dont l'aspect anatomopathologique est caractéristique.

- [0052] Par « inflammation », on entend ici l'ensemble des mécanismes réactionnels de défense par lesquels l'organisme reconnaît, détruit et élimine toutes les substances qui lui sont étrangères. La réaction inflammatoire est la réponse à une agression d'origine exogène (cause infectieuse, traumatique) ou endogène (cause immunologique, par exemple une réaction d'hypersensibilité ou une autre cause, par exemple le syndrome d'ischémie- reperfusion). La réponse inflammatoire est habituellement composée d'une phase d'initiation qui fait suite à un signal de danger d'origine exogène ou endogène et qui met en jeu des effecteurs primaires, d'une phase d'amplification avec la mobilisation et l'activation d'effecteurs secondaires et d'une phase de résolution et de réparation qui tend à restaurer l'intégrité du tissu agressé. La réaction inflammatoire est ainsi, le plus souvent, une réponse adaptée strictement contrôlée par de multiples systèmes régulateurs, dont, par exemple, les cellules Tregs. Toutefois, si la réponse inflammatoire est inadaptée ou mal contrôlée ; elle peut devenir agressive. Dans certains cas, l'inflammation peut devenir chronique : par exemple, la tuberculose entraîne une inflammation chronique.
- [0053] Les termes « traiter », « traité », « traitement », ainsi que les termes analogues, tels qu'ils sont utilisés ici, se réfèrent à la réduction ou l'amélioration des symptômes d'un trouble (par exemple, l'inflammation liée à une maladie respiratoire, notamment la tuberculose) et/ou les symptômes associés avec celui-ci chez un sujet. On notera que, bien que ce ne soit pas exclu, le traitement d'un trouble ou un état ne nécessite pas que la pathologie, la condition ou les symptômes qui lui sont associés soient complètement éliminés.
- [0054] Les termes « prévenir », « prévention », ainsi que les termes analogues, tels qu'ils sont utilisés ici, se réfèrent à la suppression du risque d'apparition d'un trouble (par exemple, l'inflammation liée à une maladie respiratoire, notamment la tuberculose) et/ou les symptômes associés avec celui-ci chez un sujet.
- [0055] On entend ici par « sujet » n'importe quel mammifère pouvant bénéficier du traitement décrit ici, y compris l'homme, le chien, le chat, les équidés, les bovins, les caprins, les porcs, les ovins et les primates non-humains. Plus spécifiquement, on appelle ici « patient » un sujet humain. Ledit patient peut appartenir à n'importe quelle classe d'âge, c'est-à-dire le patient peut être un enfant, un adolescent ou un adulte. Alternativement, le sujet peut être un mammifère non-humain, tel qu'un chien, un chat ou un équidé.
- [0056] Les présentes compositions pharmaceutiques comprennent outre la souche I-5314, un ou plusieurs excipients pharmaceutiquement acceptables.
- [0057] Par « excipient pharmaceutiquement acceptable », on entend ici un excipient dont l'administration à un individu ne s'accompagne pas d'effets délétères significatifs. Les excipients pharmaceutiquement acceptables sont bien connus de l'homme du métier.

[0058] Tel qu'il est utilisé ici, l'expression « excipient pharmaceutiquement acceptable » comprend tous les solvants, tampons, solutions salines, milieux de dispersion, revêtements, agents antibactériens et antifongiques, agents isotoniques et retardant d'absorption, et analogues qui sont physiologiquement compatibles. Les excipients sont choisis selon la forme pharmaceutique et le mode d'administration souhaité, parmi les excipients habituels qui sont connus de l'homme du métier. Le type de support sera ainsi choisi en fonction de la voie d'administration prévue. Dans divers modes de réalisation, le support est approprié pour une administration intraveineuse, intrapéritonéale, sous-cutanée, intramusculaire, topique, transdermique, orale, ou par aérosol. Ainsi, dans des modes particuliers de réalisation, la présente souche est formulée dans des véhicules pharmaceutiquement acceptables, tels que des solutions, suspensions, comprimés, comprimés dispersibles, pilules, gélules, poudres, formulations à libération prolongée ou élixirs, pour administration orale ou dans des solutions ou suspensions stériles pour administration parentérale, ainsi que des patchs transdermiques et des inhalateurs de poudre sèche. Les véhicules pharmaceutiquement acceptables comprennent les solutions ou dispersions aqueuses stériles et des poudres stériles pour la préparation extemporanée de solutions ou dispersions injectables stériles. L'utilisation de milieux et agents pour des substances pharmaceutiquement actives est bien connue dans la technique. Les techniques et les méthodes bien connues de la personne du métier seront ainsi utilisées pour préparer des compositions pharmaceutiques contenant la souche I-5314 ou les extraits de celle-ci (voir par exemple, Ansel (1985) Introduction to Pharmaceutical Dosage Forms, 4th Ed., p. 126). Les procédés pour préparer des compositions pharmaceutiques, notamment des compositions pharmaceutiques administrables par voie orale ou par inhalation, seront connus ou évidents pour l'homme du métier et sont décrits plus en détail dans, par exemple, « *Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, Pa. (1985)* », et le 18^e et 19^e éditions de ce manuel.

[0059] Les présentes compositions sont administrées au patient à une dose thérapeutiquement efficace. Le terme « dose thérapeutiquement efficace » tel qu'utilisé ici se réfère à la quantité nécessaire pour observer une activité thérapeutique ou préventive sur l'inflammation liée à la maladie respiratoire, notamment la tuberculose, en particulier la quantité nécessaire pour observer une amélioration des symptômes. La quantité de bactérie I-5314 à administrer ainsi que la durée du traitement sont évaluées par l'homme du métier selon l'état physiologique du sujet à traiter, ainsi que la voie d'administration utilisée. La souche bactérienne utilisée peut être administrée sous la forme d'une dose unique ou de doses multiples.

[0060] L'homme du métier saura ainsi choisir au mieux les voies et modes d'administration de la composition, ainsi que les posologies et formes galéniques optimales, selon les

critères généralement pris en compte dans la fabrication d'un médicament ou l'établissement d'un traitement pharmaceutique ou vétérinaire. De préférence, ces composés seront administrés par voie systémique, en particulier par voie intraveineuse, par voie intramusculaire, intradermique, intra-péritonéale ou sous-cutanée, par voie orale, ou par voie topique (au moyen de gel, aérosols, gouttes, etc.). Les formes unitaires d'administration appropriées comprennent les formes par voie orale telles que les comprimés, les gélules molles ou dures, les poudres, les granules et les solutions ou suspensions orales, les formes d'administration sublinguale, buccale, intra-trachéale, intraoculaire, intranasale, par inhalation, les formes d'administration topique, transdermique, sous-cutanée, intramusculaire ou intraveineuse, les formes d'administration rectale et les implants. Pour l'application topique, on peut utiliser les composés selon l'invention dans des crèmes, gels, pommades ou lotions.

[0061] Il sera particulièrement avantageux d'administrer la composition par voie entérale, orale, parentérale (par exemple sous-cutanée, intradermique, ou intramusculaire) ou mucosale (par exemple intranasale, sublinguale, intravaginale, transcutanée). De manière plus préférée, la composition pharmaceutique sera administrée à plusieurs reprises, de manière étalée dans le temps. Son mode d'administration, sa posologie et sa forme galénique optimale peuvent être déterminés selon les critères généralement pris en compte dans l'établissement d'un traitement adapté à un patient comme par exemple l'âge ou le poids corporel du patient, la gravité de son état général, la tolérance au traitement et les effets secondaires constatés.

[0062] Dans les présentes compositions pharmaceutiques, le principe actif ou les principes actifs sont généralement formulés en unités de dosage. Par exemple, quand la bactérie I-5314 vivante est administrée, l'unité de dosage contient au moins 10^2 ufc, préférentiellement au moins 10^3 ufc, préférentiellement au moins 10^4 ufc, préférentiellement au moins 10^5 ufc, préférentiellement au moins 10^6 ufc, plus préférentiellement au moins 10^7 ufc, encore plus préférentiellement au moins 10^8 ufc, le plus préférentiellement au moins 10^9 ufc, par unité de dosage. Selon un autre mode de réalisation, l'unité de dosage contient entre 10^2 et 10^9 ufc, avantageusement entre 10^5 et 10^9 ufc, de préférence de 10^7 à 10^9 ufc par unité de dosage, pour les administrations quotidiennes, une ou plusieurs fois par jour. Par ailleurs, quand des extraits bactériens sont administrés au patient, l'unité de dosage contient 2,5 à 500 mg, avantageusement de 10 à 250 mg, de préférence de 10 à 150 mg par unité de dosage, pour les administrations quotidiennes, une ou plusieurs fois par jour. Bien que ces dosages soient des exemples de situations moyennes, il peut y avoir des cas particuliers où des dosages plus élevés ou plus faibles sont appropriés, de tels dosages appartiennent également à l'invention. Selon la pratique habituelle, le dosage approprié à chaque patient est déterminé par le médecin selon le mode d'administration, l'âge, le poids et la réponse dudit patient.

[0063] L'invention sera décrite plus précisément au moyen des exemples ci-dessous.

