UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN

SCIENZE VETERINARIE

INDIRIZZO: Patologia e Scienze Veterinarie **(XXVII CICLO)**

Immunopathological aspects in Human and Bovine Tuberculosis: - Immunopathological Changes in IRF-8$^{-/-}$ mice during *Mycobacterium tuberculosis* Infection - Histopathological and Biomolecular Evaluations in Cattle Tuberculin Skin Test positive slaughtered according to The Regional Eradication Program

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ABSTRACT

Among diseases caused by *Mycobacteria*, tuberculosis remains an unsolved issue in humans and animals, causing economic and public health repercussions. How the host immune system responds at initial stage of infection is the key factor for progression of disease or for the establishment of latent infection. This thesis is focused on two fundamental aspects in the immunopathology of tuberculosis: firstly, in lungs of IRF-$8^{-/-}$ mice histopathologic alterations in both innate and adaptive immune response were observed, after experimental infection of animals with *Mycobacterium tuberculosis* (Mtb). Secondly, the presence of mycobacteria in carcass of TST positive cattle was investigated during sporadic cases of bTB in Sardinia. To investigate the immune events histochemistry, immunohistochemistry and molecular analysis have been applied.

IRF-8 KO mice Mtb-infected showed severe tissue damage in the lungs correlated with an uncontrolled growth of granulomas. In addition, in these animals, an altered inflammatory exudate was observed, constituted by a large number of neutrophils with poor recruitment of lymphocytes, resulting in a higher susceptibility to Mtb infection.

In TST cattle, discovering mycobacteria within the carcass using collateral diagnostic methods (histopathology and PCR) have increased the diagnosis of infection of 5% in post-mortem examination.

Therefore, for different purposes, investigating the immunopathology in tuberculosis in humans and cattle is even more important in order to perform an earlier diagnosis, aimed to improve better treatment and eradication of disease.

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CHAPTER 1

Immunopathological Aspects in Human and Bovine Tuberculosis

1.1 General introduction- *Mycobacterium tuberculosis* complex

Tuberculosis (TB), an ancient disease, remains one of the leading causes of global morbidity and mortality among infectious diseases largely due to HIV pandemics and multidrug-resistant TB[1]. Almost one-third of world population is considered infected by Mtb[2,3], and the infection is the major cause of mortality along bacterial disease, reaching 9 million new cases per year with almost 2 million deaths until 2012[4].

In cattle, bovine tuberculosis (bTB) is an enormous economical problem in food industry, on farm and trade, with important zoonotic aspect, and its eradication represents a great challenge all over the world[5,6].

Mycobacterium tuberculosis complex

Mycobacterium tuberculosis complex is a name used to identify a group of mycobacteria having genetic similarity. Bacteria belonging this group are considered very ancient, in particular *Mycobacterium tuberculosis* (Mtb) is considered resulting from "old" progenitors in Africa 3 million years ago[7].

M. tuberculosis complex include other mycobacteria: *M. africanum, M. canetti*, and *M. microti*, with *M. caprae* and *M. pinnipedii*. In addition, also *Mycobacterium bovis* (*M. bovis*), causative agent of bovine tuberculosis, belongs *M. tuberculosis* complex. Mtb

[1] Chen et al., "IL-2 Simultaneously Expands Foxp3+ T Regulatory and T Effector Cells and Confers Resistance to Severe Tuberculosis (TB): Implicative Treg–T Effector Cooperation in Immunity to TB."
[2] Esmail et al., "The Ongoing Challenge of Latent Tuberculosis."
[3] Manabe and William R. Bishai, "Latent Mycobacterium Tuberculosis– Persistence, Patience, and Winning by Waiting."
[4] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[5] Müller et al., "Zoonotic Mycobacterium Bovis– Induced Tuberculosis in Humans."
[6] F.J. Reviriego Gordejo and Vermeersch, "Towards Eradication of Bovine Tuberculosis in the European Union."
[7] Gutierrez et al., "Ancient Origin and Gene Mosaicism of the Progenitor of Mycobacterium Tuberculosis."

and *M. bovis* are very close relatives, in fact genetic studies revealed that *M. bovis* shared almost 99% genetic similarity with Mtb[8,9,10].

Mycobacteria are facultative intracellular bacteria and the Mtb complex bacteria grow slowly in culture (up to 20 days)[11]. They cannot considered as Gram positive, because the peculiar inner structure of bacterial wall. A complex called MA-AG-PG formed the inner part of cell wall, and it is constituted by covalent linkage of mycolic acids, arabinogalactan and peptidoglycan, respectively (Fig. 1). This cell wall can retain Carbon fuchsine even in the presence of acidic alcohol, hence mycobacteria are designed as acid fast bacteria (AFB)[12].

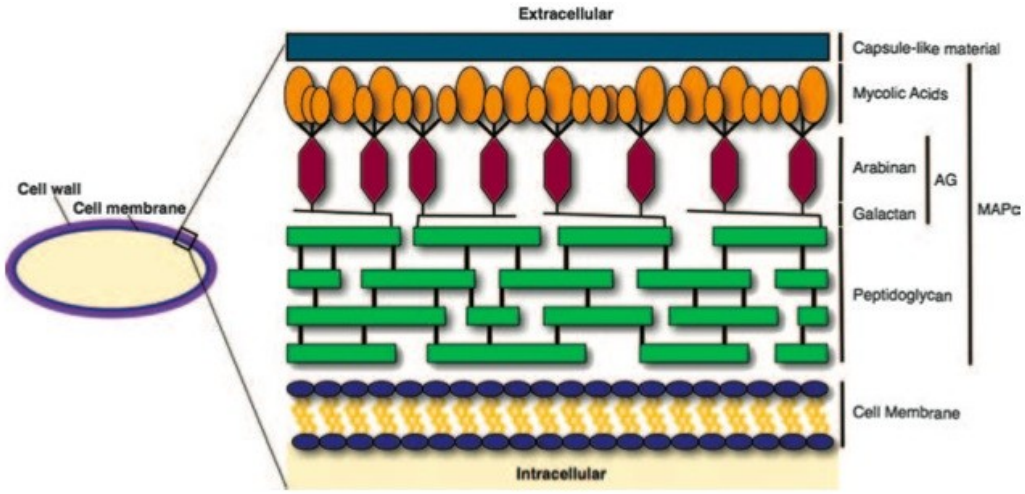

Figure 1. Components of mycobacterial wall[13]

[8] Brosch et al., "A New Evolutionary Scenario for the Mycobacterium Tuberculosis Complex."
[9] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[10] Alvarez, Estrada-Chavez, and Mario Alberto Flores-Valdez, "Molecular Findings and Approaches Spotlighting Mycobacterium Bovis Persistence in Cattle."
[11] Gengenbacher and Kaufmann, "Mycobacterium Tuberculosis: Success through Dormancy."
[12] Hett and Eric J. Rubin, "Bacterial Growth and Cell Division: A Mycobacterial Perspective."
[13] Ibid.

In bacterial infections, including tuberculosis, progression of disease and prognosis depend on bacterial, host and external influences[14].

The virulence factors of Mtb are represented by bacterial wall components, but also by several proteins [15] . In addition to cord factor, sulpholipids, mycosides and lipoarabinomannan[16], the mycobacterial protein ESAT-6 is increasingly being studied in order to discover vaccine or new diagnostic tools in bTB[17,18,19]. Interestingly, in cattle, progression of pathology is not infectious-dose dependent[20].

In mice and humans, several genes were identified to be involved in resistance against mycobacterial infections. The main gene NRAMP1 give birth to a membrane protein called "natural resistance-associated membrane-protein 1" (NRAMP1), which is expressed in macrophages [21] but in literature its protective role appears to be controversial. Nevertheless, several studies on Mtb infection identified NRAMP1 correlated to susceptibility of infected animals[22,23]. Nevertheless, there is lack of information of genetic resistance of cattle to *M. bovis* infection[24]. In addition, also immune suppression in hosts could influence the progression of disease. These events could be ACTH-cortisone release or corticosteroid injection, pregnancy, and other

[14] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[15] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[16] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[17] Vordermeier et al., "Correlation of ESAT-6-Specific Gamma Interferon Production with Pathology in Cattle Following Mycobacterium Bovis BCG Vaccination against Experimental Bovine Tuberculosis."
[18] Pollock et al., "Immune Responses in Bovine Tuberculosis."
[19] Alvarez, Estrada-Chavez, and Mario Alberto Flores-Valdez, "Molecular Findings and Approaches Spotlighting Mycobacterium Bovis Persistence in Cattle."
[20] Johnson et al., "Low-Dose Mycobacterium Bovis Infection in Cattle Results in Pathology Indistinguishable from that of High-Dose Infection."
[21] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[22] North et al., "Consequence of Nramp1 Deletion to Mycobacterium Tuberculosis Infection in Mice."
[23] Richard Bellamy, "The Natural Resistance-Associated Macrophage Protein and Susceptibility to Intracellular Pathogens."
[24] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."

concomitant infections such a BVDV in cattle[25]. In humans, the major role in immunosuppression leading severe progression of disease is played by HIV infection[26,27].

[25] Ibid.
[26] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[27] Andrea M. Cooper, "Cell Mediated Immune Responses in Tuberculosis."

1.2 Pathogenesis of tuberculosis in human and in cattle

For Mtb the favourite host is represented by humans, and for *M. bovis* cattle. Therefore, this species represent the model of infection for human and cattle tuberculosis, respectively.

Pathogenesis of TB is articulated in several steps:

- Primary infection
- Post-primary (or chronic organic) infection
- Generalisation

The primary infection is the first phase post-infection. Mycobacteria infected the host mainly through inhalation of bacilli contained in droplets from infected individuals[28,29,30]. Infected cattle can spread mycobacteria also with infected urine and faeces, resulting in indirect transmission through infected field. Hence, an oral transmission of disease is possible, especially in calves after ingestion of infected milk. Oral transmission represents, also, the main zoonotic route of *M. bovis* infection for humans[31]. Genital and vertical transmission is very rare, but experimental infection revealed the involvement of tonsils[32,33]. Then, mycobacteria reach alveolar spaces, where are phagocytised by macrophages and neutrophils and dendritic cells, which migrate to lymph nodes to present antigens[34].

[28] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[29] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[30] Menzies and Neill, "Cattle-to-Cattle Transmission of Bovine Tuberculosis."
[31] O'Reilly and Dabornt, "The Epidemiology of Mycobacterium Bovis Infections in Animals and Man: A Review."
[32] Menzies and Neill, "Cattle-to-Cattle Transmission of Bovine Tuberculosis."
[33] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[34] Domingo, Vidal, and Marco, "Pathology of Bovine Tuberculosis."

Lesions at this phase of disease consist in involvement of the tissue infected firstly. In humans and in cattle "classically" infected aerogenally, the lesions are confined in respiratory tract, including both lungs and local lymph nodes, resulting in granulomatous lymphadenitis and polmonitis[35]. This combination is known as primary complex or primary Ranke's complex[36,37,38] and tubercle is the classic gross lesion. Tubercles are granulomatous inflammation nodules showing yellowish colour, circumscribed and encapsulated. Often, tubercles can contain caseous necrosis and/or mineralization[39].

Infected individuals could: 1. Die at this stage; 2. Overcome primary infection and develop active tuberculosis; 3. Remain latent infected.

The post-primary infection derives from hematogenous dissemination of bacteria occurred during the first phase, and if the immune response of the host is ineffective, the infection generalise. This event is called early generalisation. Moreover, if generalisation occur after reinfection is called late generalisation [40] . During generalisation stages, infected animals/people could be anergic and may result false negative at screening diagnostic tests[41]. The tubercles become larger with a large caseous necrotic feature, or they can appear fibrotic, mineralised and confluent in the involved organ. In cattle, the most common feature of generalisation is represented by large number of small tubercle that is known as miliary tuberculosis. This aspect could be present on the sierosal surface, and it is observed mainly in cattle than in other

[35] Pollock et al., "Pathogenesis of Bovine Tuberculosis: The Role of Experimental Models of Infection."
[36] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[37] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[38] Domingo, Vidal, and Marco, "Pathology of Bovine Tuberculosis."
[39] Ibid.
[40] Ibid.
[41] Schiller et al., "Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of Their Relevance for Disease Control and Eradication."

species. In humans, the most common feature of post-primary infection is represented by production of cavities, resulting in a massive damage of lungs[42].

[42] Hunter, "Pathology of Post Primary Tuberculosis of the Lung: An Illustrated Critical Review."

1.3 Innate response and granuloma formation

The immune response to *M. tuberculosis* is complex and incompletely characterized, hindering development of new diagnostics, therapies and vaccines[43].

Studies on the pathogenesis of tuberculosis recognize two distinct immune mechanisms in the host that are involved in the disease, specifically required for controlling intracellular bacterial growth. The first mechanism is a cytotoxic delayed-type hypersensitivity (DTH), in which non-activated macrophages filled of mycobacteria are killed; this kind of response is responsible of most of the tissue damage in lung during the infection, resulting in liquefaction of the caseous lesions. The second mechanism is a cell-mediated immunity (CMI), in which activated macrophage organize to form tuberculous granuloma, ingest mycobacteria and kill them. In tuberculosis, a delicate balance between CMI and DTH represents the real protective mechanism[44].

In tuberculosis, immunity is mainly cell/mediated (CMI), but also humoral immunity could be observed. Gadol et al. (1974)[45] demonstrated that both CMI and antibody production are route of immunization-dependent. The main route of infection is respiratory tract, in which upper respiratory tract, including lungs, and local lymphoid organs are involved firstly[46].

At cellular level, immune response against mycobacteria starts when alveolar macrophages and dendritic cells become infected, resulting in inflammatory signals for

[43] Andrea M. Cooper, "Cell Mediated Immune Responses in Tuberculosis."
[44] Arthur M. Dannenberg Jr., "Roles of Cytotoxic Delayed-Tipe Hypersensivity and Macrophage-Activating Cell-Mediated Immunity in the Pathogenesis of Tuberculosis."
[45] Gadol, J. E. Johnson III, and Waldman, "Respiratory Tract Cell-Mediated Immunity: Comparison of Primary and Secondary Response."
[46] Domingo, Vidal, and Marco, "Pathology of Bovine Tuberculosis."

recruitment of blood monocytes and macrophages. The infected dendritic cells migrate to lymph nodes promoting the initiation of adaptive immunity[47].

Briefly:

1. Infection of macrophages and dendritic cells in lungs
2. Recruitment additional cells of innate immunity: monocytes, neutrophils
3. Migration of DCs to local lymph nodes and priming naïve T cells (Start of adaptive immunity)
4. Recruitment of activated T cells in the site of infection
5. Formation of granuloma

1.3.1 Innate immunity in TB: Mycobacterial sensing, starting innate immunity, protective mechanism

Mtb sensing

At early stage of infection innate immunity is necessary to limit mycobacterial growth and to protect tissues from damaging. There are three types of cells involved: macrophages, DCs, PMNs and Natural Killer (NK) cells[48,49].

Once bacilli come in alveolar space they are up-taken and recognized by mononucleated phagocytes and PMN through specialized molecules called pattern recognition receptors (PRRs) present on cell surface or in the cytosol.

PRRs involved in Mtb recognition are:

- Tool Like Receptors (TLRs)
- NOD-like receptors
- Mannose-binding lectins

[47] Huynh, Joshi, and Brown, "A Delicate Dance: Host Response to Mycobacteria."
[48] Korbel, Schneider, and Schaible, "Innate Immunity in Tuberculosis: Myths and Truth."
[49] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."

TLRs are molecules situated on the cell surface and handle surface recognition. Ten classes of TLRs have been identified, but TLR2, TLR4 and TLR9 are the most important in mycobacterial sensing. In particular, TLR2 recognises all mycobacterial wall component[50], including liparabinomannan (LAM) and lypopolisaccharide (LPS). TLR2 plays several roles: inducing production of TNF-α by neutrophils, stimulating production of several inflammatory cytokines (especially IL-12p40) by macrophages and promoting interaction with ESAT-6. Also, it induces repression of MHC II in response to IFN. In addition, TLR4 and TLR9, which bind CpG dinucleotides in bacterial DNA, are less involved.