LEGENDES DES FIGURES

[0064] **Figure 1 : Protocole expérimental utilisé pour étudier l'effet de l'administration de bactéries isolées du microbiote pulmonaire sur l'infection par *Mycobacterium tuberculosis* (Mtb).** Des souris C57BL/6 femelles âgées de 6 semaines reçoivent par voie intranasale (i.n.) 10^7 bactéries (*L. salivarius* CNCM I 4968, ou *L. animalis* CNCM I ou *L. rhamnosus* CNCM I 4967 ou du PBS) dans 25µL de PBS 3 fois par semaine pendant 2 semaines. Selon les expériences elles sont ensuite sacrifiées (Figure 2.) ou infectées par 10^3 UFC de Mtb (autres figures). Pour les souris infectées, l'administration de lactobacilles est poursuivie jusqu'au sacrifice à raison de 2 fois par semaine (groupes traités avant et après infection, noté « av/ap »). Alternativement les lactobacilles peuvent n'être administrés qu'après infection (groupe CNCM I 5314 ap) et non avant. Après sacrifice, les poumons sont utilisés soit entiers pour une analyse en histologie de l'immunopathologie soit homogénéisés pour déterminer la charge bactérienne et caractériser la réponse immunitaire locale par cytométrie en flux.

[0065] **Figure 2 : Modification du système immunitaire pulmonaire de souris non infectées par des bactéries isolées du microbiote pulmonaire.** Des souris C57BL/6 reçoivent par voie intranasale 10^7 bactéries (*L. salivarius* CNCM I 4968 ou *L. animalis* CNCM I 5314 ou *L. rhamnosus* CNCM I 4967 ou PBS) dans 25 µL de PBS 3 fois par semaine pendant 2 semaines. Après sacrifice, une suspension cellulaire est obtenue par dissociation enzymatique et mécanique des poumons. Les proportions de sous-populations de lymphocytes T CD4+ sont déterminés par cytométrie en flux. **A.** Stratégie d'analyse: Après exclusion des doublets et des cellules mortes, les lymphocytes T CD4+ sont sélectionnés. La proportion de différentes sous-population est déterminée en sélectionnant les cellules exprimant un facteur intracellulaire spécifique d'intérêt (panel du bas) et pas son isotype contrôle (panel du haut). **B.** Proportion de lymphocytes T CD4+ exprimant le facteur Foxp3 (appelés Treg), produisant du TNF- α (cellules de type Th1) ou de l'IL-17 (Th17). Les graphiques représentent la médiane obtenue en groupant 3 expériences ayant chacune 4-7 souris. Statistique : test de Kruskal-Wallis : * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$

[0066] **Figure 3 : Impact de l'administration de bactéries isolées du microbiote pulmonaire sur l'infection par Mtb.** Des souris C57BL/6 reçoivent par voie intranasale 10^7 bactéries

(*L. salivarius* CNCM I 4968 ou *L. animalis* CNCM I 5314 ou *L. rhamnosus* CNCM I 4967 ou PBS) dans 25 µL de PBS 3 fois par semaine pendant 2 semaines. Elles sont ensuite infectées par 1000 bactéries de la souche H37Rv de Mtb par voie intranasale. Après infection les souris reçoivent les bactéries pulmonaires comme précédemment 2 fois par semaine pendant 30 jours. Après sacrifice les poumons sont dissociés pour

estimer la charge bactérienne ou fixés pour évaluer les dommages tissulaires. **A.** Estimation de la charge bactérienne mesurée par étalement sur milieu gélosé d'un broyat de poumon. Chaque graphique représente une expérience indépendante comparant 2 groupes de souris traitées avec un groupe contrôle, n=3-7 souris par groupe. **B.** Dommages tissulaires estimés par observation de colorations Hematoxylin-Eosine (HE) réalisées sur des coupes histologiques de poumons fixés en formol 10% et inclus en paraffine. Images représentatives de 2 expériences indépendante n=3 souris par groupe.

[0067] **Figure 4 : Caractérisation de l'impact de l'administration de *L. animalis* sur l'infection par *Mtb*.** Des souris C57BL/6 reçoivent du PBS (barres blanches) ou 10^7 de *L. animalis* par voie intranasale avant et après infection (groupe CNCM I 5314 av/ap, barres grises) par 1000 UFC de la souche H37Rv de *Mtb* par voie intranasale, ou uniquement après infection (groupe CNCM I 5314 ap, barres hachurées). **A.** Estimation de la charge bactérienne 42 jours après infection, mesurée par étalement sur milieu gélosé d'un broyat de poumon. Le graphique montre une expérience représentative de 2 expériences indépendantes, n=6 souris par groupe. **B.** Dommages tissulaires estimés par observation (panel de gauche) et quantification des infiltrats leucocytaires (panel de droite) sur colorations Hematoxylin-Eosine réalisées sur des coupes histologiques de poumons fixés en formol 10 % et inclus en paraffine 42 jours après infection. Le pourcentage d'infiltration correspond au ratio de l'aire occupée par des infiltrats leucocytaires par rapport à l'aire totale des poumons. 2-5 expériences comprenant chacune 4-5 souris par groupe sont représentées. **C.D.** Caractérisation des lymphocytes T CD4⁺ pulmonaires présents 42 jours après infection par cytométrie en flux. Les proportions de LT CD4⁺ exprimant différents facteurs de transcription (**C.**) (T-bet, caractéristique des Th1, ROR γ t pour les Th17 et Foxp3 pour les Treg) ou produisant des cytokines (**D.**) après stimulation avec phorbol 12-myristate 13-acetate (PMA) et Ionomycine en présence de Monensin et Brefeldin A (IFN- γ et TNF- α pour les Th1, IL-17 pour les Th17, IL-10 et TGF- β pour les Treg) sont représentées. **E.F.G.** Caractérisation des lymphocytes Treg exprimant Foxp3. L'origine des Tregs, naturels (nTreg, exprimant le facteur Helios, haut) ou induits (iTreg, qui ne l'expriment pas, bas) (**E.**), leur prolifération (caractérisée par l'expression de l'antigène Ki67) (**F.**) et production de cytokines (**G.**) est détectée par cytométrie en flux comme en C et D. Statistiques : graphiques correspondants à 1-4 expériences indépendantes poolées avec 4-7 souris par groupe pour les panels C et D. La médiane de chaque groupe est représentée et un test de Kruskal-Wallis compare les souris traitées aux souris contrôles : * p<0,05 ; ** p<0,01 ; **** p<0,0001.

EXEMPLES

Matériels et Méthodes

[0068] Souches bactériennes, milieux, conditions de croissance

[0069] Les souches bactériennes pulmonaires ont été isolées d'homogénats de poumons de souris avec un homogénéiseur (Ultraturax (IKA) ou Tissu Lyser (Qiagen)). Elles ont ensuite été cultivées sur milieu yhBHI, M17, MRS, ou Mannitol Sel Agar (BD biosciences) pendant 24 à 48h à 37°C sous conditions aérobie ou 5 jours à 37°C dans une chambre Freter sous conditions anaérobie. Les souches isolées ont été congelées à -80°C dans 16% de glycérol. L'identité de chaque souche a été confirmée par spectrophotométrie de masse et séquençage par PCR de l'ARN 16S. Les souches sélectionnées ont été déposées à la Collection Nationale des Cultures de Microorganismes (CNCM). Les trois souches utilisées ici sont un *Lactobacillus animalis* (souche de l'invention) déposée sous la référence CNCM I 5314, un *Lactobacillus rhamnosus* (CNCM I 4967) et un *Lactobacillus salivarius* (CNCM I 4968). Ces bactéries sont cultivées en milieu liquide MRS 24h à 30°C (pré-culture) ou 3-4h à 37°C (pour les ins-tillations) sans agitation.

[0070] Modèle murin d'infection par la tuberculose et traitement probiotique

[0071] Toutes les expériences réalisées sur des animaux ont été approuvées par le Ministère de l'enseignement supérieur et de la recherche après examen par le Comité d'Ethique Régional (Agréments APAFIS 5704). Les souris utilisées sont des femelles C57BL/6 âgées de 6 à 8 semaines provenant des élevages de Charles River Laboratories.

[0072] Pour chaque administration *in vivo* d'un des lactobacilles, une suspension bactérienne contenant 4.0×10^8 CFU/mL a été préparée en tampon phosphate salin (PBS) à partir de cultures fraîches en phase exponentielle. Les souris reçoivent 25 μ L de PBS contenant 1.0×10^7 CFU ou 25 μ L de PBS (groupe contrôle) par voie intranasale (i.n.) sous anesthésie gazeuse (isoflurane 4%, Virbac Danmark). Cette opération est répétée 3 fois par semaine pendant 2 semaines puis les souris sont soit sacrifiées (expériences sur les souris non infectées) soit infectées (procédures décrites ci-après), et reçoivent à nouveau l'administration des bactéries commensales 2 fois par semaine jusqu'au sacrifice. Dans certaines expériences, l'administration des lactobacilles est réalisée uniquement après infection et non pas avant et après infection (voir Figure 1).

[0073] Une culture fraîche de la souche H37Rv de Mtb (cultivée en milieu liquide 7H9 (Difco) supplémenté en glycérol 0,5%, ADC (Middlebrook) 10% et tyloxapol 0.05%) est utilisée pour infecter les souris. Chaque souris reçoit par voie i.n. 1.0×10^3 CFU de Mtb dans 25 μ L de PBS sous anesthésie isoflurane. Les souris sont sacrifiées par dis-location cervicale (sous anesthésie isoflurane) après 42 jours d'infection.

[0074] Analyses histologiques

[0075] Les poumons entiers de souris dédiées aux analyses histologiques sont utilisés. Ils sont gonflés puis fixés pendant 5 jours à 4°C avec une solution contenant 10% de

formol (Formalin solution, Sigma-Aldrich) et inclus en paraffine. Des colorations hématoxyline-éosine (HE) sont réalisées pour visualiser les infiltrats leucocytaires qui sont quantifiés, après numérisation, sur le logiciel CaseViewer (3DHISTECH).

[0076] Préparation des homogénats pulmonaires et détermination de la charge bactérienne

[0077] Les poumons entiers des souris ont été prélevés de manière stérile, homogénéisés avec un gentleMACS dissociator avant (tubes C, cycle m_lung_01, Miltenyi) et après (cycle m_lung_02) 30 min d'incubation à 37°C avec de la collagénase D (2 mg/mL, Roche) et de la DNase I (0,1mg/mL, Roche). Une part de cet homogénat est diluée en série en PBS puis étalée sur milieu gélosé 7H10 (Difco) supplémenté en peptone et OADC (Middlebrook). Après 2-3 semaines, le dénombrement des colonies de Mtb obtenues permet d'estimer la charge bactérienne pulmonaire. Le reste des homogénats est passé au travers de filtre 70 µm pour détruire les agrégats, et centrifugé à 329 x g pendant 5 min. Les surnageants sont passés 2 fois au travers de filtres 0,2 µm et stockés à -80°C pour l'analyse des cytokines présentes dans les poumons. Les globules rouges présents dans le culot sont lysés pendant 5 min avec une solution contenant 150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA (pH 7.2), neutralisée par ajout de milieu RPMI contenant 10% du sérum de veau fœtal (SVF). La suspension cellulaire ainsi obtenue est filtrée au travers d'un filtre 40 µm (élimination des agrégats de globules rouges lysés) pour les analyses de cytométrie en flux.

[0078] Cytométrie en flux

[0079] L'analyse des différentes populations de lymphocytes T auxiliaires CD4⁺ est réalisée grâce à la détection par cytométrie en flux de facteurs de transcription et de cytokines caractéristiques de ces sous population par marquages des cellules présentes dans la suspension cellulaire obtenue comme décrit dans la section précédente. Une partie de la suspension cellulaire est incubée dans du RPMI contenant 50 ng/mL de Phorbol Myristate Acetate (PMA, (Sigma Aldrich) et 500 ng/mL d'ionomycine (Sigma-Aldrich) pour induire la production de cytokines par les lymphocytes ainsi que de la Brefeldin A (Golgi plug 1/1000, BD Biosciences) et du Monensin (Golgi Stop 1/2000, BD Biosciences) pour ségréger ces cytokines dans l'appareil de Golgi pendant 4h à 37°C et 5% CO₂. Le reste de la suspension cellulaire, utilisée pour le marquage de facteurs de transcription, est conservée dans du Cell Staining Buffer (CSB, Biolegend) 4 h à 4°C. Le marquage extracellulaire de ces deux fractions est réalisé en CSB 30 min à 4°C dans le noir en utilisant un anticorps anti-Cluster Differentiation 16 /32 (CD16/CD32, Biolegend) pour limiter les marquages aspécifiques, un marqueur de viabilité (live/dead fixable blue dead cell stain kit, Invitrogen), un anti-CD45.2 BV711 (clone 104, BD Biosciences), un anti-CD3 FITC (17A2, Biolegend) ou anti-T cell Receptor beta (TCRb) Alexa 700 (H57-597, Biolegend) et un anti-CD4 BV786 (Sk3, BD Biosciences). Pour le marquage intracellulaire les cellules sont ensuite fixées 30

min à température ambiante (TA), perméabilisées 15 min à TA (Foxp3 / transcription factor staining buffer set, eBioscience) et incubées 45 min à TA avec un panel d'anticorps comprenant un anti-RORgt PE-CF594 (Q31-378, BD Biosciences), un anti-T-bet PE-Cy7 (eBio4BIO, eBiosciences), un anti-Foxp3 APC (FJK-16s, eBioscience), un anti-Helios APC-eFluor 780 (22F6, eBiosciences), un anti-Ki67 Alexa 700 (SolA15, eBioscience) ou un anti-interleukin 10 (IL-10) FITC (JES5-16E3, BD Biosciences), un anti-IL-17 PE (TC11-18H10, BD Biosciences), un anti-IFN γ PE-Dazzle (XMG1.2, Biolegend), un anti-TNF α PE Cy7 (MP6-XT22, BD Biosciences), l'anti-Foxp3 APC et un anti-TGF- β BV421 (TW7-16B4, BD Biosciences). Pour les expériences réalisées sur des souris infectées par Mtb, les cellules sont fixées 2h en paraformaldéhyde (PFA) 4% à TA. Les données sont acquises avec un FACS LSRII ou Fortessa (BD Biosciences) et analysées sur le logiciel FlowJo V10. Les doublets (FSH-H vs. FSC-W et SSC-H vs. SSC-W) et les cellules mortes (live/dead positives) sont exclues au début de chaque analyse.

[0080] Analyse statistique

[0081] L'analyse statistique des résultats a été réalisée avec le logiciel GraphPad Prism 7. Sur les graphiques, chaque point représente une souris différente. La médiane de chaque groupe est représentée par les barres. Un test de Mann-Whitney (comparaison de 2 groupes) ou de Kruskal-Wallis (comparaison de 3 groupes) a été utilisé pour comparer les valeurs. La significativité est représentée par : * p<0.05; ** p<0.01; *** p<0.001; and **** p<0.0001.

Résultats

[0082] L'identification de bactéries du microbiote pulmonaire capables de modifier la réponse immunitaire au cours de l'infection par Mtb s'appuie sur une banque de bactéries primo-colonisatrices du poumon de souris isolées et identifiées par l'équipe du Dr Langella (Probiote – MICALIS – INRA), et notamment par Aude Remot et Muriel Thomas. L'une de ces bactéries, une souche d'*Enterococcus sp.* déposée à la CNCM sous le numéro I-4969 est capable de moduler la susceptibilité des souris à l'asthme allergique (voir WO 2017/12987 et [8]). Parmi ces bactéries, 3 sont des lactobacilles (*L. salivarius*, *L. animalis* et *L. rhamnosus*) qui possèdent un statut d'organismes GRAS (Generally Recognised As Safe) et ont été déposés à la CNCM sous les numéros : CNCM I 4968, CNCM I 5314 et CNCM I 4967, respectivement.

[0083] Pour déterminer le potentiel probiotique de ces 3 lactobacilles pour la prévention et le traitement de la tuberculose, différents protocoles résumés en Figure 1 ont été employés dans un modèle murin. L'administration de 10⁷ bactéries est réalisée par voie intranasale pendant deux semaines avant sacrifice des souris (Figure 2) ou infection par *Mycobacterium tuberculosis* (Mtb) (autres figures). Dans ces expériences les bactéries sont administrées avant et après infection (groupes av/ap) ou seulement après infection

(groupe ap, Figure 4).

- [0084] Dans un premier temps, la capacité des bactéries à modifier le système immunitaire local de souris non-infectées a été évaluée par l'analyse en cytométrie en flux de l'expression de marqueurs intracellulaires caractéristiques de différentes sous-populations de lymphocytes T auxiliaires CD4⁺ (stratégie d'analyse en Figure 2A), orchestrateurs clés de la réponse immunitaire anti-Mtb: les lymphocytes Th1 (produisant du TNF- α), Th17 (produisant l'IL-17A) et T régulateurs (Treg exprimant Foxp3) [16, 17, 18]. Nos résultats montrent que les trois lactobacilles isolés du microbiote pulmonaire ont des capacités immunomodulatrices fortes, avec un profil anti-inflammatoire (même en dehors d'un contexte infectieux). La souche CNCM I 5314 de *L. animalis* est celle qui induit la plus forte diminution des Th1, augmentation des Th17 et des Treg pulmonaires (Figure 2B), alors que ces trois espèces appartiennent au même genre bactérien.
- [0085] Pour déterminer si ces bactéries (et notamment la souche de *L. animalis*) induisent une réponse anti-inflammatoire suffisante pour diminuer l'immunopathologie associée à la tuberculose, les bactéries ont été administrées comme précédemment par voie intranasale, 15 jours avant infection par Mtb puis tout au long de l'infection (Figure 1). Dans ce modèle, les trois bactéries ne modifient pas la charge bactérienne de Mtb (Figure 3A). En revanche, *L. animalis* CNCM I 5314 semble conférer une protection importante contre l'infiltration leucocytaire des poumons (moins de surface pulmonaire occupée par des cellules immunitaires) menant à l'immuno-pathologie à des stades tardifs de l'infection (et pas les deux autres bactéries) (Figure 3B).
- [0086] Pour mieux caractériser l'effet protecteur de la souche CNCM I 5314, la même expérience a été répétée (groupe CNCM I 5314 av/ap) en incluant des analyses permettant de déterminer la composition de l'infiltrat immunitaire des poumons. De plus, la capacité de cette bactérie à exercer son effet protecteur dans une stratégie de traitement (par opposition à l'approche prophylactique décrite précédemment) a été évaluée en ajoutant un groupe pour lequel l'administration de la bactérie ne commence qu'après l'infection (groupe CNCMI 5314 ap) (détail des différents groupes dans la Figure 1). Ces expériences confirment que l'effet anti-inflammatoire induit par cette bactérie ne permet pas à Mtb de se multiplier de manière incontrôlée (Figure 4A) alors qu'il accorde une protection significative (pour le groupe ayant reçu la bactérie en pré-traitement, CNCM I 5314 av/ap) contre l'immuno-pathologie induite par l'infection avec une réduction des infiltrats leucocytaires (Figure 4B). Bien que l'infiltration pulmonaire de lymphocytes Th1 pro-inflammatoires (exprimant T-bet et produisant du TNF- α ou de l'IFN- γ) varie peu entre les groupes, la souche CNCM I 5314 induit une augmentation à la fois des lymphocytes Th17 (exprimant ROR γ t et produisant de l'IL-17A) et des Treg (exprimant Foxp3, produisant de l'IL-10 et du TGF- β) 42 jours