NOD-like receptors are situated within cytosol and lead endosomal recognition. In mycobacterial infection NOD2 is the most important. NOD2 senses muramyl dipeptide (MDP) in host cell cytosol from live mycobacteria. NLRs are very important because of leading inflammasome formation and their involving in secretion of pro-inflammatory cytokines.

Mannose-binding lectins are the PRRs main promoters of Mtb uptake: DC-sign, Dectin-1, Mincle. Dectin1 senses lipoglycans constituting mycobacterial wall in order to activate secretion of TNF-α, IL-6, IL-1β, IL-12, IL-23 and IL-17. Further, Mincle is the ligand for dimycolate and this induces the pro-inflammatory response.

Interestingly, both TLRs and NOD induce innate pro-inflammatory responses[51,52].

Mycobacterial sensing by phagocytes is necessary to drive T-cells activation. The

[16] Korbel, Schneider, and Schaible, "Innate Immunity in Tuberculosis: Myths and Truth."
[50] Underhill et al., "Toll-like Receptor-2 Mediates Mycobacteria-Induced Proinflammatory Signaling in Macrophages."
[16] Korbel, Schneider, and Schaible, "Innate Immunity in Tuberculosis: Myths and Truth."
[23] Dorhoi, Reece, and Kaufmann, "For Better or for Worse: The Immune Response against Mycobacterium Tuberculosis Balances Pathology and Protection."
[51] Huynh, Joshi, and Brown, "A Delicate Dance: Host Response to Mycobacteria."
[52] Reinout van Crevel, Tom H. M. Ottenhoff, and Jos W. M. van der Meer, "Innate Immunity to Mycobacterium Tuberculosis."

production of cytokines subsequently to mycobacterial detection can leads Th1, Th17 or T regulatory response. After recognizing and internalizing of bacteria, in APCs cytosol starts the activation of a molecular complex pathway called inflammasome, mediated by NLR proteins family. Binding pathogen associated molecular pattern activates first NLRs, which trig the formation of inflammasome in APCs cytosol. Mtb infection promotes the activation of NLRP3 inflammasome through the effector proteins caspase 1 (also knows as the IL-1β-converting enzyme) and caspase 5. However, the main ligand for NLRP3 activation remains unknown.

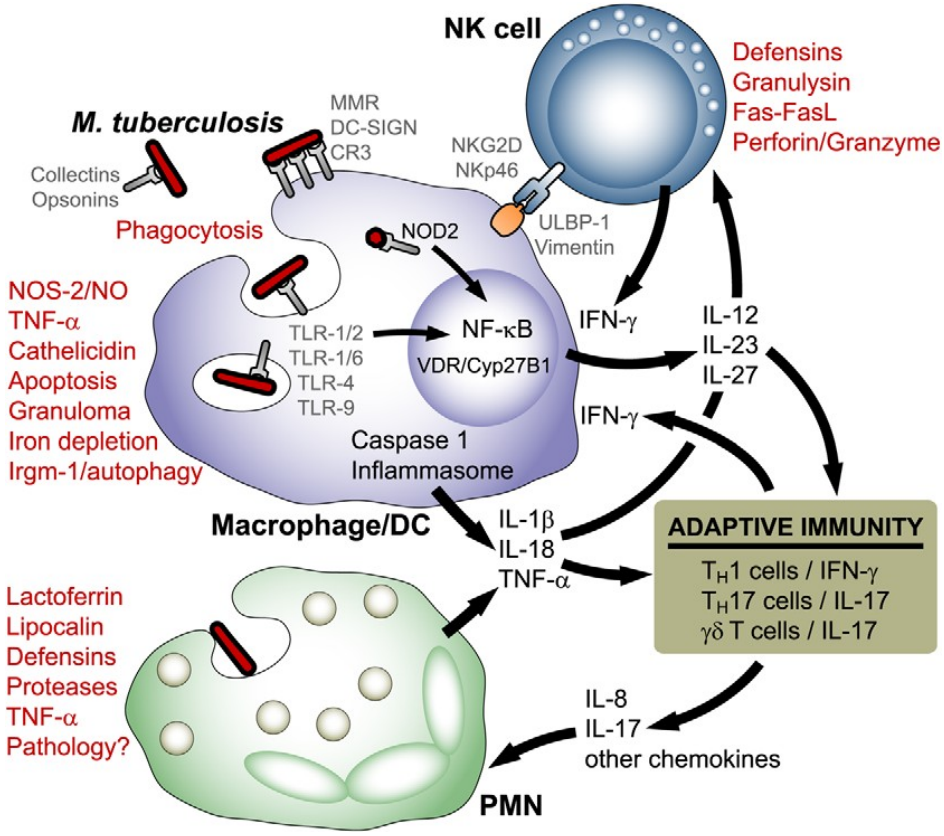

Figure 2. **Innate immunity to tuberculosis**[53].
Macrophages, DCs and neutrophils sense mycobacteria through membrane-receptors, thus bacteria are phagocytized. TLRs and NOD2 recognise Mtb, induce inflammasome activation via caspase 1. Activated inflammasome trigs secretion of IL-1β and other cytokines such as IL-12 and IL-18. Doing so, they drive the immune response to an adaptive type, sustained mainly by Th1 (control macrophage activity through IFN-γ), Th17 and γδ T cells that secrete IL-17 to activate PMNs. In red: mediator expressed by cells; CR3, complement receptor 3; DC-SIGN, DC-specific intercellular-adhesion-molecule-3-grabbing non-integrin; MMR, macrophage mannose receptor; TLR, Toll-like receptors; ULBP-1, UL16-binding protein 1; VDR, vitamin D receptor; Cyp27B1, vitamin D-1 hydrolase Cyp27B1.(Korbel DS et al., 2008)

[53] Korbel, Schneider, and Schaible, "Innate Immunity in Tuberculosis: Myths and Truth."

The importance of inflammasome formation is important for secreting specific cytokines, in particular IL-1β[54]. In fact, the production of IL-1β is decreased in infected macrophages or DCs NLRP3$^{-/-}$, where inflammasome is not activated[55]. Inflammasome is also important in limiting bacterial burden and in producing other cytokines, controlling NO production and in the formation of granuloma[56,57,58].

In course of tuberculosis infection, the increase of IFN-γ trigs the secretion of high amount of Nitric Oxide (NO), which down-regulates NLRP3 inflammasome and subsequently reduces IL-1β production. Participation of NO is important for suppressing tissue damage deriving from this continuous innate immunity activation[59]. In addition, also the ZN-metalloprotease of Mtb can inhibit NLRP3-inflammasome activation[60].

1.3.2 Protective mechanisms: killing of mtb and apoptosis

In PMN, apoptosis is induced directly by oxidative processes and TLR-2 pathway in presence of cytokine milieu in pleural space. Through that process, TLR-2, instead of activate PMNs induces an anti-inflammatory response, increases production of transforming growth factor-β (TGF-β) and E2 and decrease of IL-6/IL-8/IL-12/TNF-α.

[54] Huynh, Joshi, and Brown, "A Delicate Dance: Host Response to Mycobacteria."
[55] Aleman, "Neutrophil Apoptosis in the Context of Tuberculosis Infection."
[56] E Mortaz et al., "Interaction of Pattern Recognition Receptors with Mycobacterium Tuberculosis."
[57] Master et al., "Mycobacterium Tuberculosis Prevents Inflammasome Activation."
[58] Briken, Ahlbrand, and Shah, "Mycobacterium Tuberculosis and the Host Cell Inflammasome: A Complex Relationship."
[27] Ibid.
[29] Master et al., "Mycobacterium Tuberculosis Prevents Inflammasome Activation."
[59] Hernandez-Cuellar et al., "Cutting Edge: Nitric Oxide Inhibits the NLRP3 Inflammasome."
[60] Briken, Ahlbrand, and Shah, "Mycobacterium Tuberculosis and the Host Cell Inflammasome: A Complex Relationship."

By doing so, TLR-2 signalling leads apoptosis in neutrophils[61]. Macrophages can ingest both apoptotic and necrotic infected PMN but necrosis is not a great mechanism to control mycobacterial infection[62]. Moreover, $CD4^+$ and CD8+T cells perform apoptosis of infected cells to control bacterial growth[63,64].

High amount of TNF-α are produced in apoptotic cells during Mtb infection in a similar way has been seen in non-apoptotic cells. In addition, apoptosis causes tissue damage and promotes proliferation and dissemination of mycobacteria[65].

1.3.3 Innate immunity and granuloma formation

As previously described, alveolar macrophages are the first cells to be involved in the innate phase of inflammatory process. These cells are deficient to control Mtb but play a protective role[66].

Granuloma is the histopathologic hallmark in tuberculosis and represents the result of immune response that arises during mycobacterial infection. This forms around infected macrophages, which are required for stimulating the granuloma formation. It is a kind of cell-mediated immunity and in lung this type of response is stimulated more effectively when antigen arrive through respiratory route. Resulting a complex cell-interaction based on production by involved inflammatory cells of several cytokines,

[61] Aleman, "Neutrophil Apoptosis in the Context of Tuberculosis Infection."
[62] Lowe et al., "Neutrophils in Tuberculosis: Friend or Foe?"
[63] Prezzemolo et al., "Functional Signatures of Human CD4 and CD8T Cell Responses to Mycobacterium Tuberculosis."
[64] Canaday et al., "CD4+ and CD8+ T Cells Kill Intracellular Mycobacterium Tuberculosis by a Perforin and Fas/Fas Ligand-Independent Mechanism."
[65] Ciaramella et al., "Proinflammatory Cytokines in the Course of Mycobacterium tuberculosis–Induced Apoptosis in Monocytes/Macrophages."
[66] Flynn, Chan, and Lin, "Macrophages and Control of Granulomatous Inflammation in Tuberculosis."

required to recruit inflammatory cells in the site of infection[67].

There are different stages in the maturation of granuloma[68]:

1. Stage of symbiosis (early primary tubercle): blood-derived macrophages that are present in the infection focus are non-activated and with a cytoplasm ideal for the survival of ingested mycobacteria. They accumulate in the lung and mycobacteria can multiply within them.
2. Caseous necrosis (three weeks of age): macrophages are partly activated and lymphocytes start to accumulate in the tubercle. The formation of caseum begins when the non-activated macrophages with mycobacteria within are killed in the cytotoxic immune response activated by tuberculin-like products). Live mycobacteria are present within the caseum.
3. Chronic granuloma in resistant host (four to five weeks of age): Macrophages that are present in the tubercle are activated by lymphocytes, they are competent and have a cytoplasm unadapt to growth of mycobacteria within.
4. Chronic granuloma in susceptible host (four to five weeks of age): Tubercle has a large caseous necrotic centre, whereas most of the macrophages present are non-activated and incompetent. In such macrophages mycobacteria can multiply within because of ideal cytoplasmic environment of phagocytes.
5. Stage of cavitation: when the caseous-liquefied center is discharging into the bronchial tree, allowing the dissemination of bacteria[69].

Granulomas show different features in active phase or in latent phase of the disease. Active aged granuloma appears, grossly, as classical "caseous granuloma",

[67] Guirado and Schlesinger, "Modeling the Mycobacterium Tuberculosis Granuloma – the Critical Battlefield in Host Immunity and Disease."
[68] Arthur M. Dannenberg Jr., "Roles of Cytotoxic Delayed-Tipe Hypersensivity and Macrophage-Activating Cell-Mediated Immunity in the Pathogenesis of Tuberculosis."
[69] Ibid.

characterized by the caseous necrosis in the centre of the lesion. Microscopically, Mtb leads the disposition of macrophage-infected population surrounded by T cells population with scattered B cells forms the peculiar compact tuberculous granuloma. Sometimes neutrophils can be present in such granuloma. Even though other type of granulomas exist (non-necrotizing; necrotic neutrophilic; fibrotic), most of all in chronic granuloma or during latent phase of TB, the typical chronic granuloma appears with a necrotic and/or mineralized central area where mycobacteria can die or switch in a dormant state in order to become reactivated[70]. Although granuloma has a protective role[71] and is required for naïve T cells priming[72], specific functions of the peculiar structure remain unknown, as unknown is whether dormant mycobacteria survive within macrophages or extracellularly[73].

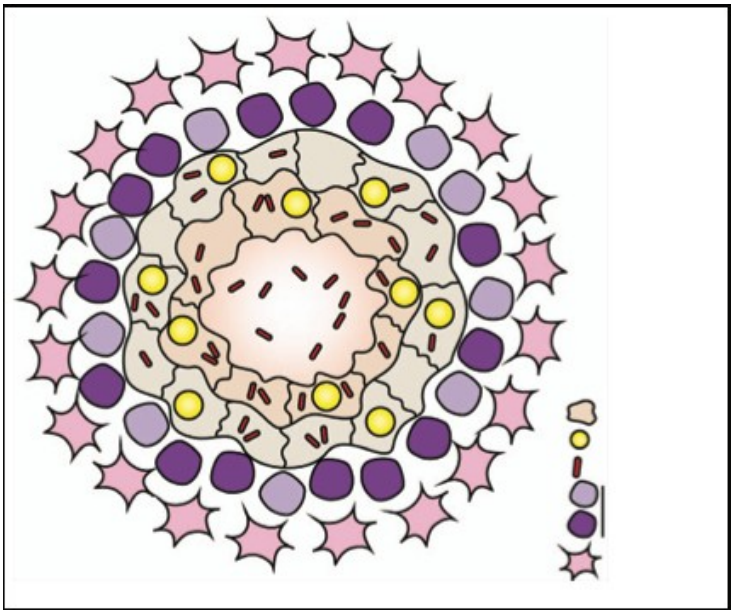

Figure 3. Classical feature of tuberculous granuloma. Bacteria stay in the caseous center of the lesion or within infected macrophages, multinucleated giant cells and epithelioid macrophages. These cells are arranged in rings,

[70] Houben, Nguyen, and Pieters, "Interaction of Pathogenic Mycobacteria with the Host Immune System."
[71] Lalita Ramakrishan, "Revisiting the Role of the Granuloma in Tuberculosis."
[72] Day et al., "Secondary Lymphoid Organs Are Dispensable for the Development of T-Cell-Mediated Immunity during Tuberculosis."
[73] Houben, Nguyen, and Pieters, "Interaction of Pathogenic Mycobacteria with the Host Immune System."

surrounding the center, and show a foamy aspect (with lipid bodies shown in yellow). Various subsets of lymphocytes (shown in two grades of violet) and fibroblasts (shown in pink) occupy the outer of the granuloma[74].

The granuloma response influences bacterial control and outcome of pathologic process. Inflammatory granuloma evolves in active tuberculosis in the presence of one of these combinations:

1. High bacterial numbers, many macrophages, both classically and alternatively activated, T helper cells 1 (Th1), neutrophils and regulatory T cells (Treg);

2. High bacterial load, large amount of alternatively activated macrophages, Tregs, few Th1 and classically activated macrophages;

3. Low bacterial load, predominance of classically activated macrophages and Th1 cells, few Tregs;

Latent granuloma can be populated by macrophages classically and alternatively activated in balanced proportion and few T cells or with abundant numbers of Tregs, Th17 and Th1[75].

In cattle, early lesions at the site of infection could be observed by 7 day post-infection (gross lesions are evident by 14 day post-infection). Within the granuloma $WC1^+$ and $CD2^+$T cells can be detected and AFB increase in number. Encapsulation of granuloma remains incomplete at day 41 post-infection but central necrosis and mineralization can be present[76].

[74] Huynh, Joshi, and Brown, "A Delicate Dance: Host Response to Mycobacteria."
[75] Flynn, Chan, and Lin, "Macrophages and Control of Granulomatous Inflammation in Tuberculosis."
[76] McNair, Welsh, and Pollock, "The Immunology of Bovine Tuberculosis and Progression toward Improved Disease Control Strategies."