après infection (Figure 4C). Cependant une analyse plus poussée révèle que l'augmentation des Treg observée avec l'administration de *L. animalis* (que ce soit avec une approche prophylactique ou de traitement) n'est pas liée à une augmentation de Treg conventionnels (Foxp3 + ROR γ t⁻), mais à celle d'une population récemment décrite, double positive Foxp3 + ROR γ t⁺ appelée bi-Treg. Ces cellules possèdent à la fois des fonctions pro-inflammatoires et anti-inflammatoires et produisent de l'IL-17 mais aussi de l'IL-10, du TGF- β et de l'IL-35 (non analysée ici) qui en font des régulateurs clés de l'inflammation [17, 18]. Elles pourraient donc être responsables de la diminution des infiltrats leucocytaires (observés en 4B) et donc de l'effet prometteur de cette bactérie pour la prévention et le traitement de la tuberculose. La proportion de Th17 (Foxp3⁻ ROR γ t⁺) semble également augmentée. Dans le cas d'un environnement riche en IL-10 et TGF- β (comme c'est le cas ici), l'activité des Th17/IL-17 est biaisée et ces cellules exercent des fonctions anti-inflammatoires et protectives vis-à-vis des dommages tissulaires, principalement via la production d'IL-22 (production non mesurée dans nos expériences), suggérant un rôle bénéfique de ces cellules dans notre contexte [16, 17]. Les Treg augmentés par *L. animalis*, et en particulier les biTreg n'expriment pas le facteur de transcription Helios, indiquant qu'ils sont induits au niveau des muqueuses (iTreg) par opposition aux Treg d'origine naturelle (nTreg) c'est-à-dire générés dans le thymus, suggérant que cet effet est spécifique et lié à la tolérance périphérique (Figure 4E). L'augmentation de ces cellules ne semble pas liée à une plus forte prolifération puisque l'expression de l'antigène Ki67 est diminuée (Figure 4F). Elles semblent pouvoir avoir les effets pro- et anti-inflammatoires décrits dans la littérature puisque les Tregs observés produisent de l'IL-17, de l'IL-10 mais surtout du TGF- β (dont la production est également plus forte dans les deux groupes de souris ayant reçus l'administration de *L. animalis*) (Figure 4G).

[0087] Nous montrons ici pour la première fois qu'une souche bactérienne du microbiote pulmonaire (*L. animalis* CNCM I 5314) est capable de modifier la réponse immunitaire à Mtb notamment par une induction de biTreg qui pourraient mieux contrôler la balance immunitaire (pro-/anti- inflammatoire) et ainsi réduire l'immunopathologie induite par l'infection. Ces résultats présentent ainsi la souche *L. animalis* (CNCM I-5314) isolée du microbiote pulmonaire comme un probiotique prometteur pour la tuberculose. Les résultats préliminaires obtenus avec le groupe « CNCM I 5314 ap » suggèrent également des applications pour le traitement de cette maladie. D'autres maladies respiratoires étant liées à une inflammation, nous supposons que son rôle bénéfique ne se limiterait pas à la tuberculose.

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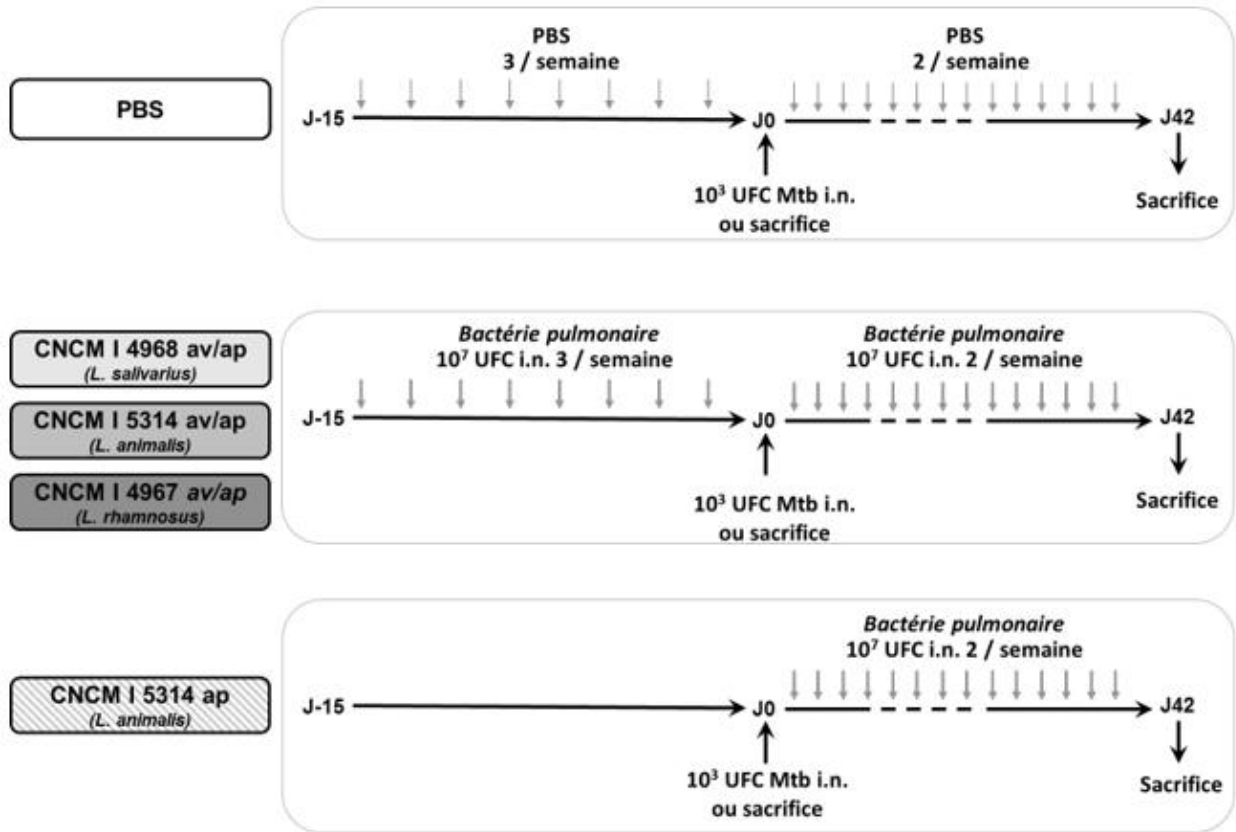
Revendications

- [Revendication 1] Bactérie *L. animalis* pour son utilisation dans le traitement et/ou la prévention de l'inflammation liée à une maladie respiratoire.
- [Revendication 2] Bactérie *L. animalis* pour son utilisation selon la revendication 1, caractérisée en ce que ladite bactérie est inactivée.
- [Revendication 3] Bactérie *L. animalis* pour son utilisation selon l'une quelconque des revendications 1 ou 2, caractérisée en ce que ledit traitement et/ou prévention comprend une diminution de l'infiltration leucocytaire et une augmentation des populations pulmonaires lymphocytes Th17 que de lymphocytes Tregs.
- [Revendication 4] Bactérie *L. animalis* pour son utilisation selon la revendication 3, caractérisée en ce que les lymphocytes Tregs sont des lymphocytes iTregs.
- [Revendication 5] Bactérie *L. animalis* pour son utilisation selon la revendication 4, caractérisée en ce que les lymphocytes iTregs sont des lymphocytes biTregs.
- [Revendication 6] Bactérie *L. animalis* pour son utilisation selon l'une quelconque des revendications 1 à 5, caractérisée en ce que la maladie respiratoire est la tuberculose.
- [Revendication 7] Bactérie *L. animalis* pour son utilisation selon l'une quelconque des revendications 1 à 5, caractérisée en ce que ladite bactérie est la souche de *L. animalis* déposée à la Collection nationale des cultures de microorganismes (CNCM, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France) sous le numéro I-5314.
- [Revendication 8] Souche de *L. animalis* déposée à la Collection nationale des cultures de microorganismes (CNCM, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France) sous le numéro I-5314.
- [Revendication 9] Composition pharmaceutique comprenant la souche de la revendication 8 et au moins un excipient pharmaceutiquement acceptable.
- [Revendication 10] La composition de la revendication 9 caractérisée en ce que la souche est inactivée.
- [Revendication 11] La composition de la revendication 10 caractérisée en ce que la souche est inactivée par la chaleur.
- [Revendication 12] La composition de la revendication 10 caractérisée en ce que la souche est présente sous forme d'extraits.