1.4 Cells involved in granulomatous inflammation

Inflammatory cells have a common hematopoietic origin. They derive from multipotent hematopoietic stem cells, which differentiate in myeloid and lymphoid hematopoietic progenitors, driving the development of lymphoid and myeloid cells lineage respectively.

Granulocytes differentiate from Common myeloid progenitor from hematopoietic cell progenitor[77].

Briefly, cells involved in granuloma formation are:

- Phagocytic cells (Dendritic cells and macrophages)
- Lymphocytes
- Neutrophils

1.4.1 Phagocytic cells (Dendritic cells and macrophages)

Dendritic cells (DCs) and macrophages take part of mononuclear phagocytic system (MPS), which comprises hematopoietic progenitors, blood monocytes and tissue macrophages. These cells share several common features: stellate morphology, endocytic activity, expression of enzymes detectable by histochemical staining and non-specific uptake ability[78].

Conventionally, macrophages are considered to be differentiated both from blood monocytes in tissues and from a common myeloid progenitor (CMP) in bone marrow. DCs may derive from CMP, from an earliest lymphoid progenitor[2] and, upon inflammatory stimuli, from blood monocytes[79]. Nevertheless, in several murine organs, lung included, it was defined two distinct lineages of macrophages and DCs: one

[77] Takeuchi and Furue, "Dendritic Cells—Ontogeny."
[78] David A. Hume, "The Mononuclear Phagocyte System."
[79] Geissmann et al., "Development of Monocytes, Macrophages, and Dendritic Cells."

originated from hematopoietic stem cells, and another developed in yolk sac[80].

The development of all subsets of DCs is controlled by several transcription factors: IRF-8, IRF-4, Id2, Zbtb46, E2-2, Batf3, the STATs, RelB, Ikaros, Notch RBP-J and PU.1. Although a unique predominant DCs regulator remains unidentified, the receptor for Flt3 ligand plays an important role in specification of DCs[81], as well as the STATs that regulates DCs functions[82].

DCs population can be divided in two major categories: conventional DCs (cDCs) and plasmocytoid DCs (pDCs). On the basis of their tissue location, DCs are denominated blood DCs if they reside in blood and lymphoid organs, and tissue DCs those resident in non-lymphoid tissues (skin, solid organs, mucosa). Based on expression of cell surface antigens, generally, in mouse there are two main cDCs subsets: $CD8^+$ and $CD8^-$ DC, specialised in activation of Th1 and Th2 T cells differentiation respectively, through cross-presenting antigens constitutively and/or after their activation. By secreting large amount of IL-12p70, $CD8^+$ DCs recruit T cells driving a Th1 type response, but also play an important role as regulatory cells compared to $CD8^-$ DCs[83].

In mouse lung have been identified: $CD11b^+CD103^+$ $Langerin^+$ DCs (intraepithelial subset); $CD11b^{hi}$signal-regulatory protein α^+CX_3CR1 DCs (lamina propria subset); $CD11b^-$ and $CD11b^+$ subsets. $CD11b^+$ DCs is specialized in presenting antigen to $CD4^+$T cells, whereas antigen presentation to $CD8^+$ T cells is carried out by $CD103^+$DCs. In addition $Ly6C^+$ monocyte-derived DCs (inflammatory DCs), different

[80] Schulz C et al., "A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells."
[81] Perié L and Naik SH, "Toward Defining a 'lineage' – The Case for Dendritic Cells."
[82] Haiyan SL and Watowich SS, "Diversification of Dendritic Cell Subsets, Emerging Roles for STAT Proteins."
[83] Shortman and Heath, "The CD8+ Dendritic Cell Subset."

from cDCs, can be detected in inflammation[84].

In humans, DCs are divided in two subset showing phenotypic and functional similarities with murine DCs: $CD141^+$ DCs (resembling mouse $CD8^+$DCs) that express Tool Like Receptor 3 (TLR3) and produce Interleukin 12 (IL-12); $CD1c^+$DCs (corresponding murine $CD11b^+$DCs resident in lung parenchyma) express all TLRs except TLR9[85-86].

Mature macrophages can be found in several organs: liver, lung, brain, bone, spleen and lymph nodes. They are located within connective tissue and around the basement of small blood vessels. In the lung, in particular, there are two macrophages subset bearing different functions: interstitial and alveolar macrophages, in interstitial connective and within alveolus respectively. Whereas alveolar macrophages are more involved in non-specific antimicrobial defence, interstitial macrophages have a role in regulation of inflammation and in driving specific immune response, as suggested by large amount of C3 receptor expression and IL-1 and IL-6 secretion in that cells[87].

Guirado et al (2013)[88] have classified four subsets of macrophages, distinguishable for their culture under different conditions:

- Type I: macrophage classically activated (CAM or M1), characterised for expression of class I major histocompatibility complex (MHC I)
- Type II: macrophage innate activated. In this group are included type I and type II macrophages; these cells product TNF-α, IL-1β, IL-6, reactive oxygen

[84] Hu W and Pasare C, "Location, Location, Location: Tissue-Specific Regulation of Immune Responses."
[85] Schlitzer and Ginhoux, "Organization of the Mouse and Human DC Network."
[86] Chistiakov DA, "Myeloid Dendritic Cells: Development, Functions, and Role in Atherosclerotic Inflammation."
[87] Laskin DL, Weinberger B, and Laskin JD, "Functional Heterogeneity in Liver and Lung Macrophages."
[88] Guirado, Schlesinger, and Kaplan, "Macrophages in Tuberculosis: Friend or Foe."

molecules, IL-10 and IL-12

- Alternatively activated macrophages (AAM or M2): these cells are characterised for high expression of Pattern Recognition Receptors (PRRs) and production of anti-inflammatory cytokines. They do not secrete pro-inflammatory cytokines and reactive oxygen products, but are involved in tissue repair and humoral immunity
- Deactivated macrophages, which secrete anti-inflammatory cytokines.

In tuberculosis infection, with dendritic cells, alveolar macrophages represent the first obstacle for Mtb[89]. When Mtb comes in the airways, DCs recognize it through TLR9 hence start IL-12 production, migrate to mediastinal lymph nodes, especially $CD11b^{+}$ DCs, and present antigens to T cells. The major DCs subset founded in the lung during Mtb infection is $CD11c^{hi}$ $CD11b^{hi}$ DCs that is the most representative subset containing bacteria in lymph nodes. During tuberculosis infection, DCs located in the lung have three main functions: antigen uptaking; migration to mediastinal lymph nodes and antigen presentation[90].

On the other hand, especially macrophages play a dual role: firstly, they product NO and reactive oxygen intermediates (ROI) to kill Mtb directly; secondly, through TNF production, they induce apoptosis in macrophages infected and contribute to the granuloma formation. –Although RNI is considered to be a major antimicrobial system even in tuberculosis, the role of ROI in such infection remains unclear, but cannot be excluded. However, compared to DCs, macrophages are less inducers of Th1

[89] Ibid.
[90] Plantinga, Hammad, and Lambrecht, "Origin and Functional Specializations of DC Subsets in the Lung."

response[91,92].

Macrophages are the promoters in granuloma formation and the most represented cells within[93]. This macrophage population, in mice, express F4/80 antigen, which represent the best marker for recognise them within granuloma, as well as multinucleated giant cells[94]. Alveolar macrophages that encounter Mtb for first are considered M2 type macrophages and their main function is to uptake Mtb. Doing so, they perform an immune-regulatory activity by preserving alveolar structure and promoting adaptive immunity through anti-inflammatory and pro-inflammatory cytokines production[95,96].

1.4.2 Lymphocytes

NK cells

NK cells main activities are producing pro-inflammatory cytokines and killing transformed/virus-infected cells. In tuberculosis infection, NK cells kill directly Mtb-infected monocytes and macrophages, and indirectly through stimulation of CD8 T cells; induce apoptosis in order to control mycobacterial growth; kill FoxP3 Treg cells activated by Mtb[97].

CD4 T cells

In tuberculosis, CD4 T cells are considered the most important cells in the protective immune response. Firstly, these cells contribute with production of IFN-γ in order to

[91] JoAnne L. Flynn and John Chan, "Immunology of Tuberculosis."
[92] Guilliams, Lambrecht, and Hammad, "Division of Labor between Lung Dendritic Cells and Macrophages in the Defense against Pulmonary Infections."
[93] Flynn, Chan, and Lin, "Macrophages and Control of Granulomatous Inflammation in Tuberculosis."
[94] Gordon and Plűddemann, "Tissue Macrophage Heterogeneity: Issues and Prospects."
[95] Rajarama et al., "Macrophage Immunoregulatory Pathways in Tuberculosis."
[96] Guirado, Schlesinger, and Kaplan, "Macrophages in Tuberculosis: Friend or Foe."
[97] Korbel, Schneider, and Schaible, "Innate Immunity in Tuberculosis: Myths and Truth."

sustain CMI and interact with dendritic cells through CD40-CD40L signalling. CD40L on T cells surface interacts with CD40 receptor on dendritic cells, resulting in co-stimulation and increase of antigen presentation; such interaction can be involved in CD4 T-cells ability to stimulate CD8 T cells functions. Further, CD4 T cells are also important to promote a proper B cell activity[98].

Zhu J and Paul WE[99] reviewed four subtypes of CD4 T-helper (CD4 Th) cells, each with different immune function: T-helper cells 1 (Th1), in immunity caused by intracellular pathogens; T-helper 2 (Th2), in immunity caused by extracellular parasites, helmints and in allergic response[100]; T-helper 17 (Th17), in immunity caused by extracellular bacteria; Treg, involved in self tolerance and regulation of immune response. These subsets can be distinguishable on the basis of cytokines production. The differentiation of naïve CD4 T-cells is driving and regulate by specific transcription factors and cytokines/STAT signalling.

Th1 cells: these cells product IFN-γ, in order to activate macrophages and to give a positive feedback for Th1 response, and IL-12 for CD4 T cell memory response. The master transcription gene is T-bet. Development of Th1 responses is dependent upon IL-12p70[101]. Differentiation from naïve CD4 T cells starts when APC are activated producing IL-12. This cytokine induces IFN-gamma/STAT 1 signalling in CD4 naïve cells, resulting in up-regulation of T-bet and in increase of IFN-gamma production and IL-12Rβ2 expression. Thus, in these, activated CD4 T cell starts IL-2/STAT4 signalling which is typical in CD4 Th1 subtype.

Th2 cells are the major source of IL-10 and in less amount other interleukins. Firstly IL-

[98] Rodríguez-Pinto, "B Cells as Antigen Presenting Cells."
[99] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults."
[100] Okoye and Wilson, "CD4+ T Helper 2 Cells – Microbial Triggers, Differentiation Requirements and Effector Functions."
[101] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis."

4, necessary for positive feedback in Th2 differentiation and mediation of IgE switch in B-cells. Secondly, Th2 cells product cytokines necessary in allergic response: IL-5, IL-9 and IL-13 for recruitment of eosinophil granulocytes, stimulating mucin production in epithelial cells and induction of airway hypersensitivity. Further, they product IL-25, which acts as enhancer in secretion of IL-4, IL-5 and IL-13. In vivo development of Th2 response is completely depending on GATA-3. The transcription factor is up-regulated by production of IL-4 through activation of IL-4/STAT6 signalling. Collaborating with Gfi-1, GATA-3 expression can select the growth of GATA-3^{high} Th2 cells.

Th17 cells product IL-17, which induces secretion of IL-6, IL-8 by macrophages and activates neutrophil granulocytes. For the positive feedback in Th17 differentiation, also IL-21 is produced by these cells and, acting in the presence of IL-6 from activation of TGF-β. Thus, naïve CD4 T cells product IL-21 that induces expression of IL-23R and of the transcription gene ROR-γt, driving differentiation in Th17 phenotype. IL-21 replaces IL-6 in this pathway in order to enhance Th17 response, which is maintained by IL-23/STAT3 signalling[102]. Massive recruitment of neutrophils, associated with extensive lung pathology, can be observed in mice after re-exposure to Mtb. This phenomenon is related to an enhanced Th17 response supported by elevated IL-23 expression[103].

The main cytokine product by Treg is TGF-β, necessary in Th17 but also for Treg differentiation. In fact, in absence of pro-inflammatory cytokines, TGF-β induces expression of FoxP3 in naive CD4 T-cells. Survival of Treg is regulated by IL-2/STAT5 pathway.

In Mtb infection Treg population can expand from a pool of pre-existing FoxP3 cells

[102] Lyakh et al., "Regulation of Interleukin-12/interleukin-23 Production and the T- Helper 17 Response in Humans."
[103] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis," -17.

and can be converted into a IL-17 secreting cells in the presence of pathogen components or IL-6. This plasticity can confer to converted Treg cells an important role in regulation of inflammation and in control of bacterial growth. Moreover, FoxP3 Tregs may limit T-cell accumulation in the lung and restrict subsequent effector immune responses[104]. Conversely, development of Th17 cells is promoted through an early secretion of IL-6 and IL-23[19]. Despite IL-23/Th17 pathway is considered not required for protection in Mtb infection, it can be involved in granuloma formation and inflammatory response[105].

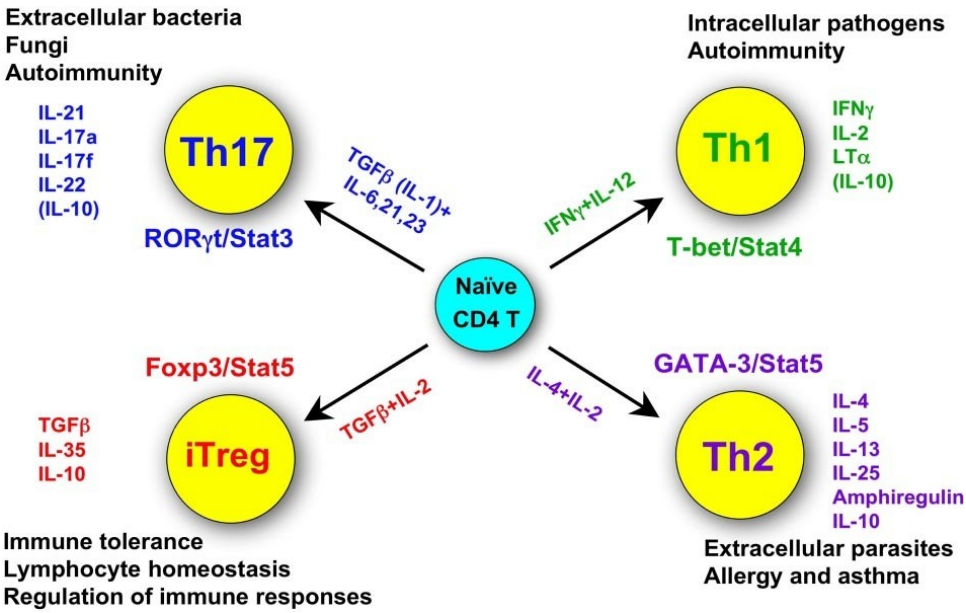

Figure 4. Pattern of differentiation and function of T helper cells from naïve CD4 T cells described by Zhu et al., 2008[106].

CD8 T cells

Put aside old classification of CD8 T cells, which distinguished cytotoxic and suppressor CD8 T cells, recently two subset are defined: Type 1 (Tc1) and Type 2

[104] Redford, Murray, and O'Garra, "The Role of IL-10 in Immune Regulation during M. Tuberculosis Infection."
[105] Khader and Gopal, "IL-17 in Protective Immunity to Intracellular Pathogens."
[106] Zhu and Paul, "CD4 T Cells: Fates, Functions, and Faults."

(Tc2). Differently than CD4 T cells classification, specific functions and cytokine patterns performed by these subtypes are to be defined. $CD8^{+}$T cells can recognize antigens through MHC Class I or in a non-classical way using CD1 molecules.

On CD4 T cell response, CD8 T cells have an immune-regulatory role, characterized by the down-regulation of CD4 T cells proliferation, both through direct and indirect suppression. Direct suppression can be performed by secretion of several cytokines or by activation-induced cell-death. An indirect suppression can be possible not only by inhibiting and decreasing stimulatory functions of APCs, but also through inhibition in expression of CD40L or IL-2 in CD4 T cells, blocking co-stimulation between them and APCs.

Another function of CD8 T cells is to stimulate DCs maturation. It is reported to occur via activation of survival factor for DCs, tumour necrosis factor-related activation-induced cytokine (TRANCE). About CD40L/CD40 interaction, which is important for producing IL-12 by APCs, not only CD4 T cells can express CD40L but also CD8-type 2 T cells can do so[107].

CD8 T cell response start when T cells are activated by infected and/or activated macrophages and suppressor activity can be stimulate by activated CD4 T cells. They are divided in two major subsets: MHC Class I-Restricted CD8 T cells and Non-classically Restricted CD8 T cells. Such classification depends on which molecules provide to antigens presentation, MHC class I or other molecules called CD1[108]. Studies revealed that T cells recognize several proteins secreted by bacteria in culture, named culture filtrate proteins (CFPs). The major early secretes antigens are Ag 85B and

[107] Vurmanovic-Stejic et al., "Specificity, Restriction and Effector Mechanisms of Immunoregulatory CD8 T Cells."
[108] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis."

ESAT-6 (early secreted antigenic target-6) both in vitro and in vivo[109].

However, the main function of CD8 T cells consists in lysis of infected macrophages and DCs, namely via perforin-granulysin pathway. In addition these cells produce INF-γ, required to activate macrophage activation[110].

B cells

B cells are the main cells involved in humoral immunity. B cells are classified as B effector-1 (Be1), B effector-2 (Be-2) and regulatory B10 cells, on the pattern of cytokine secretion. In particular, Be-1 are stimulate in the presence of Th1 cells to secrete IFN-c, IL-12. Moreover, Th2 primed Be-2 cells that produce IL-2, lymphotoxin, IL-4, IL-13. Both Be-1 and Be-2 cells can secrete TNF-α, IL-10 and IL-6[111]. A co-operation between B cells and T cells is demonstrated, especially in self-tolerance, but the mechanisms behind are still unknown[112]. Interestingly, B cells have the function of presenting antigen to other cells, especially naïve $CD4^{+}$T cells, although dendritic cells are considered the main cells in that activity.[113][114]

Kozakiewicz et al. (2013)[115] recently reviewed the role of B cells and humoral immunity in TB. Even though cell-mediated immunity plays the major role in defence against Mtb and humoral is considered inefficient, B cells can participate in the development of immune response. B cells, in fact, could enhance T cells activity or not, it depends by the subset of B cells involved, whether Be1 and Be2 or regulatory B cells.

[109] Winslow et al., "Early T-Cell Responses in Tuberculosis Immunity."
[110] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis."
[111] Lund and Troy D. Randall, "Effector and Regulatory B Cells: Modulators of CD4+ T Cell Immunity."
[112] Mitchison, "T-cell–B-Cell Cooperation."
[113] Rodríguez-Pinto, "B Cells as Antigen Presenting Cells."
[114] Rodríguez-Pinto and Moreno, "B Cells Can Prime Naive CD4+ T Cells in Vivo in the Absence of Other Professional Antigen-Presenting Cells in a CD154-CD40-Dependent Manner."
[115] Kozakiewicz et al., "The Role of B Cells and Humoral Immunity in Mycobacterium Tuberculosis Infection."

In particular, regulatory B cells regulate T cells activity through the production of IL-10 and TGF- β, but their functions need to be investigated. Conversely, there exists a phenomenon called "antibody-dependent enhancement (ADE) of microbial infection", in which legation of FcγR with parasites antibody leads an increase of IL-10 secretion and a decrease of IL-12[116]. In Mtb infection, even though FcγRIIB-deficient mice showed increased capability to control the infection, the role in vivo of this mechanism in TB is unknown. Studies on mouse models with B cells deficiency revealed that Mtb infected mice develop a strong protective immunity, whereas in other works, in the absence of B cells, mice develop a chronic tuberculosis, resulting from an inflammatory progression. From these works arise the peculiar "phase-specific" function of B cells during tuberculosis: during acute phase, B cells contribute to granulomatous response. On the contrary, during chronic phase, B cells aggregates promote the maintenance of immunity in order to prevent reactivation. However, how humoral immunity influences the development of immunity in TB through production of cytokines (in particular of IL-10) remain unclear.

Lymphocytes in bovine TB

Lymphocytes are also present in immune response against *M. bovis*, in cattle. All of the main subtypes are involved, in particular $CD4^+$ and $CD8^+$ T cells[117]. However, at early stage after lesion development in the site of infection, γδ+ T cells play an important role[118,119]. This particular T cell subtype is very important in lesion development[120], but

[116] Ibid.
[117] Blanco et al., "Increased IL-17 Expression Is Associated with Pathology in a Bovine Model of Tuberculosis."
[118] Pollock et al., "Immune Responses in Bovine Tuberculosis."
[119] Cassidy et al., "Lymphocyte Subtypes in Experimentally Induced Early-Stage Bovine Tuberculous Lesions."

especially they act as a bridge between innate and adaptive immune. In particular, they are involved in stimulation of macrophages via secretion of IL-12 and other cytokines including IFN-γ. Moreover, $\gamma\delta^+$ T cells have the function to recruit other T cells subtypes in order to promote adaptive immunity[121]. In order of appearance, $\gamma\delta^+$ T cells are involved firstly, followed by $CD4^+$T cells and, later, by $CD8^+$T cells. Anyway, $CD4^+$T cells predominate the immune scenario, especially through Th1 response, and they are the most producer of IFNγ. Whereas these T cells subtype sustain macrophage stimulation via secretion of IFNγ, $CD8^+$T cells are involved in lysis of infected cells[122,123].

1.4.3 Neutrophils

PMNs are recruited early in the site of Mtb infection, attracted by macrophages, but the specific mechanisms of their attraction remain elusive.

In tuberculosis, PMNs have both a protective and destructive roles. These cells can phagocyte and kill intracellular pathogens such as Mtb. In fact, PMNs may kill phagocytised bacteria through cationic proteins stored in cytosolic granules, control bacterial load and transport mycobacteria to draining lymph nodes. In the acute phase of Mtb infection, neutrophil are, with macrophages, the most cell present in inflammatory site, but they subsequently decrease toward the chronic phase. Also neutrophil contribute in the granuloma formation, putting in action a neutrophil-mediated regulation in part dependent on chemokine signalling through CXCR3 and CXCL9, which is produced widely by neutrophils in early stages of infection. Neutrophil

[120] Cassidy et al., "Early Lesion Formation in Cattle Experimentally Infected with Mycobacterium Hovis."
[121] Smyth et al., "In Vitro Responsiveness of γδT Cells from Mycobacterium Bovis-Infected Cattle to Mycobacterial Antigens: Predominant Involvement of WC1+ Cells."
[122] Villarreal-Ramos et al., "Investigation of the Role of CD8+ T Cells in Bovine Tuberculosis In Vivo."
[123] Liebana E. et al., "Cellular Interactions in Bovine Tuberculosis: Release of Active Mycobacteria from Infected Macrophages by Antigen-Stimulated T Cells."

neutralization and block of CXCL9 result in deficient granuloma development[124].

On the other hand, PMNs contribute to immunopathology of TB in several ways. First of all, when PMNs are the dominant cells present in inflammation animal infected with Mtb show increased susceptibility to TB and suppressed early cytokine production such as IL-1 and iNOS. In addition, granulocytes are involved in bronchogenic dissemination of Mtb [125]. Evidence from genetically susceptible mice suggests that neutrophils contribute to pathology during infection with *M. tuberculosis*[126]. Our studies support a role for neutrophils in the pathogenesis of TB, which may result from over-activation by IFN-c and type I IFNs[127]. Moreover, neutrophils play a role in crosstalk with T cells.

The specific role of neutrophils during bTB is not completely understood, although at the early stage of disease they are involved in phagocytosis of mycobacteria[128,129].

[124] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis."
[125] Dorhoi, Reece, and Kaufmann, "For Better or for Worse: The Immune Response against Mycobacterium Tuberculosis Balances Pathology and Protection."
[126] Lowe et al., "Neutrophils in Tuberculosis: Friend or Foe?"
[127] Berry et al., "An Interferon-Inducible Neutrophil-Driven Blood Transcriptional Signature in Human Tuberculosis."
[128] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[129] Domingo, Vidal, and Marco, "Pathology of Bovine Tuberculosis."

1.5 Cytokine pattern in tuberculosis

Based on their functions, cytokines are divided in two categories: pro-inflammatory and anti-inflammatory, inducing inflammatory response or inhibiting it respectively. The balance between these pro- and anti-inflammatory cytokines is very important in determining the outcome of the disease. Susceptibility to tuberculosis infection is expressed by alterative balance of pro- and anti-inflammatory cytokines[130].

Briefly, pro-inflammatory cytokines are: IL-1β, TNF-α, IL-6, IL-12 and IL-18. In addition, among these there is IL-10 that has both pro-inflammatory and anti-inflammatory function.

IL-1β: produced by macrophages after activation of inflammasome; its main activity is attraction of other phagocytes and stimulate T-cells proliferation. Up-regulation of IL-2 and IL-2R expression is the dominant activity, but IL-1β augmented production of other pro-inflammatory cytokines such as IFN-γ, IL-6 and TNF-α[131,132]. However, by inducing of IL-2 and IL-2R expression in CD4 T-cells, IL-1β leads a proliferation of CD4 T-cells population. Moreover, IL-12 production by macrophages promotes IFN-γ secretion by CD4 Th1 cells, which, in turn, sustains macrophages activation and their production of TNF-α and reactive oxygen and nitrogen radicals, for killing and elimination mycobacteria in tuberculosis[133,134].

TNF-α: Tumor necrosis factor alpha (TNF-α) is produced mainly by monocytes and activated macrophages[135]. TNF has an important role during acute Mtb infection,

[130] Dinarello, "Proinflammatory Cytokines."
[131] Dinarello and Fantuzzi, "Interleukin-18 and Host Defense against Infection."
[132] Sharma and Bos, "Role of Cytokines in Immune Response to Pulmonary Tuberculosis."
[133] Bekker et al., "Immunopathologic Effects of Tumor Necrosis Factor Alpha in Murine Mycobacterial Infection Are Dose Dependent."
[134] Hamza, Barnett, and Li, "Interleukin 12 a Key Immunoregulatory Cytokine in Infection Applications."
[135] Parameswaran and Patial, "Tumor Necrosis Factor-A Signaling in Macrophages."

specifically in early cell recruitment and in the production of early cytokine in order to establishing and maintaining granulomatous response. TNF improve host immune response. Firstly, by activating macrophages to kill mycobacteria, allows a protective control of bacterial load. Secondly, the cytokine drives the formation of well-organised granuloma, because of the proper accumulation of monocytes, macrophages and lymphocytes within. On the other hand, high dose of TNF can have detrimental effects on lung architecture, compromising tissue functionality. In fact, after the inoculum with high dose of BCG-TNF, Mtb-infected-mice died rapidly despite the activity of TNF in controlling bacterial growth. These results highlight that the TNF positive or negative effects on lung are dose-dependent[136]. However, experiments on $TNF^{-/-}$ mice confirmed that in deficient animal the recruitment of immune cells is delayed, as well as the secretion of cytokine such as MIP-1β, MIP-2 and MCP[137].

Interestingly, a synergism of IL-1β and TNF-α is reported and it is responsible of the increase in prostaglandin-E2 production, lowering the threshold of pain in the site of inflammation. On the other hand, IL-1β alone is not involved in programmed apoptotic program like other cytokines are, e.g. TNF-α[138]

IFN-γ. In tuberculosis, NK cells and also both CD4 and CD8 T-cells produce this cytokine.

IFN-γ interplays with TNF-α for activating anti-microbicidal effects by macrophages in vitro, especially secretion of nitric oxide and related nitrogen intermediate products[139].

IL-10. IL-10 is widely produced by Th2 cells and macrophages (after activation through

[136] Bekker et al., "Immunopathologic Effects of Tumor Necrosis Factor Alpha in Murine Mycobacterial Infection Are Dose Dependent."
[137] Roach et al., "TNF Regulates Chemokine Induction Essential for Cell Recruitment, Granuloma Formation, and Clearance of Mycobacterial Infection."
[138] Dinarello, "Proinflammatory Cytokines."
[139] JoAnne L. Flynn and John Chan, "Immunology of Tuberculosis."

TLRs[140]), but also Th1, Th17, B cell, neutrophils, macrophages and $CD11b^+$ DCs subsets can secrete it in a few amounts. IL-10 acts mainly on APCs (DCs included) and its activity is mainly suppresses protective Th1 response through indirect action on APCs (both macrophages and DCs). Firstly, IL-10 inhibits DCs functions necessary for Th1 differentiation such as secretion of IL-12. Secondly, IL-10 blocks TNF-α secretion in APCs and other pro-inflammatory cytokines. Interestingly, IL-10 interacts with IL-10R on Tregs enhancing FoxP3 expression in these cells in a positive feedback pathway. Moreover, by blocking secretion of reactive oxygen and nitrogen species (RONS) and various signalling pathway, IL-10 suppresses phagocytosis and microbial killing into phagocytes. Further, IL-10 down-regulates MHC molecules and blocks the antigen presentation function in APCs[22]. In fact, several studies on IL-10 deficient mice reveal that in in these animals a granulomatous response arise with recruitment of large amount of T cells and F4/80 macrophages. In these animals clearance of mycobacteria was faster and correlated to an augmented inflammatory response[141].

IL-12. IL-12 is considered as a pro-inflammatory cytokine that connects innate and adaptive immunity[142]. It is produced widely by APCs activated and neutrophils through TLRs, after their activation[143,144]. IL-12 is constituted of two subunits, IL-12p35 and IL-12p40, which is been considered to have protective activity in mycobacterial infection model[145]. IL-12 is required to trigger a protective immune response in tuberculosis and to connect innate and adaptive immunity. IL-12 is considered the dominant IFN-γ-

[140] Saraiva and O'Garra, "The Regulation of IL-10 Production by Immune Cells."
[141] Jacobs et al., "Increased Resistance to Mycobacterial Infection in the Absence of Interleukin-10."
[142] Giorgio Trinchieri, "Interleukin-12: A Proinflammatory Cytokine with Immunoregulatory Functions That Bridge Innate Resistance and Antigen-Specific Adaptive Immunity."
[143] Hamza, Barnett, and Li, "Interleukin 12 a Key Immunoregulatory Cytokine in Infection Applications."
[144] Lyakh et al., "Regulation of Interleukin-12/interleukin-23 Production and the T- Helper 17 Response in Humans."
[145] Hamza, Barnett, and Li, "Interleukin 12 a Key Immunoregulatory Cytokine in Infection Applications."

inducing factor (in NK cells) and, through this activity, it stimulates naïve CD4 T cells to differentiate in Th1 T cells[146].

These functions are demonstrated in animal model, in which the absence of IL-12p40 is found to be associated with incapability from DCs to migrate to lymph nodes and to activate naïve CD4 T cells population because of the consequent decrease in IFN-γ secretion. DCs from animal IL-12p40 deficient do no express the chemokine receptor CCR7[147].