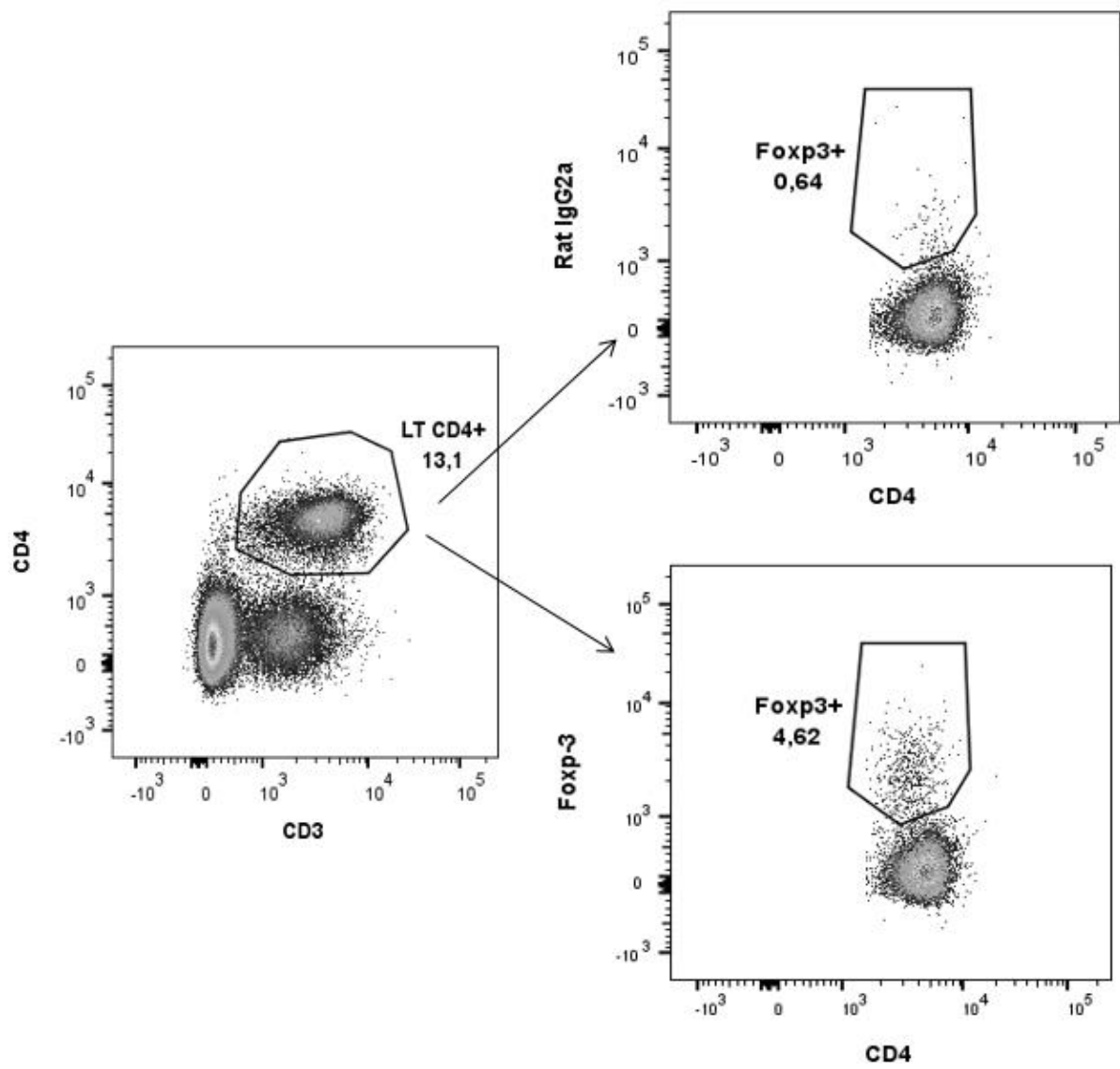
Abrégé

La présente invention a pour objet l'utilisation de la bactérie *Lactobacillus animalis* dans le traitement des maladies respiratoires chez les humains tout comme chez les animaux. Il est notamment décrit une souche particulière de cette bactérie et des compositions pharmaceutiques la comprenant.

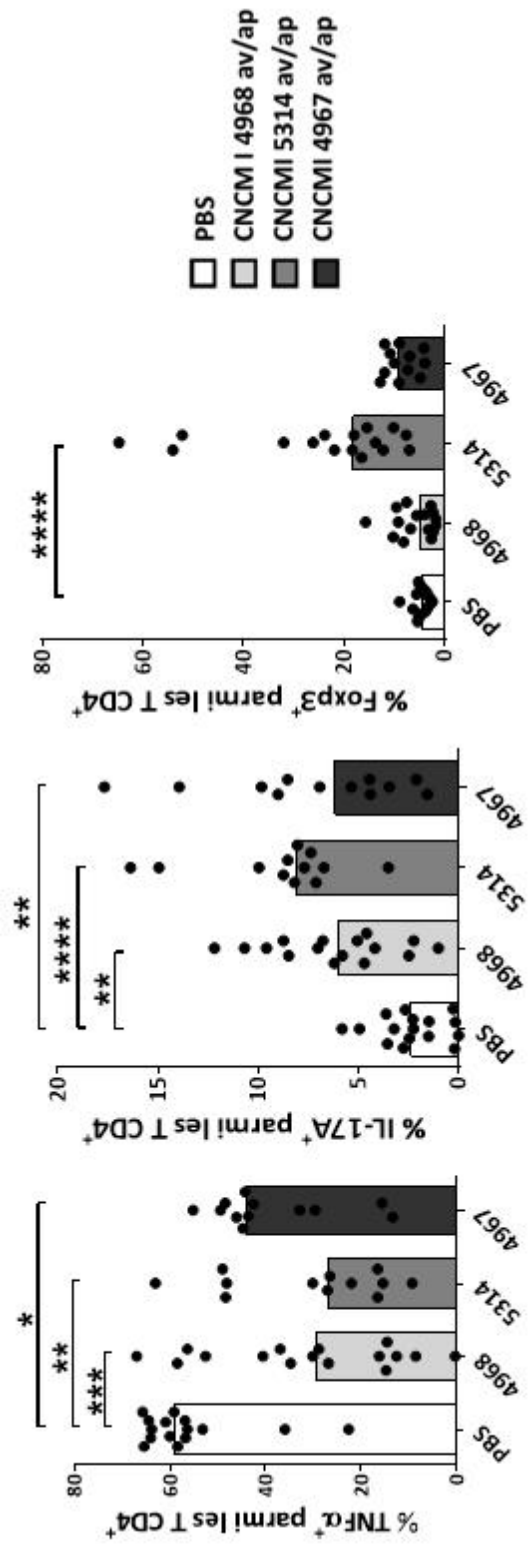
[Fig. 1]



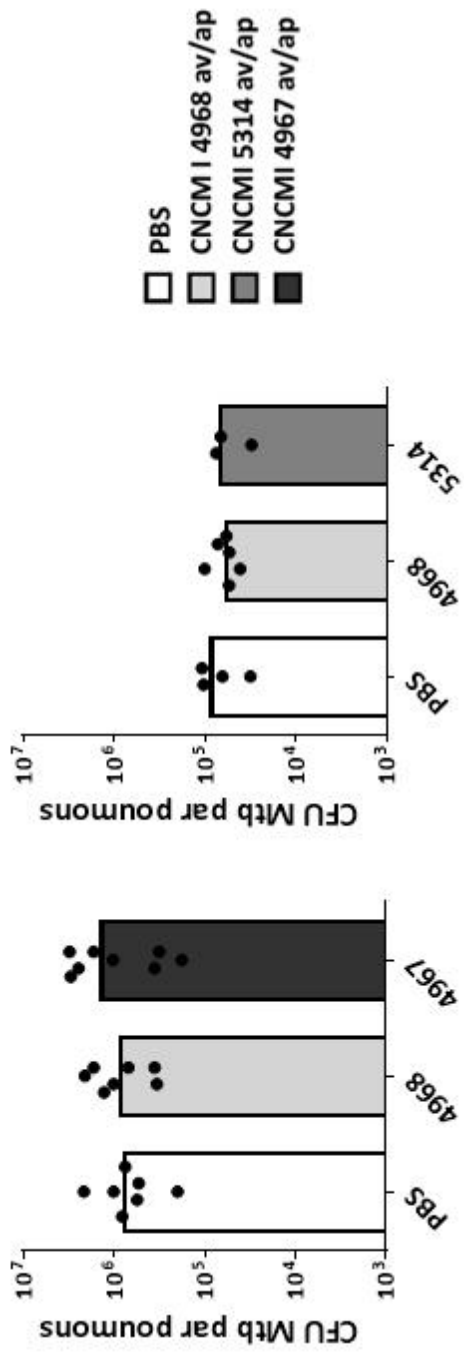
[Fig. 2A]