IL-18. IL-18 can be present in several cells: macrophages, DCs, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts. IL-18 has effects particularly on Th1 cells, on these it acts in synergism with IL-12. Briefly, its function on lymphoid cells is supporting T cells and NK cells development and cytokine secretion in order to enhance cytotoxicity. Studies on IL-18 deficient mice showed NK having less cytolytic ability than wild type mice[148]. Interestingly, the receptor for IL-18 (IL-18R) recruits a cytosolic protein called MyD88. When this protein is absent, in mice deficient Th1 cells do not respond to IL1 or IL-18 stimuli, suggesting a function of MyD88 in both IL-1 and IL-18 signalling. Moreover, a synergism between IL-12 and IL-18 is needed in stimulation of IFN-γ production by marrow-derived macrophages in mice, although Th1 cells remain the major source of IFN-γ. In particular, on macrophages IL-12 up-regulated IL-18R and only in this condition IL-18 have the capability to stimulate IFN-γ secretion[149].

IL-17. Mainly γδT cells produce IL-17, also during Mtb infection. IL-17 is a pro-inflammatory cytokine acting as promoter in recruitment of protective cells and in

[146] Lyakh et al., "Regulation of Interleukin-12/interleukin-23 Production and the T- Helper 17 Response in Humans."
[147] Urdahl, Shafiani, and Ernst, "Initiation and Regulation of T-Cell Responses in Tuberculosis."
[148] Gracie, Robertson, and McInnes, "Interleukin-18."
[149] Dinarello and Fantuzzi, "Interleukin-18 and Host Defense against Infection."

organization of cellular population in the lung, favouring granuloma formation. In particular, IL-17 stimulates recruitment of PMNs and their survival. Excessive exposure of PMNs, because of their consequent longer survival, causes in them changes in phenotype and homeostasis, resulting in immunopathology. Anyway there is suggested a role for IL-10 to regulate Th17 response in tuberculosis[150].

At the beginning of infection, in mouse lung, Mtb induces the production of peculiar chemokines, which are required to drive the correct spatial localization of immune cells within the granuloma. At 12 day post-infection, in the lung CXCL3 and CXCL5 expression is detectable and may be involved in the recruitment of PMNs, which bear CXCR2, the specific receptor. Especially CCL19, CCL21 and CXCL13 are important in T cells priming and in disposition of these cells. In fact, specific receptors are expressed in lymphatic endothelium, in follicular DCs and in B cells adjacent to the granuloma[151].

Witchell et al. (2010)[152] investigated on cytokine-expression in cattle during bTB and they found an important increase of IL-10 expression, suggesting a pro-inflammatory immune activity in cattle.

[150] Torrado and Cooper, "IL-17 and Th17 Cells in Tuberculosis."
[151] Slight and Khader, "Chemokines Shape the Immune Responses to Tuberculosis."
[152] Witchell et al., "Time Dependent Expression of Cytokines in Mycobacterium Bovis Infected Cattle Lymph Nodes."

1.6 Adaptive immunity in tuberculosis – maintenance of inflammation

The adaptive response in tuberculosis is important to control bacterial load and maintenance of inflammation and also the granuloma structure[153,154]. Nevertheless, it is slow to start and when T cells arrive in the inflammation site, phagocytes populate the lesion[155]. The priming of T cells in local lymph nodes occur between 12-21 days post-infection[156], when naïve CD4 T-cells are activated by antigens presented by APCs on MHC class II molecules, which are most involved than MHC class I in mycobacterial antigen-presentation[157]. This cell-interaction is based on production of several cytokines by involved inflammatory cells, required to recruit inflammatory cells in the site of infection, cytokine production, and recruitment of antigen-specific immune cells[158].

Maintenance of inflammation

The early CMI characteristic immune response in mycobacterial infections is based on activity of mononucleated cells and in minor part by neutrophils. At the beginning of infection, in the lung, alveolar macrophages and dendritic cells (DCs) recognise and uptake mycobacteria through phagocytosis, before migration to local lymph nodes, and present antigens to naïve T cell triggering innate response[159,160].

In host defence against Mtb, controlling bacterial burden and killing mycobacteria represented pivotal points. Phagocytic cells, especially macrophages and neutrophils,

[153] Guirado and Schlesinger, "Modeling the Mycobacterium Tuberculosis Granuloma – the Critical Battlefield in Host Immunity and Disease."
[154] Bozzano, Francesco Marras, and Maria, "Immunology of Tuberculosis."
[155] Robinson, Orme, and Cooper, "The Onset of Adaptive Immunity in the Mouse Model of Tuberculosis and the Factors That Compromise Its Expression."
[156] Flynn, Chan, and Lin, "Macrophages and Control of Granulomatous Inflammation in Tuberculosis."
[157] Russell, "Mycobacterium tuberculosis: here today, and here tomorrow."
[158] Guirado and Schlesinger, "Modeling the Mycobacterium Tuberculosis Granuloma – the Critical Battlefield in Host Immunity and Disease."
[159] Pozzi, Maciaszek, and Rock, "Both Dendritic Cells and Macrophages Can Stimulate Naive CD8 T Cells In Vivo to Proliferate, Develop Effector Function, and Differentiate into Memory Cells."
[160] Hume, "Macrophages as APC and the Dendritic Cell Myth."

perform both activities through a receptor/mediated phagocytosis[161]. Unfortunately, mycobacteria have the capability to contrast that mechanism to reduce acidification of phagosome and prevent its fusion with lysosomes, apparently through lack of H^{p}-ATPase pumps on phagocytic vacuoles, resulting in an increasing of intracellular pH. In addition, Mtb products large amounts of ammonia with two enzymatic systems, urease and glutamine synthetase, both associated to the mycobacterial pathogenicity and the ability to persist in infected tissues[162,163]. Such mechanisms allow the mycobacteria to survive and multiply within infected cells[164]. Mtb decrease the Ca^{+} concentration within the phagosome, which needs this element for its maturation[165]. Further, mycobacteria inhibit cellular apoptosis in several myeloid cells, especially in neutrophils. This event, not only could cause delayed acquisition of Mtb by DCs, but also cause a delayed priming of naïve $CD4^{+}$T cells. Finally, inhibition of neutrophil apoptosis leads a delayed $CD4^{+}$ and $CD8^{+}$T cells immune response.[166,167]

T cell response is crucial and it is predominant going toward the chronic phase of disease, and it is important to specify that detection of T cell response appears to be subsequent to bacterial dissemination[168]. This occurs because such response follows antigen presentation in mediastinal lymph nodes, after infection of dendritic cells and alveolar macrophages. Live mycobacteria can be found in lymph nodes 9-11 days post-infection, after DCs migration in there and before this step, specialized T cells cannot be

161 Deretic and Fratti, "Mycobacterium Tuberculosis Phagosome."
162 Houben, Nguyen, and Pieters, "Interaction of Pathogenic Mycobacteria with the Host Immune System."
163 JoAnne L. Flynn and John Chan, "Immunology of Tuberculosis."
38 Urdahl, Shafiani, and Ernst, "Initiation and Regulation of T-Cell Responses in Tuberculosis."
164 Deretic and Fratti, "Mycobacterium Tuberculosis Phagosome."
165 Houben, Nguyen, and Pieters, "Interaction of Pathogenic Mycobacteria with the Host Immune System."
166 Urdahl, Shafiani, and Ernst, "Initiation and Regulation of T-Cell Responses in Tuberculosis."
167 Blomgran et al., "Mycobacterium Tuberculosis Inhibits Neutrophil Apoptosis, Leading to Delayed Activation of Naive CD4 T Cells."
168 Wolf et al., "Initiation of the Adaptive Immune Response to Mycobacterium Tuberculosis Depends on Antigen Production in the Local Lymph Node, Not the Lungs."

activated. In other words, T cell response is activated when granulomatous inflammation is becoming to form in the lung[169]. In fact, T cells can be detected in the lungs at 17-day post infection, highlighting that priming occurs mainly in lymph nodes, where recognition of bacterial antigens is a pivotal event for starting adaptive immunity in tuberculosis. There are three hypotheses to justify delayed T cell response, all about the consideration that T cells activation is dependent on antigen availability. The first is that few bacteria cannot generate MHC/peptide complexes in sufficient amounts to activate T cells. The second hypothesis is an incognita, because how activated DCs acquire antigens is unclear. Moreover, it remains unknown if bacterial infection of DCs is required or if these cells can capture antigens passively. The third reason is that Mtb infection on APCs compromises their capability to present antigens[170,171]. During the earliest stages of infection, Mtb is located in a privileged site and even Th1 cells are not able to recognize pathogens antigens. For maintaining protection and control of infection, a constant expression of IL-12 is necessary to sustain an effective Th1 cells response[172].

[169] Winslow et al., "Early T-Cell Responses in Tuberculosis Immunity."
[170] Ibid.
[171] Wolf et al., "Initiation of the Adaptive Immune Response to Mycobacterium Tuberculosis Depends on Antigen Production in the Local Lymph Node, Not the Lungs."
[172] Urdahl, Shafiani, and Ernst, "Initiation and Regulation of T-Cell Responses in Tuberculosis."

1.7 Human tuberculosis vs Bovine tuberculosis: Latency/persistence and reactivation

During infection, when environmental condition (within macrophages or extracellularly) are not favourable to bacterial growth and replication (such hypoxia, reduced pH), bacilli enter in dormant phase and the infected host is designed as "latent infected"[173]. Around 50 genes (such as sigF and acr genes[174]) are identified as involved in bacterial response to stressful conditions could be involved also in regulation of dormancy, during which mycobacteria reduced or arrest metabolism and stop their replication[175,176,177,178]for long period of time, years or even decades.

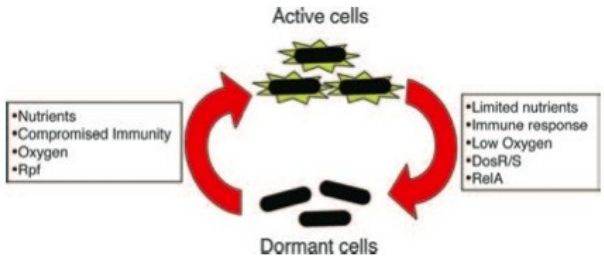

Figure 5. Environmental conditions lead switch of bacteria from active state to dormant state. Also, into the squares are listed nutrients and proteins involved in bacterial life cycle transition[179].

Latent infected animals/people do not show any symptoms of disease, but infection is still present and could be reactivated, resulting in active tuberculosis when immune system are suppressed. Experiments on mice suggested that dormancy of bacteria is inducted when a switch from Th1 to Th2 immune response occurs in infected animals,

[173] Gengenbacher and Kaufmann, "Mycobacterium Tuberculosis: Success through Dormancy."
[174] Parrish, Dick, and William R. Bishai, "Mechanisms of Latency in Mycobacterium Tuberculosis."
[175] Esmail et al., "The Ongoing Challenge of Latent Tuberculosis."
[176] Cossu et al., "Expression Profiling of Mycobacterium Tuberculosis H37Rv and Mycobacterium Smegmatis in Acid-Nitrosative Multi-Stress Displays Defined Regulatory Networks."
[177] Hett and Eric J. Rubin, "Bacterial Growth and Cell Division: A Mycobacterial Perspective."
[178] Alvarez, Estrada-Chavez, and Mario Alberto Flores-Valdez, "Molecular Findings and Approaches Spotlighting Mycobacterium Bovis Persistence in Cattle."
[179] Hett and Eric J. Rubin, "Bacterial Growth and Cell Division: A Mycobacterial Perspective."

but the immune pathway behind latency remains unknown[180].

Latent tuberculosis is considered a huge problem in diagnosis of infection, both in humans and cattle, because the risk of reactivation is constant. Potentially, all stressful events could restore the environment favourable for resuscitation of mycobacteria. In particular, HIV infection for humans and other viral infection for cattle (such BVDV and bovine leucosis) represent the classical predisposing factors for leading reactivation of tuberculosis[181].

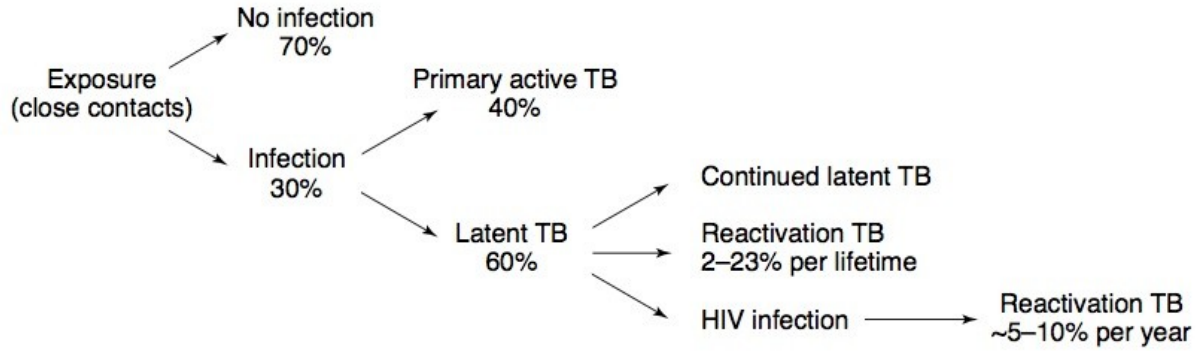

Figure 6. Outcome of human TB schematized by Parrish et al. (1998)[182]. After exposure to Mtb, 30% of people develop infection. Whether 40% of infected individuals enter in primary phase of TB, 60% become latent infected that could develop active TB after reactivation. Only 2-23% per lifetime along immunocompetent patients reactivate TB, while HIV infected patients have a higher rate of TB reactivation (5-10% per year).

1.8 Animal models for tuberculosis

Literature of tuberculosis is mainly about the pathology in humans. Nevertheless, samples of people with active TB are difficult to obtain. The use of animal models is necessary to investigate immune pathway behind the disease. Mouse is the most used specie for human TB, even though lesions in mice lungs are less organized and are

[180] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[181] Alvarez, Estrada-Chavez, and Mario Alberto Flores-Valdez, "Molecular Findings and Approaches Spotlighting Mycobacterium Bovis Persistence in Cattle."
[182] Parrish, Dick, and William R. Bishai, "Mechanisms of Latency in Mycobacterium Tuberculosis."

lacking of the classical structure seen in humans[183][184]. Also guinea pigs are largely used as animal model for TB, but because of their high susceptibility to Mtb infection, they are not used in experiments on latent tuberculosis[185]. For this purpose, bovine model could be an excellent model to use in order to discover new vaccines using latency antibody[186].

For studying bTB, in countries where bTB is endemic experimental infection on bovine models are largely used. On the contrary, in areas with sporadic cases of bTB it is preferred investigating the natural infection occurred in cattle during these outbreaks.

[183] Flynn, Chan, and Lin, "Macrophages and Control of Granulomatous Inflammation in Tuberculosis."
[184] Slight and Khader, "Chemokines Shape the Immune Responses to Tuberculosis."
[185] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."
[186] Ildiko Van Rhijn et al., "Bovine Tuberculosis as a Model for Human Tuberculosis: Advantages over Small Animal Models."

CHAPTER 2

Immunopathological Changes in Irf-8$^{-/-}$ Mice during *Mycobacterium Tuberculosis* (Mtb) Infection

2.1 Introduction – Interferon regulatory factor family

Interferon regulatory factor family (IRFs) is a group of growing (or maturation) transcription factors with a broad variety of activities. All members of the family share a DNA binding domain homology. IRF family consists in nine members: IRF-1, IRF-2, IRF-3, IRF-4 (Pip/LSIRF/ICSAT), IRF-5, IRF-6, IRF-7, IRF-8/ICSBP[187].

In particular, IRF-4 and IRF-8, also known as Interferon consensus sequence-binding (ICSBP), is the only IRFs restrictively expressed in lymphocyte and monocyte/macrophage lineage. IRF-8 plays a role in immune regulation with the main activity in proliferation and differentiation of immune cells at the level of myeloid progenitors. Mice lacking in IRF-8 are defective in TH1 cytokine production, antigen-presenting cells and in promotion of T cells differentiation. These alterations cause an increased susceptibility to viral, bacterial and parasitic infection. Moreover, IRF-$8^{-/-}$ mice show a syndrome similar to human chronic leukemia, in which patients are highly sensitive to infection including tuberculosis, developing a severe outcome of the disease[188,189].

[187] Mamane et al., "Interferon Regulatory Factors: The next Generation."
[188] Ibid.
[189] Nguyen, Hiscott, and Pitha, "The Growing Family of Interferon Regulatory Factors."

2.2 IRF-8 and function in inflammatory cells development

IRF-8 was cloned as an interferon-γ (INF- γ)-inducible nuclear protein with the function of binding INF response motive in the major histocompatibility complex class I (MHC-I) genes.

IRF-8 is highly expressed in myeloid and monocytic cell lineage. It was detected in common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs). IRF-8 is expressed by mononuclear phagocytes, but also by monocyte-DC progenitors, common DC progenitors, CD115$^-$CDPs (plasmacytoid DC-biased progenitors), pre-classical DCs[190].

Several studies showed that mice lacking in IRF-8 have more susceptibility to Malaria, Salmonella and Mycobacterial infection[191], as well as developing of chronic myeloid leukemia-like syndrome (CML-like disease). Gene ontology analysis identified 232 genes involved in the immune response in the 30 days following infection with Mtb. In this list, besides several cytokines such as INF-g, TNF and IL -12 (IL- 12p40), there is also IRF-8[192]. This study showed that IRF-8 mRNA expression is inducted in response to MTB infection, in an up-regulation system.

2.2.1 IRF-8 and phagocytic cells

In DC development, IRF-8 is required until DCs final activation to become CD8α$^+$DCs, plasmocytoid DC and Langherans Cells, and it is necessary in each step of their maturation. In fact CD8α$^+$DCs and pDCs express largely IRF-8, which regulates their

190 Tamura, Kurotaki, and Koizumi, "Regulation of Myelopoiesis by the Transcription Factor IRF8."

191 Turcotte et al., "Icsbp1/IRF-8 Is Required for Innate and Adaptive Immune Responses against Intracellular Pathogens."

192 Marquis et al., "Disseminated and Rapidly Fatal Tuberculosis in Mice Bearing a Defective Allele at IFN Regulatory Factor 8."

differentiation via the Fl3L/STAT3 pathway downstream [193] and through activation of transcription factors *Id2* and *Bcl6* required for the proper development of $CD8\alpha^{+}$DCs and $CD4^{+}$ $CD8\alpha^{+}$DCs respectively.

In $Icsbp^{-/-}$mice $CD8\alpha^{+}$DCs are reduced in all lymphoid organs, appear bigger. In addition $CD8\alpha^{+}$DCs show very little endocytic ability, displaying defect in capturing antigens. These cells express low levels of co-stimulatory antigens CD40, CD80 and CD86, ICAM-1 and MHC class I and class II, which are both indicative of incomplete activation of $CD8\alpha^{+}$DCs in deficient mice[194].

IRF-8 is required for the development of different subset of DCs [195], $CD8^{+}$DCs, plasmocytoid DCs, epidermal DCs and dermal DCs[196,197,198,199].

Tamura et colleagues (2005) confirmed that IRF-8 plays a role in DCs development and it is essential for stimulating proper production of IL-12p40 and INF-γ in these cells. These functions in development and activity of DCs result in a compromised T-cells stimulation, and subsequently in a T cells proliferative response. In fact, $IRF\text{-}8^{-/-}$ DCs promote Th2 response.

Compared to WT, spleen-resident DCs from $ICSBP^{-/-}$ mice have shown less expression of MHC molecules, reduced endocytic activity (in $CD8^{+}$DCs) and, in addition, they

[193] Gabriele and Ozato, "The Role of the Interferon Regulatory Factor (IRF) Family in Dendritic Cell Development and Function."
[194] Schiavoni et al., "ICSBP Is Essential for the Development of Mouse Type I Interferon-Producing Cells and for the Generation and Activation of CD8α+ Dendritic Cells."
[195] Moore and Anderson, "Dendritic Cell Development: A Choose-Your-Own-Adventure Story."
[196] Schiavoni et al., "ICSBP Is Essential for the Development of Mouse Type I Interferon-Producing Cells and for the Generation and Activation of CD8α+ Dendritic Cells."
[197] Aliberti et al., "Essential Role for ICSBP in the in Vivo Development of Murine CD8+ Dendritic Cells."
[198] Tsujimura, Tamura, and Keiko Ozato, "Cutting Edge: IFN Consensus Sequence Binding Protein/IFN Regulatory Factor 8 Drives the Development of Type I IFN-Producing Plasmacytoid Dendritic Cells."
[199] Schiavoni et al., "ICSBP Is Critically Involved in the Normal Development and Trafficking of Langerhans Cells and Dermal Dendritic Cells."

failed to perform a proper MHC-II antigen presentation to T-cells[200].

2.2.2 IRF-8 and lymphocytes development

B cells

In association with IRF-4, IRF-8 plays an important role for pre-B-cell development, germinal center (GC) reaction and plasma cell differentiation. Both regulatory factors promote light chain transcription, activation and rearrangement; also, IRF-8 is important, but not essential, for GC reaction, which appeared less organized in its absence[201].

Starting from HSCs IRF-8, in synergy with PU.1, drives differentiation in MP (Myeloyd Progenitors) and in CLP (Common Lymphoid Progenitors). Then IRF-8 and PU.1, together, activate EBF expression to make up Small Pre-B cells, which are regulated by IRF-8 and IRF-4 to become Immature B cells[202].

2.2.3 IRF-8 and neutrophils

Studies on IFR-$8^{-/-}$mice improved knowledge about functions of IRF-8 in innate and adaptive immunity. In fact, BXH-2 mice carry a mutation (C915T) in the ICSBP1 gene. In BXH-2 mice with CML –like disease, alteration of lymphoid organs such splenomegaly and lymphadenopathy are evident macroscopically, and massive infiltration of $Mac1^{+}/Gr1^{+}$ myeloid granulocytic precursors is associated. In addition, cytometric analysis on cellularity of spleen, lymph nodes and bone marrow from $ICSBP^{-/-}$ mice show increased frequency of $Mac1^{+}/Gr1^{+}$ myeloid granulocytic, both

[200] Mattei et al., "ICSBP/IRF-8 Differentially Regulates Antigen Uptake during Dendritic-Cell Development and Affects Antigen Presentation to CD4 T Cells."
[201] Lu, "Interferon Regulatory Factor 4 and 8 in B-Cell Development."
[202] Wang and Herbert C. Morse III, "IRF8 Regulates Myeloid and B Lymphoid Lineage Diversifcation."

mature and precursors. In mutant mice also an increase of granulocytes is detected in peripheral blood associated to increased number of plasmablast phenotype of B cells[203]. Moreover, mice lacking in IRF-8 gene show reduction of Common Dendritic cells Progenitors (CDP) and increase of Granulocyte-Macrophage Progenitors (GMP), not only in frequency but also in absolute number[204].

Cell culture study demonstrates that IRF-8 blocks neutrophil development and promotes differentiation of DC expressing CD11c and producing IL-12 after stimulation with LPS. IRF-8 is involved both in lymphoid and in myeloid pathway; *Klf4* is necessary to block neutrophil production[205].

2.2.4 IRF-8 and cytokine production/expression

The main role of IRf-8 in cells cytokine production is to stimulate INF gene transcription in DCs, as demonstrate by IRF-8 recruitment to INFα and INFβ promoters in the second phase of their transcription[206]. In ICSBP$^{-/-}$ mutant mice the ability of T cells to produce IFN-γ is compromised and, after LPS stimulation, also spleen cells showed low production of IFN-γ respect to WT, probably due to decreased expression of IL-12p40 in these animals. The reduced production of IL-12p40 in IRF-8 deficient mice is another aspect to confirm IRF-8 is involved in production of this cytokine. Several studies have demonstrated that both mice and human bearing IRF-8 deficiency express low or not detectable levels of IL-12 production[207,208,209], and, in humans, this

[203] Holtschke et al., "Immunodeficiency and Chronic Myelogenous Leukemia-like Syndrome in Mice with a Targeted Mutation of the ICSBP Gene."
[204] Becker et al., "IRF-8 Extinguishes Neutrophil Production and Promotes Dendritic Cell Lineage Commitment in Both Myeloid and Lymphoid Mouse Progenitors."
[205] Ibid.
[206] Tailor et al., "Type I Interferon Induction in Dendritic Cells Requires IRF-8 That Effects the Feedback Phase of Transcription."
[207] Giese et al., "Interferon (IFN) Consensus Sequence-Binding Protein, a Transcription Factor of the IFN Regulatory Factor Family, Regulates Immune Responses In Vivo through Control of Interleukin 12 Expression."

defect give birth a paediatric disease charaterized by absence of DCs in blood and production of Il-12 and IFN-γ in response to BCG[210].

[208] Turcotte et al., "Icsbp1/IRF-8 Is Required for Innate and Adaptive Immune Responses against Intracellular Pathogens."
[209] Schiavoni et al., "ICSBP Is Essential for the Development of Mouse Type I Interferon-Producing Cells and for the Generation and Activation of CD8α+ Dendritic Cells."
[210] Hambleton et al., "Mutations in IRF8 and Human Dendritic Cell Immunodeficiency."

2.3 Aim of the study

In immune response against Mtb, innate immunity plays a pivotal role. Mononuclear phagocytes, neutrophils and NK cells are deputed to start an unspecific immunity during the early stage of TB, in which mycobacteria are newly arrived into the alveolar spaces. Proper growth and maturation of these cells will serve to drift an effective inflammatory cascade, from which depends the prognosis of disease. In particular, DCs have the important function to prime naïve $CD4^{+}$T cells, resulting in driving the initiation of adaptive response that is necessary for the maintenance of inflammation and to deal with the progression of infection.

It is known that IRF-8 is a required factor for growth and differentiation of DCs, and taken the other several function of IRF-8 on both innate and adaptive immunity, the aim of this study is investigate the immune cells dynamics controlled by this factor in lungs immunity of mice during Mtb infection. By comparing IRF-$8^{-/-}$ and WT mice, we focused on several aspects of the host response in TB at 15 day and at 30 day post-infection. Supported by cytometric assay, the study has a predominant histopathological approach. Firstly, we analysed the outcome of disease at 15 day and at 30 day p.i. Secondly, we analysed the features of granulomas, by analysing their growth and the types of immune cells present within. At last, we investigate on development of lung lymphoid structures, focusing on their organization and cellular distribution in order to evaluate whether IRF-8 was correlated with changes in their structures or organization.

2.4 Materials and methods

Mice

In this study C57BL/6-IFR-8-deficient mice (IFR-$8^{-/-}$) and animal counterparts (WT-B6) were used in 6 groups each constituted by 5 animals. The animals were bred and housed at ISS.

Microorganisms

For infection studies we used pathogenic *Mycobacterium tuberculosis* Erdman strain. After growth in Middlebrook 7H9 broth supplemented with ADC and Tween 80, Mycobacteria were harvested, re-suspended in sterile PBS (pH 7.2) and stored at 280uC until use. Before infection, strain aliquots were grown on 7H10 plates supplemented with OADC for treatment of bacteria after unfreezing.

Infections and CFU assay

Experimental infection was performed in a Biosafety level-3 facility. Mice were infected per aerosol with 100 CFU Mtb/animal and maintained until they became moribund. At 8, 15 and 30 day post-infection, groups of 5 mice were sacrificed. After 21 days of incubation at 37uC in sealed plastic bags, in order to enumerate bacterial colonies in spleens and lungs, CFU assay was performed on 7H10/OADC plates.

Histopathology

The lungs (left lobes) were fixed in 4% buffered paraformaldehyde and paraffin embedded (FFPE), sectioned at 3μ and stained with hematoxylin and eosin (HE) and with Ziehl-Neelsen (ZN). Six sections from each left lobe sample were obtained, and a total of thirty of lung sections per group (six section for five lungs belonging to each group) were evaluated by light microscopy (Nikon Eclipse 80i microscope). The total surface area and the area with granulomatous lesions were measured (using Axiovision

4.4 software-Zeiss) on digital images of section (recorded with Nikon DS-L2 camera and Nikon software 3422.1001.1798.080117.); for each section and for each group averages were calculated. In addition cellular automatic count was performed with Axiovision 4.4 software-Zeiss.

Immunohistochemistry and Immunofluorescence

Immunohistochemical analysis was focused on highlight different population within granuloma lesions. We used the following antibodies:

1. CD3- S.C. Biotechnology (Lymphocyte T)*
2. CD4- BD Pharmingen (Lymphocyte T CD4+)**
3. CD8- S.C. Biotechnology (Lymphocyte T CD8+)*
4. FoxP3- eBioscience (Lymphocyte TReg)***
5. DEC205- Dendritics (Dendritic Cells)*
6. 7/4- Caltag Laboratories (Neutrophils)*
7. F4/80- Caltag Laboratories (Macrophages)*
8. CD45R- BD Pharmingen (Lymphocyte B)****

*1:100 diluted; **1:30 diluted; ***1:120 diluted; ****1:50 diluted

A positive control for each antigen was processed in each run.

Three-micron lung sections FFPE on Superfrost slides (Fisher Scientific) were

1. Deparaffinised in xylene
2. Rehydrated with graded alcol
3. Antigen retrieving: using high-temperature heating method (slides were incubated in target retrieval solution at pH 6 (Dako) for 20 minutes, in a steamer (90–95uC)

4. Endogenous peroxidase blocking: 3% hydrogen peroxide solution
5. Prevent non-specific binding: incubation in 2% BSA-PBS solution, stabilizing protein and 0.015 mol/L sodium azide (Protein Block Serum-Free, Dako)
6. Incubation o/n at 4°C with mAbs
7. Incubation with secondary Ab at r.t. for 1h
8. For IHC tissues: 45 min incubation with streptavidin–biotin–peroxidase complex (Lab Kit peroxidase, Dako) at r.t.; reaction was revealed using the chromogen 3, 39- diaminobenzidine (DAB) (DakoCytomation) and Fast Red (Sigma). Sections were counterstained with Mayer's hematoxylin and then cover-slipped in 50:50 xylene/Permount (Fisher Scientific).
9. For Immunofluorescence: incubation with Alexa FluorH 555 streptavidin (Invitrogen) and then counterstained with blue Hoechst.

The sections were recorded as digital images with Nikon DS-L2 camera and Nikon software 3422.1001.1798.080117 and Leica LAS AF (build 7266) for immunohistochemistry and immunofluorescence analysis respectively. Morphometric analysis was performed on three photo series of three independent experiments (400x) by two researchers. Using Axiovision 4.4-Zeiss (with the automated, morphometric tool of the software), positive cells were counted evaluating cellular infiltrates occupying the lesion areas, with measure of lung sections by squared micron value (25).

Statistical analysis

Normal distribution of data was checked with Shapiro-Wilk test then statistical significance was determined using Student's t-test and performed with Stata 11.2 software (StataCorp, College Station, TX, USA). P values <0.05 were considered

statistical significant.

2.5 Results

Macroscopic and microscopic evaluation revealed, in IRF-$8^{-/-}$ mice, several interesting aspects:

- Uncontrolled and disorganized growth of granulomas at late stage of infection
- Defective recruitment of T-cells and prevalence of neutrophil granulocytes during acute phase of TB
- Impaired development of lymphoid structures
- Defective maintenance of immune cell recruitment in lung granulomas

2.5.1. IRF-$8^{-/-}$ pulmonary granuloma show uncontrolled and disorganized growth

Bacterial burden and mice surviving.

The experimental infection was performed on IRF-$8^{-/-}$ and WT mice through aerogenous route with low-dose of Mtb Erdman strain.

At early stage of disease (15 day p.i.), in both groups of animal, bacterial loads levels were similar in spleen and lungs (Fig. 1A).

Conversely, at day 30 p.i., KO mice showed exponential Mtb replication (9,4 logs CFU in lungs and 7,3 logs in spleens), 2,8 log and 3-log higher compared to bacterial loads in WT lungs and spleens, respectively (Fig. 1A).