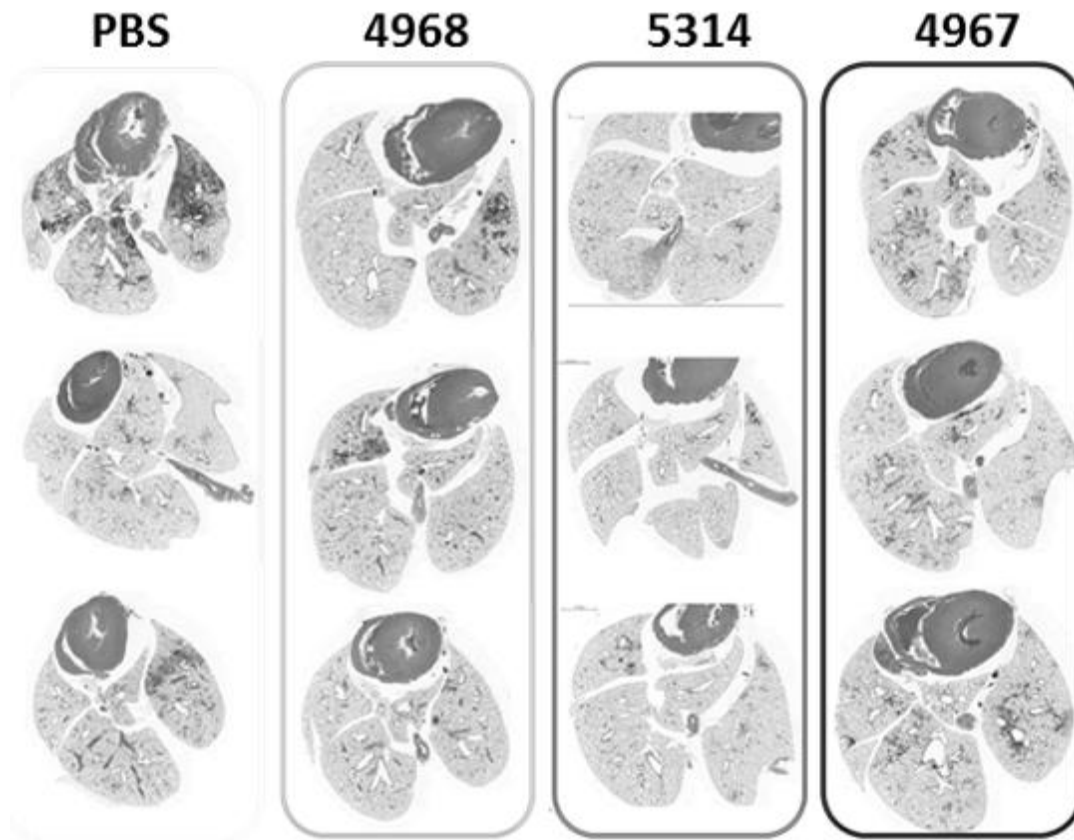
[Fig. 2B]



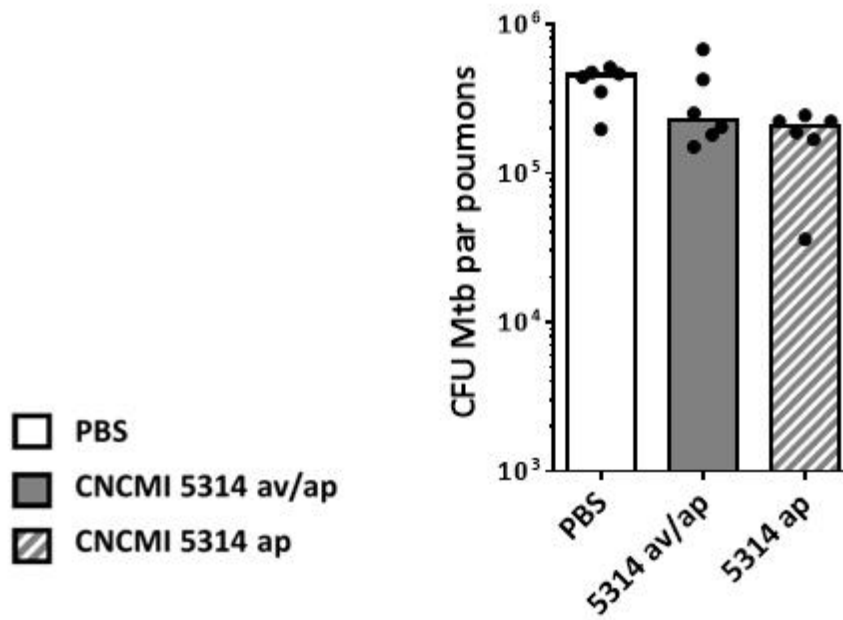
[Fig. 3A]



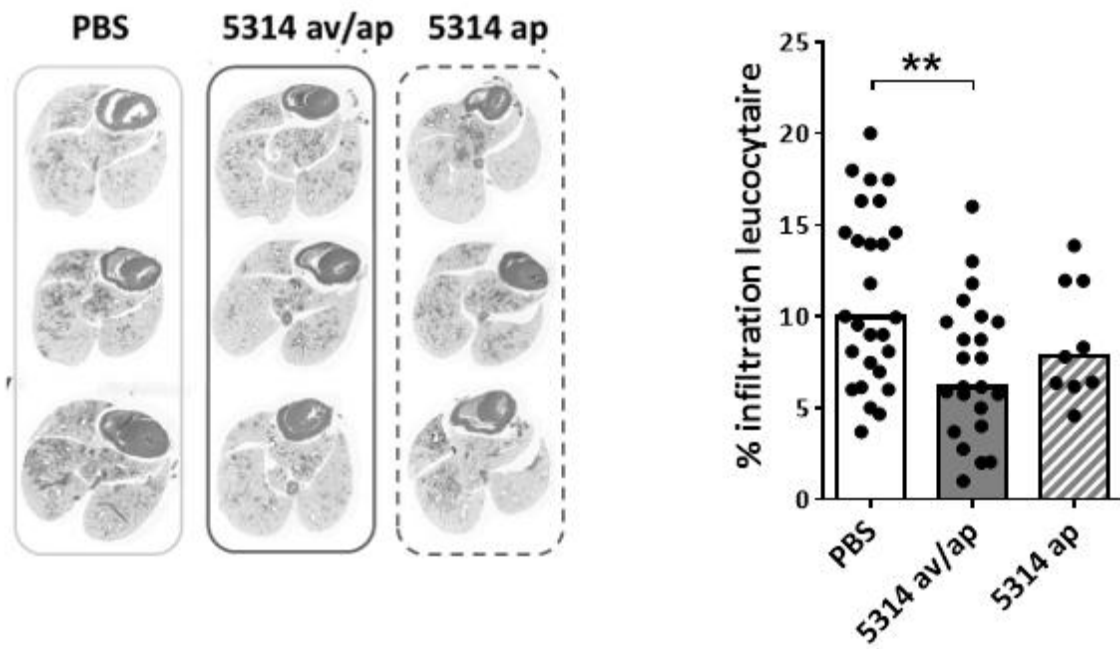
[Fig. 3B]