Moreover, deficient mice did not survive to Mtb infection unlike control animal, which developed a chronic infection and survived (Fig. 1B).

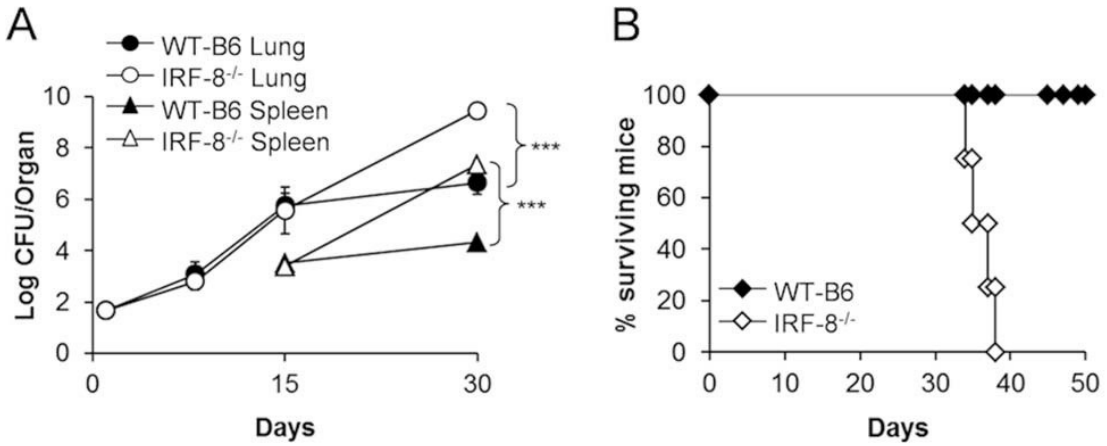

Figure 1. Susceptibility is increased in IRF-$8^{-/-}$ mice during experimental Mtb infection via aerosol route (100 CFU infected dose).
A: Bacterial loads (express in CFU), at 8, 15, 30 day p.i. in spleen and lung. Data are expressed as mean 6 SEM of each individual mouse (n = 5 mice/group). **P,0.001 IRF-$8^{-/-}$ vs. WT-B6 at day 30 p.i
B: Surviving mice.

Macroscopical findings (at day 30 p.i.).

In IRF-$8^{-/-}$ mice lungs tubercles appeared larger than those in WT controls at macroscopic evaluation at day 30 p.i. (Fig 1A). This suggests a massive cellular infiltration in the site of bacterial replication (Fig. 2A).

Microscopical findings (day 15 p.i.).

Despite in both groups of animals microscopical evaluation of WT and IRF-$8^{-/-}$ lungs at day 15 p.i. revealed few microscopical granulomatous lesions, deficient mice lungs exhibited less-organized granulomas characterized by an increased number of mononuclear inflammatory cells (Fig. 2B).

Microscopical findings (day 30 p.i.).

At day 30 p.i. IRF-$8^{-/-}$ lungs showed large granulomas barely organized and extensive areas of colliquative and coagulative necrosis within lung parenchyma. This feature was absent in WT mice, where granulomatous lesions were compact, non-necrotizing and well organized (Fig 2B). These granulomas contain inflammatory cells arranged in two main layers: the inner composed by large epithelioid cells and phagocytes and the outer

constituted by lymphocytes. Conversely, in KO mice, both inner and outer layers, forming typical tuberculous granuloma, are disrupted by the presence of massive necrosis within lung parenchyma (Fig. 2C). In addition, WT mice phagocytes within lesions appeared almost intact and containing few mycobacteria, whereas in IRF-$8^{-/-}$ animals they were foamy, lysed and containing a high bacterial load (Fig. 2B and 2D).

Quantitative analysis.

All of those findings descripted above were supported by quantitative analysis of tissue damage performed on IRF-$8^{-/-}$ mice lungs. This evaluation revealed that the numbers of granulomas in the lungs were not significantly different between the two groups of animals (Data not shown). However, at day 30 p.i., in IRF-$8^{-/-}$ mice it observed an higher median granuloma surface area (Fig 2E), an higher median total tissue surface area with lesion (Fig 2F), and area with lesion versus healthy tissue ratio percentage of lung damaged area on total lung area (Fig 2G). At day 15 p.i., quantitative analysis of tissue damage showed no difference in IRF-$8^{-/-}$ lungs versus WT. Hence, this result suggests IRF-8 deficiency have a pathogenic role occurring at late stage of infection, resulting in a detrimental role in limiting bacterial replication.

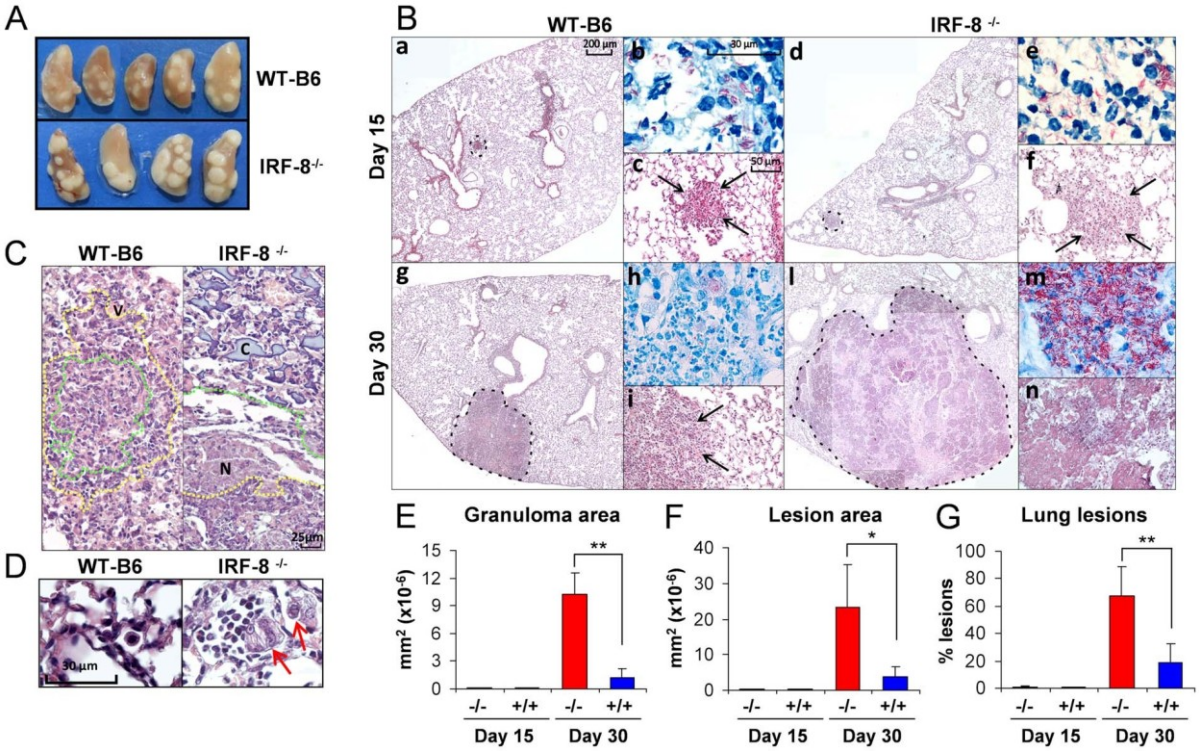

Figure 2. Uncontrolled growth of pulmonary granulomas in IRF-8$^{-/-}$ mice at late phase of Mtb infection.

A: Day 30 p.i.- Macroscopic lesions in IRF-8$^{-/-}$ and WT-B6 animals.
B: Day 15 and 30 day p.i- Histopathology, HE and Ziehl Neelsen staining. Granuloma formation (Dotted lines in panels a, d, g, l); 20X magnification. Panels c, f, i, n show a 200X magnification of the granuloma. Arrows depict granulomatous structures. ZN staining (Panels b, e, h, m); 1000X magnification.
C: Day 30 p.i.- Details of granulomatous lesions observed. Lymphocyte area (dotted yellow lines) and macrophage area (dotted green lines). V: blood vessel; N: coagulative necrosis; C: colliquative necrosis. 400X magnification.
D: Day 30 p.i.- Macrophage area in granulomas, HE staining. Foamy cells are detected at 1000X magnification
E–G: Extent of tissue damage assessed by E) median granuloma surface area; F) median total tissue surface area with lesions and G) percentage of tissue surface area with lesions with respect to total lung area. Five lungs per group were analyzed with at least three sections in two different points of the sample. Representative slides are shown. *P,0.05; **P,0.01.
doi:10.1371/journal.pone.0062751.g002

2.5.2 Defective recruitment of T lymphocytes and prevalence of neutrophils in IRF-8$^{-/-}$ granulomas during acute phase of TB

Taken the totality of CD45^{+} cells, in cytometric assay, immune cells has been labelled as: neutrophil granulocytes as Gr-1^{+}CD11b^{+}CD11c^{-}, macrophages as Single-F^{+}CD11b^{low}CD11c^{+}, Dendritic Cells (DCs) as Single-F^{+}Gr-1^{-}CD11c^{+}, T cells as CD3^{+}CD4^{+} and CD3^{+}CD8^{+} to identify CD4 and CD8, respectively. In deficient mice frequency of neutrophil granulocytes appeared increased (26,9% of total CD45^{+} cells) compared to WT animals (11,5% of total CD45^{+} cells). On the contrary, in IRF-8$^{-/-}$

animals, macrophages were decreased (5,2% of $CD45^+$ cells) and DCs, $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells appeared reduced_compared to WT mice (42,6% of total $CD45^+$ leucocytes) (Fig. 3A.). As confirmed by quantitative analysis, neutrophil granulocytes are the prevalent innate immune population in deficient mice, whereas in WT-B6 lungs macrophages were the most abundant inflammatory cells (Fig 3B). Therefore, granulocyte/macrophage ratio was 4-fold higher in KO mice compared to WT (Fig. 3C).

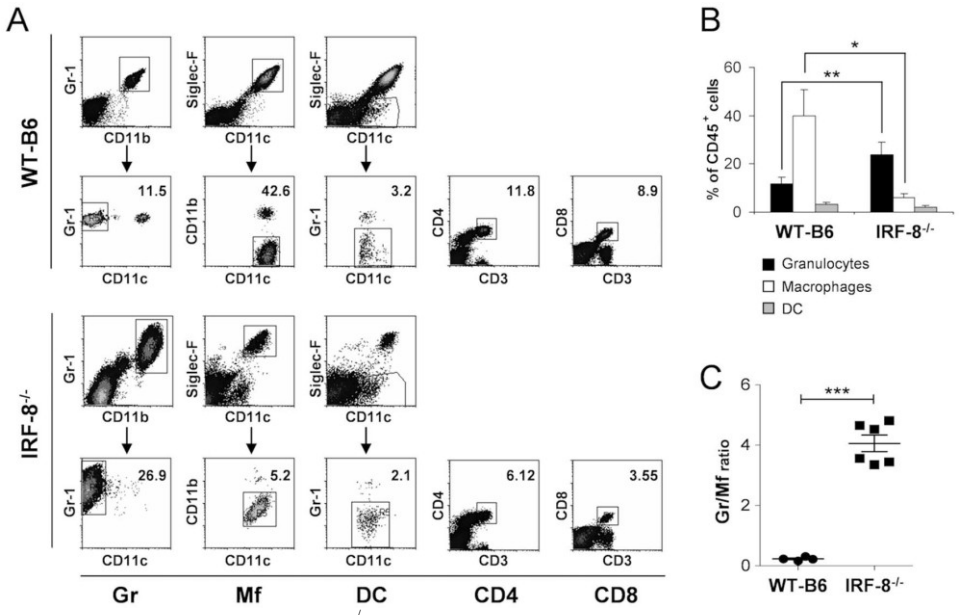

Figure 3. Immune cells distribution in IRF-$8^{-/-}$ and WT mice.
Lung cells from naïve IRF-$8^{-/-}$ and WT-B6 mice were labeled with a panel of fluorescent mAbs against the indicated surface markers. A) Representative FACS analysis of indicated cell populations. Upper panels show cells gated over total $CD45^+$ leukocytes. Plots in lower panels show cell populations gated as indicated by the arrows. Numbers represent the percent values of each indicated cell population over total $CD45^+$cells. B) Mean percentages of lung granulocytes, macrophages and DC 6SD (n = 6 mice/group). *P,0.05; **P,0.01. C) Ratio values between granulocytes and macrophages 6SEM (n = 6 mice/group). ***P,0.001. Data are representative of one experiment out of four.
doi:10.1371/journal.pone.0062751.g003

In immunofluorescence, inflammatory cells have been labelled as: neutrophils as $7/4^+$, macrophages as $F4/80^+$, dendritic cells as $DEC205^+$, T-cells as $CD3^+$, divided in $CD4^+$ and $CD8^+$ for identifying CD4 and CD8 subtypes respectively and, at last, T-regulatory cells as $FoxP3^+$.

At day 15 p.i., cellular spatial distribution in IRF-$8^{-/-}$ mice granulomas was constituted

by $CD3^+$ T-cells in the outer part of lesion, few follicular dendritic cells within and high number of $7/4^+$ neutrophils. Furthermore, at early stage (15 day p.i.), immunofluorescence revealed an efficient recruitment of DCs, macrophages, $CD4^+$ and $CD8^+$ T-cells in IRF-$8^{-/-}$ mice compared WT (Fig. 4A-C), in spite of the reduced frequency of these cells in KO animals.

At day 30 p.i., frequency of $DEC205^+$ DC and $F4/80^+$ cells remained similar in both IRF-$8^{-/-}$ and WT mice, whereas $CD4^+$ and $CD8^+$T-cells decreased in deficient mice. In addition, in IRF-$8^{-/-}$ lungs, there was an increase of $7/4^+$ neutrophils (Fig. 4A-C). In lungs of KO mice, decreased infiltration of $FoxP3^+$ T-reg cells was more evident at day 30 than at day 15 p.i.

Therefore, at late stage of infection, IRF-$8^{-/-}$ mice showed low recruitment of $CD3^+CD4^+$ and $CD3^+CD8^+$ T-lymphocytes (Fig 3B-C) in addition to uncontrolled growth of granuloma.

Moreover, $B220^+$ cells were largely detected in IRF-$8^{-/-}$ lungs at day15 p.i., whereas their presence did not highlighted in late stage of infection (data not shown).

Immune cells, constituted by T lymphocytes and phagocytic cells such as macrophage, FDCs and DCs, were early recruited in granulomatous lesions of IRF-$8^{-/-}$ mice, but their presence decreased gradually in disease progression.

In contrast, $7/4^+$ neutrophils increase persistently in association with an uncontrolled and disorganized growth of granulomas in defective mice, supporting a role of IRF-8 in controlling the functions of these cells in TB pathogenesis.