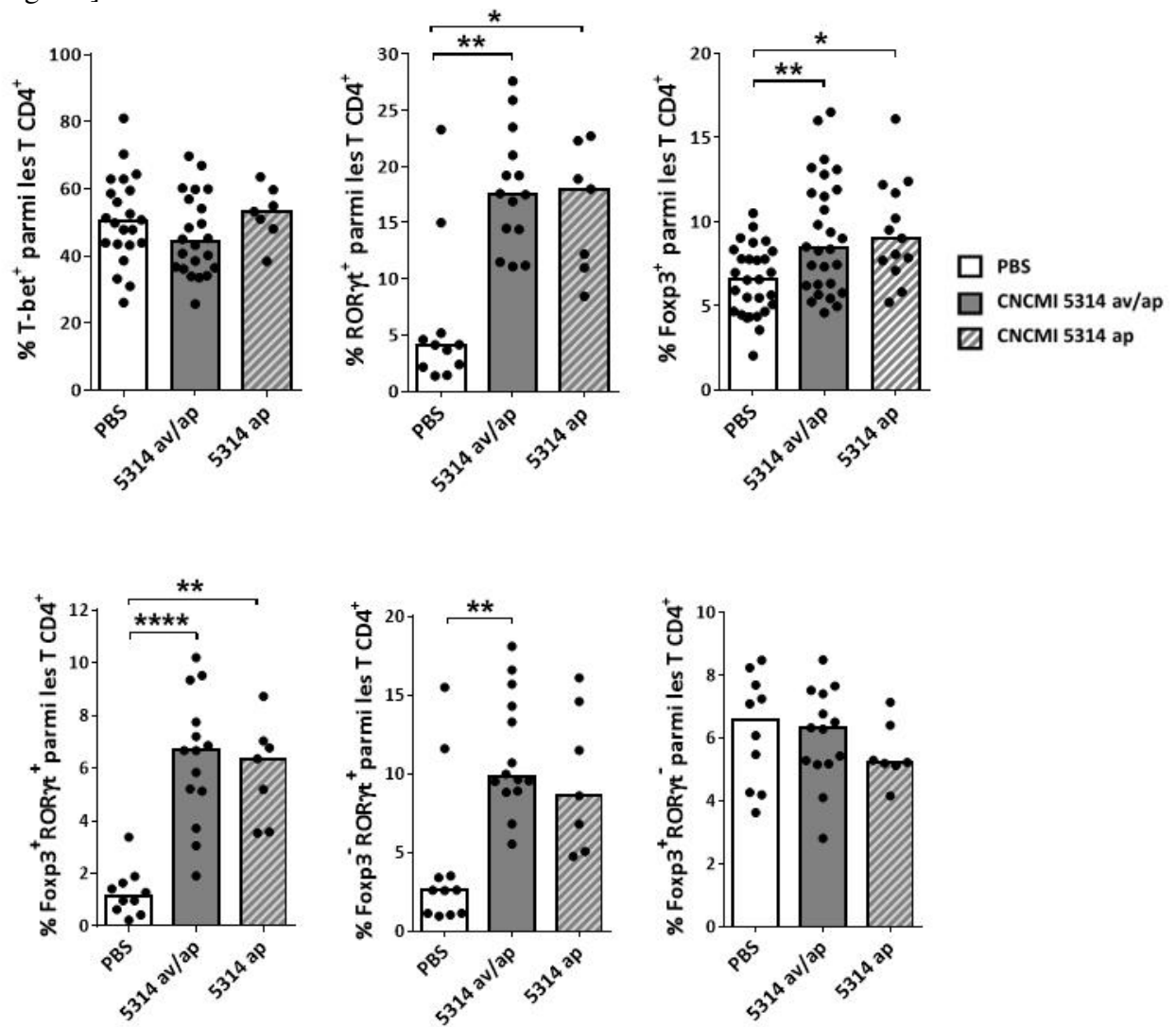
[Fig. 4A]



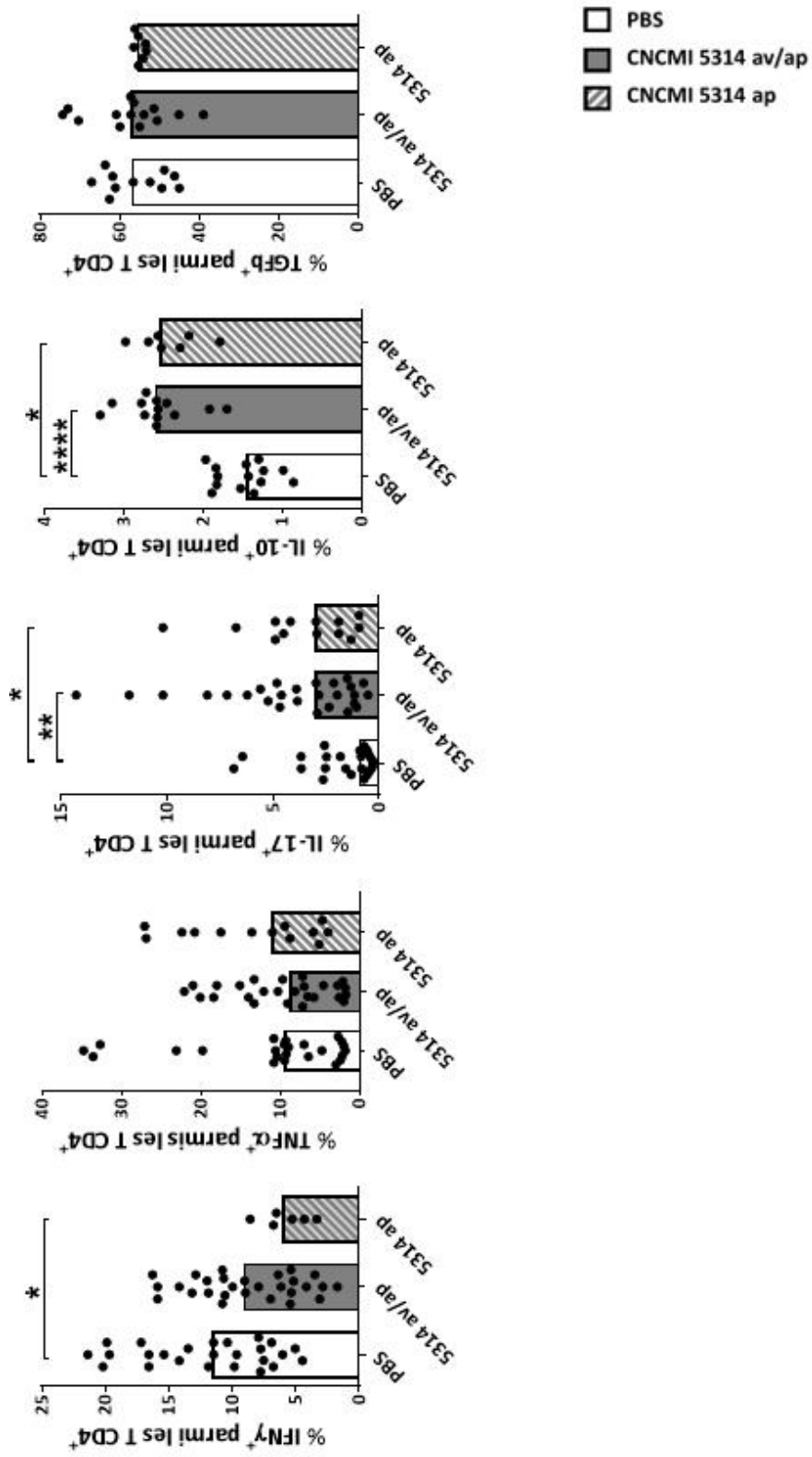
[Fig. 4B]



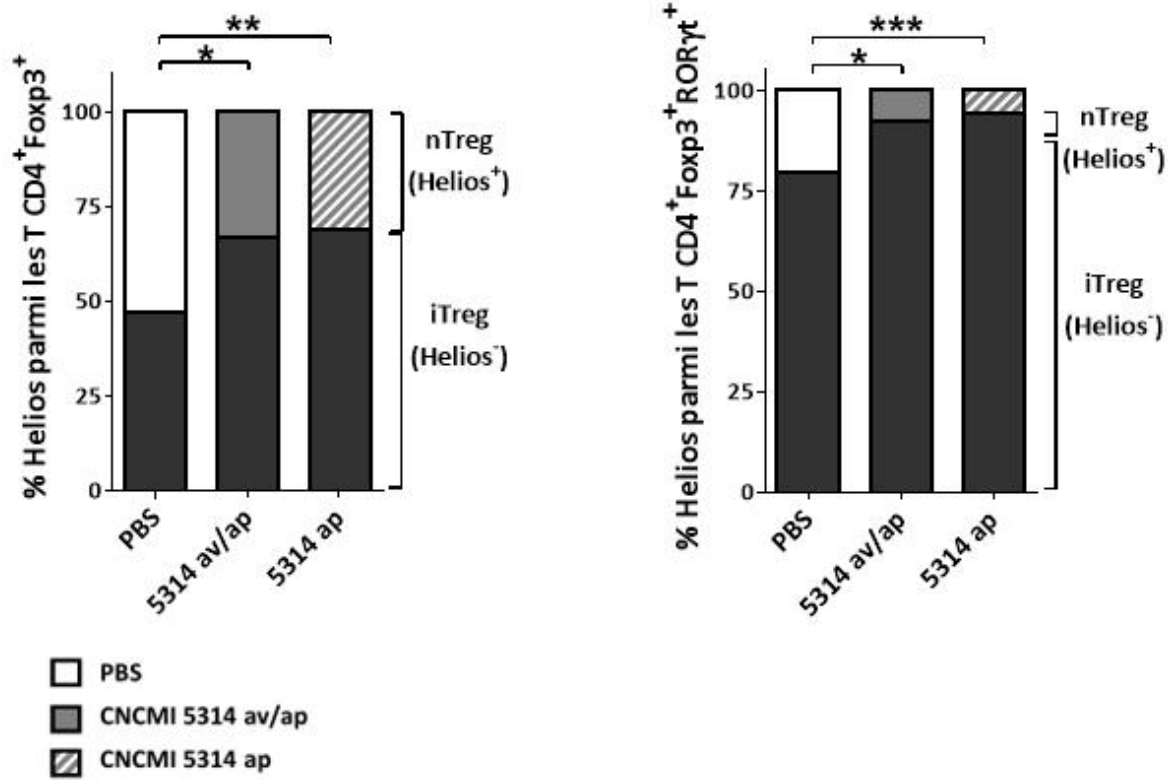
[Fig. 4C]



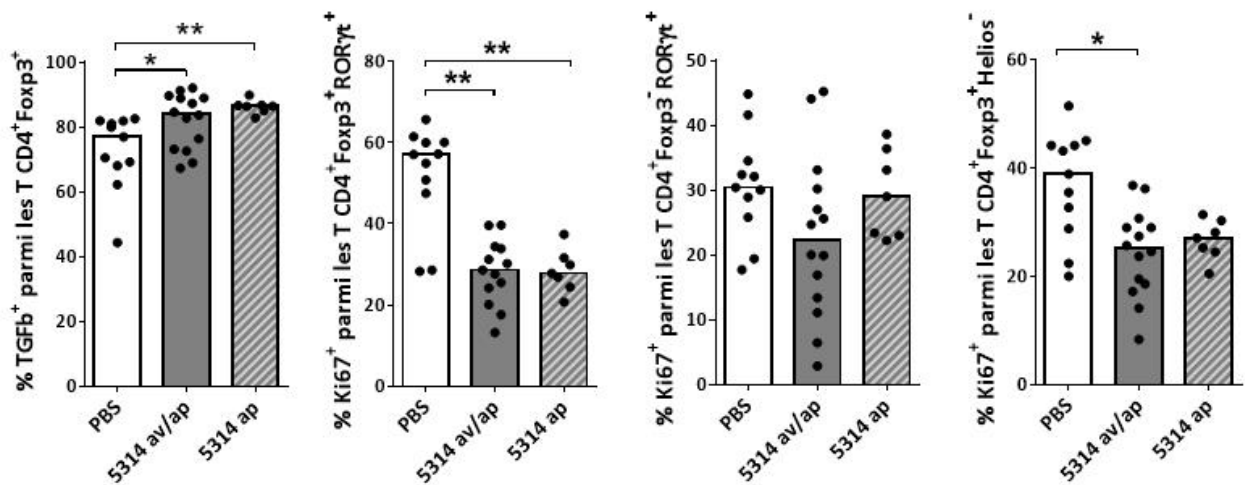
[Fig. 4D]



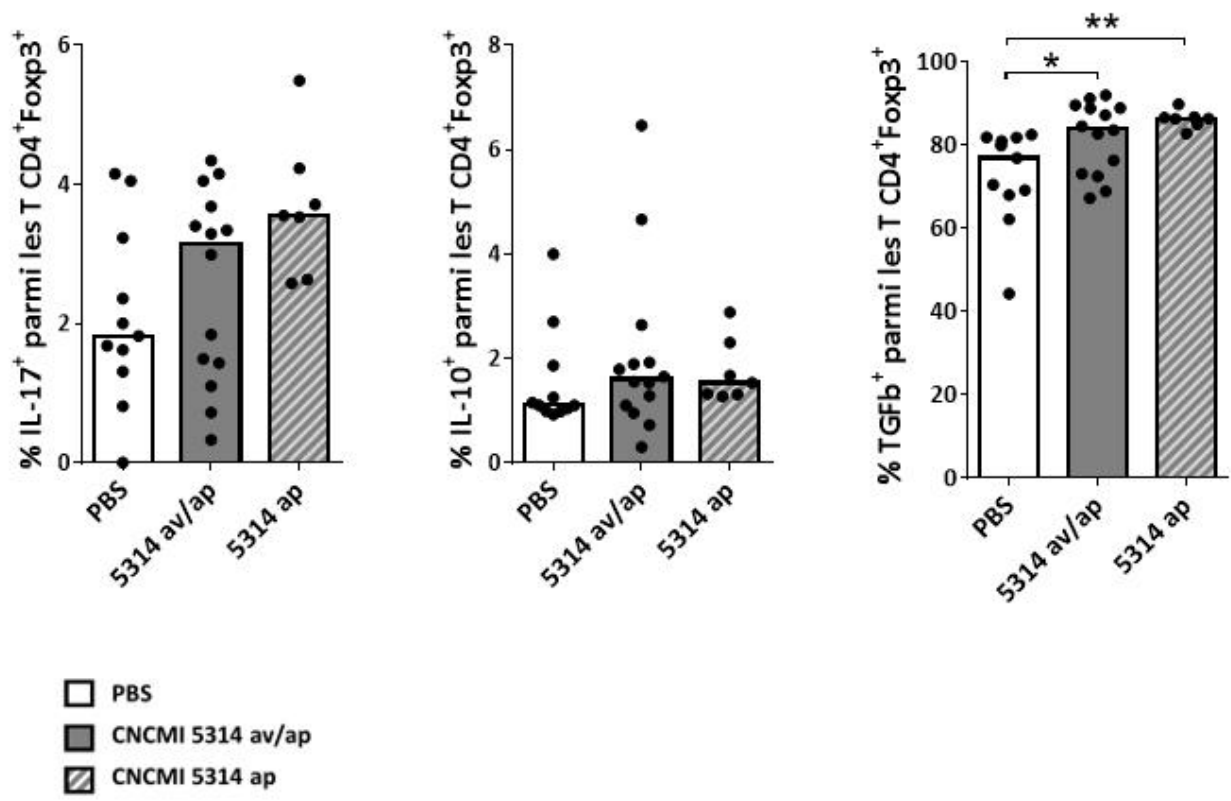
[Fig. 4E]



[Fig. 4F]



[Fig. 4G]



Résumé

Utilisation de bactéries du microbiote pulmonaire pour moduler le système immunitaire local à l'état basal et pendant l'infection par *Mycobacterium tuberculosis* chez la souris

Les muqueuses du corps humain (notamment l'intestin) sont colonisées dès la naissance par des milliards de microorganismes formant le microbiote (ou flore commensale) et qui vivent en symbiose avec lui. La survie de notre organisme (l'hôte) et de son microbiote dépend de l'état d'activation de notre système immunitaire. Alors qu'une trop faible activation nous rend susceptibles aux infections, une trop forte activation ou inflammation altère nos tissus. Certaines bactéries du microbiote intestinal, interagissent avec les cellules du système immunitaire pour moduler cette balance. Leur administration en tant que probiotique améliore de nombreuses pathologies et est envisagée pour le traitement d'infections respiratoires. L'infection causant le plus de décès dans le monde est la tuberculose, une maladie respiratoire causée par *Mycobacterium tuberculosis* et dans laquelle une hyper-activation du système immunitaire détruit les tissus pulmonaires. Dans ce projet de thèse, j'ai cherché à savoir si des bactéries du microbiote peuvent influencer la réponse du système immunitaire à la tuberculose, évaluant ainsi le potentiel d'une stratégie probiotique pour améliorer les traitements de cette maladie. En particulier, j'ai fait l'hypothèse que des bactéries commensales isolées du microbiote pulmonaire (récemment décrit) pourraient, par un effet local, fortement modifier cette réponse comme c'est le cas dans l'asthme, chez la souris. Dans un premier temps, j'ai montré que certaines bactéries du microbiote pulmonaire, administrées à des souris saines par voie intranasale, ont une forte capacité à moduler les lymphocytes T CD4⁺ des poumons tels que les Th1 ou Th17, impliqués dans l'immunité pro-inflammatoire, et les T régulateurs (Treg) réduisant l'activation immunitaire. En particulier, elles induisent la prolifération d'un sous type de Treg exprimant ROR γ t (facteur de transcription caractéristique des Th17), nommé ROR γ t⁺ Treg, récemment identifié dans l'intestin où l'activation de ces cellules par le microbiote limite les maladies inflammatoires de l'intestin comme la colite. Nous avons montré pour la première fois que ces cellules sont induites dans les poumons de souris traitées avec des bactéries isolées du microbiote pulmonaire appartenant aux genres bactériens *Lactobacillus*, *Staphylococcus* et *Neisseria* et avons caractérisé leur phénotype. Comme dans l'intestin, ces cellules semblent avoir un fort potentiel anti-inflammatoire, soutenu par leur forte expression des molécules inhibitrices CTLA-4, ou PD-1, du marqueur d'activation ICOS et de la cytokine anti-inflammatoire TGF- β associée à une faible sécrétion de cytokines pro-inflammatoires telles que le TNF- α . De façon intéressante, j'ai observé que des souches de *Lactobacillus* pulmonaires induisent les mêmes populations leucocytaires dans le modèle murin de tuberculose que dans des souris naïves, et notamment les ROR γ t⁺ Treg. Tandis qu'aucune des souches testées ne diminuent la charge bactérienne de *M. tuberculosis* dans les poumons ou la rate des souris infectées, une souche de *Lactobacillus murinus* (CNCM I-5314) qui augmente fortement les Th17 et les ROR γ t⁺ Treg, réduit l'infiltration leucocytaire pulmonaire, suggérant sa capacité à réduire l'inflammation associée à cette infection. Bien que la détermination du rôle des Th17 et des ROR γ t⁺ Treg dans ce phénotype reste à élucider, nos résultats démontrent d'ores et déjà que l'administration de bactéries commensales pulmonaires peut fortement moduler l'immunité locale même au cours d'infections comme la tuberculose. Une meilleure caractérisation des composants du microbiote pulmonaire et des mécanismes par lesquels ils interagissent avec notre système immunitaire pour maintenir la santé respiratoire devrait donc permettre l'émergence d'une nouvelle génération de probiotiques, d'origine pulmonaire, pour prévenir et améliorer le traitement des maladies respiratoires.