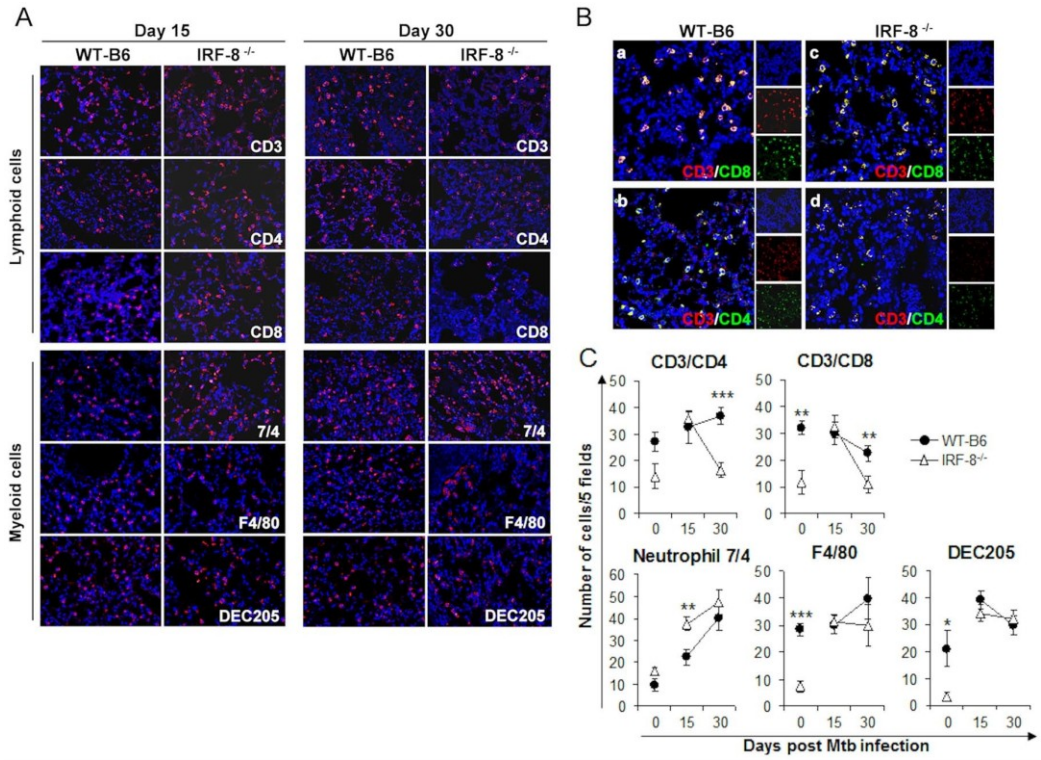

Figure 4. Immune infiltration in lungs of IRF-8$^{-/-}$ and WT mice infected with Mtb. Immunofluorescence and morphometric analysis.
A: Day 15 and 30 p.i. immune cells were labeled with mAb against indicated markers.
B: Co-localization of immune cells, with anti CD3 plus anti CD4 (b, d), and anti CD3 plus anti CD8 (a, c).
C: Morphometric analysis. Cells express indicate markers in lung sections. Data express mean cell numbers 6SD of 5 fields (1 field = 0.16 mm^2 at 400x magnification) of each slide (4–15 slides/mice; n = 3 mice/ group) analyzed in lungs of Mtb-infected IRF-8$^{-/-}$ and WT-B6 and their uninfected counterparts. One representative experiment out of two is shown. *P,0.05; **P,0.01; ***P,0.001. doi:10.1371/journal.pone.0062751.g004

2.5.3 Impaired development of LS was detected in IRF-8$^{-/-}$ mice after Mtb infection

During TB infection, both in human and in mice pulmonary granuloma development is associated with lymphoid neogenesis as secondary lymphoid organization. Anyway, mice lacking these structures showed normal T- cell development, which is granuloma-dependent[211]. Stromal follicular dendritic cells (FDCs) are necessary for the formation of germinal centers (GC)[212], and when the lymphoid structures (LS) are present, they

[211] Day et al., "Secondary Lymphoid Organs Are Dispensable for the Development of T-Cell-Mediated Immunity during Tuberculosis."
[212] Mohey Eldin M. El Shikh and Pitzalis, "Follicular Dendritic Cells in Health and Disease."

influence the development of acquired immunity in TB[213].

We evaluated LS in their structure and organization through HE staining and immune-histochemistry, labelling FDCs as FDC-M1$^+$ and B cells as B220$^+$.

Day 15 p.i. results: HE and single staining.

At day 15 p.i., HE revealed aggregates of immune cells were present in both mouse strain around vessels, bronchi and within the interstitium, resembling LS (Fig 5A). Compared to WT, IRF-8$^{-/-}$ perivascular and peribronchial LS appeared less organized and containing large B220$^+$ cells aggregates (Fig 5B). Moreover, in deficient mice FDC appeared interspersed in inflammatory infiltrate, contrary to what it observed in WT-B6 lungs, where FDC aggregates were well organized. In addition, well-defined LS in WT-B6 lungs were observed (Fig. 5C).

Day 15 p.i. results: double staining.

Double staining on F4/80$^+$ macrophages and CD3$^+$ T-cells into LS was performed (Fig. 5E). Both mice strains, at day 15 p.i., showed a similar distribution of these cells, with F4/80$^+$macrophages distributed around vessels and bronchial areas (Fig 5E, F, G). Also DEC205$^+$ DCs were similarly distributed in IRF-8$^{-/-}$ LS as in WT (Fig 5D).

Differences were observed in investigation of 7/4$^+$ neutrophils and FoxP3$^+$ Treg cells between IRF-8$^{-/-}$ and WT-B6 mice. In deficient mice 7/4$^+$ neutrophils appeared distributed in large aggregates, whereas they were absent in WT (Fig. 5H). Moreover, FoxP3$^+$ Treg cells were poorly detectable in IRF-8$^{-/-}$ mice but these cells were present in large number in controls.

213 Kashino et al., "Initiation of Acquired Immunity in the Lungs of Mice Lacking Lymph Nodes after Infection with Aerosolized Mycobacterium Tuberculosis."

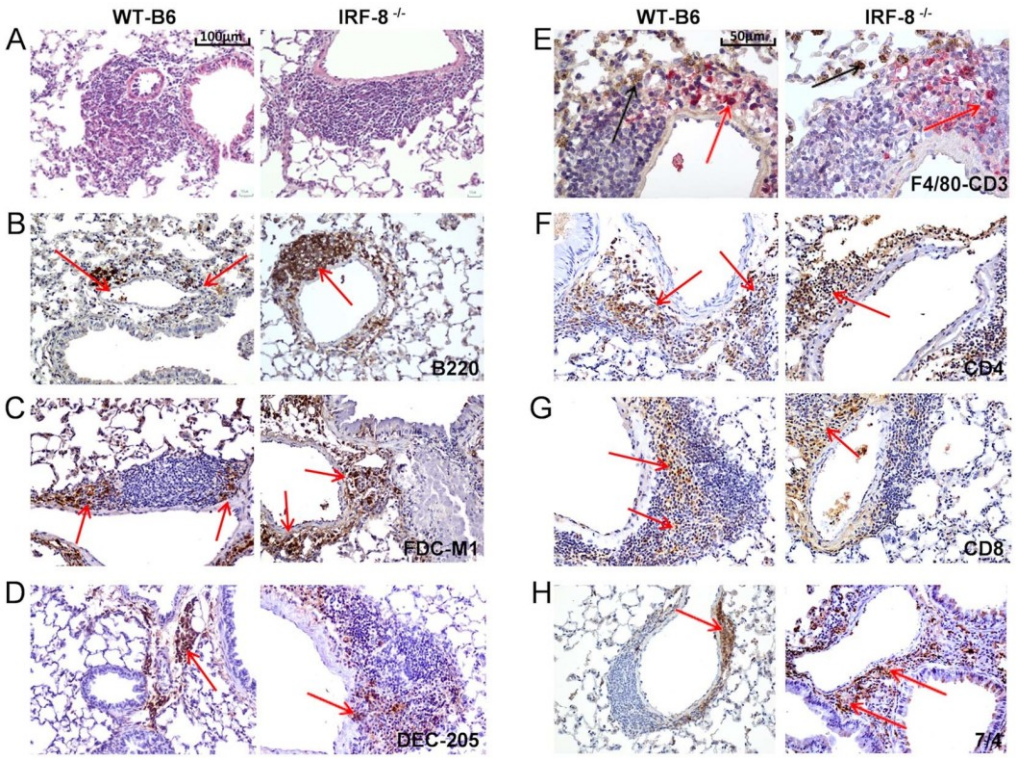

Figure 5. Lymphoid structures (LS) in IRF-$8^{-/-}$ and WT mice Mtb-infected. Immunohistochemistry analysis of lung section at 15 day p.i. shows immune cells distributed in perivascular, peribronchial and interstitial areas of lung.
A: HE staining, 200X magnification.
B-D and F-H: Staining with the indicated mAbs (brown). Red arrows depict marker-positive cells. 200X magnification.
E: Co-staining with anti-F4/80 (brown) and anti-CD3 (red) mAbs, depicted by black and red arrows respectively. 400X
Three independent experiments were performed.
doi:10.1371/journal.pone.0062751.g005

Day 30 p.i. results.

At late stage of infection, IRF-$8^{-/-}$ lungs appeared damaged with immune infiltrates, and LS were massively altered and disorganized. And, in particular, in peribronchial and perivascular LS, $B220^{+}$ cells remained still large, well-defined aggregates, surrounding vessels and bronchial tree. Conversely, in WT-B6 mice, LS remained well organized and with few $B220^{+}$ cells (Fig. 6A, B).

The total $CD3^{+}$ T cells appeared decreased in LS of KO mice compared to control counterparts (Fig. 6E-G). The other labelled immune cells ($F4/80^{+}$ macrophages, FDC and DCs) were present in LS as well-organized aggregates in WT lungs. On the contrary, all of these cells were poorly detectable in LS of KO animals (Fig. 6C-E),

where, at this stage of disease, $7/4^{+}$ neutrophils appeared still as large aggregates (Fig. 6H).

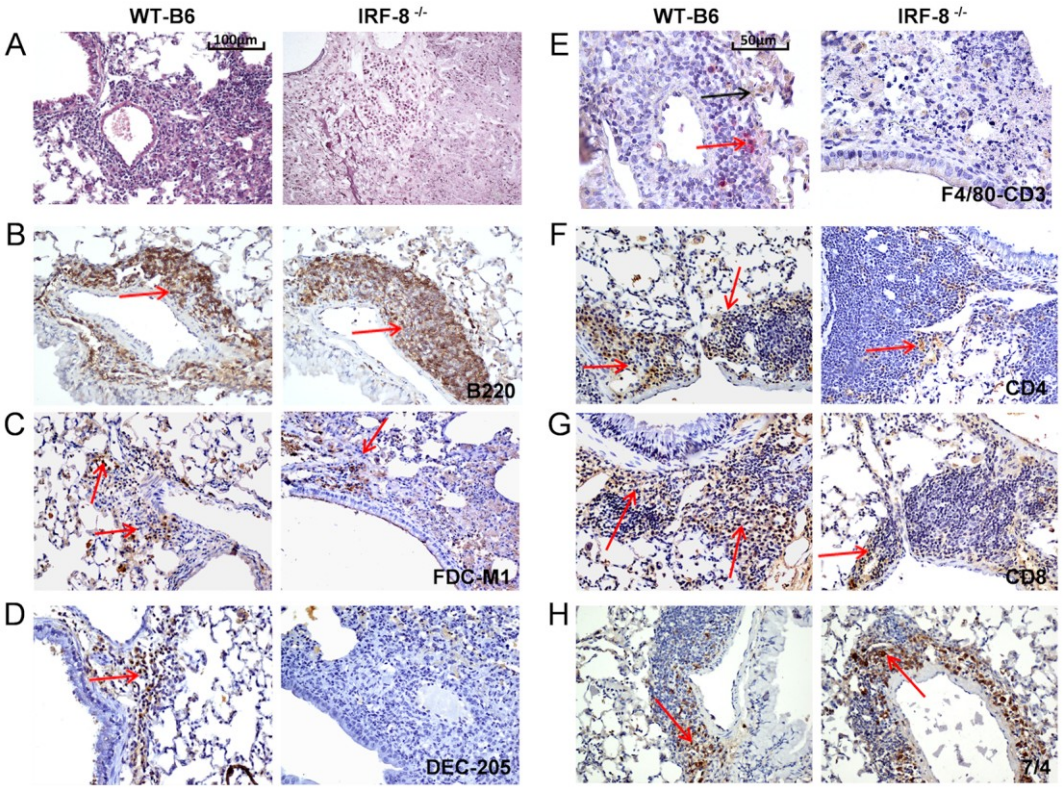

Figure 6. Altered organization of LS in lungs of Mtb-infected IRF-$8^{-/-}$ mice. Immunohistochemistry analysis at 30 day p.i.; immune cells are distributed in perivascular, beribronchial and interstitial areas of lung.
A: HE staining, 200X magnification.
B-D and F-H: Staining with the indicated mAbs (brown). Red arrows depict marker-positive cells. 200X magnification.
E: Co-staining with anti-F4/80 (brown) and anti-CD3 (red) mAbs, depicted by black and red arrows respectively. 400X
Three independent experiments were performed.

Chemokine evaluation.

We investigated whether IRF-8 deficiency influences the expression of the homeostatic chemokines CCL19 and CXCL12, which are necessary for the proper organization of LS in lungs[214].

In KO lungs, at day 15 p.i., CCL19 was detected massively, contrary of CXCL12, of which presence was lower (Fig. 7A). Therefore, at day 30 p.i., in pulmonary tissue of

[214] Carragher, Rangel-Moreno, and Randall, "Ectopic Lymphoid Tissues and Local Immunity."

IRF-8$^{-/-}$ mice did not detected CCL19 nor CXCL12, contrary to WT animals, where both chemokines were highly detected (Fig. 7B). Our results confirmed the strict correlation between organization of LS and expression of CCL19 and CXCL12 during TB.

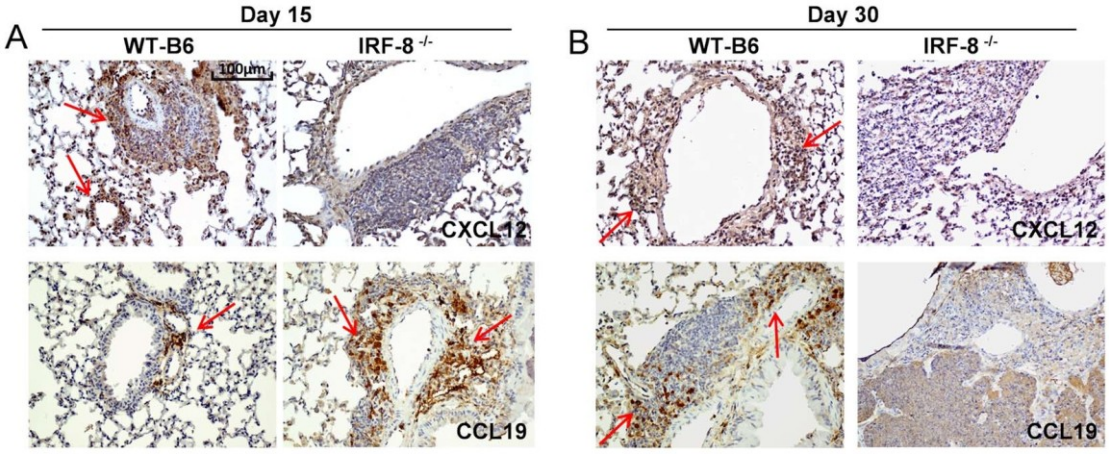

Figure 7. Correlation between cytokine expression and LS organization in lungs of Mtb-infected mice (both IRF-8$^{-/-}$ and WT). Immunohistochemistry of peribronchial and interstitial areas of lung.
A: Day 15 p.i.- 200X magnification.
B: Day 30 p.i.- 200X magnification.
Staining with mAbs against CCL19 and CXCL12 (brown). Red arrows depict marker-positive cells. Three independent experiments were performed.

2.5.4 Defective maintenance of immune cell recruitment in lung granulomas of IRF-8$^{-/-}$ mice

Finally, we evaluated the infiltrate in granulomatous lesions, in both strains.

Day 15 p.i.

Total CD3^{+} cells were detected in the outer layer of granulomas in KO mice (Fig. 8A). FDC were found in well-organized aggregates in WT mice, but their detection was poor in IRF-8$^{-/-}$ lungs (Fig. 8B). Conversely, in KO mice a high accumulation of B220^{+} cells was observed, whereas in deficient mice was detected a high accumulation of 7/4^{+} neutrophils (Fig. 8C). The chemokine CCL19 were found in the outer layer of IRF-8$^{-/-}$ granulomas (Fig. 8D).

Day 30 p.i.

In WT mice were observed well-structured granulomas with well detectable $CD3^+$ T cells, $F4/80^+$ macrophages, FDC cells and DCs. On the contrary, lesions of KO mice appeared poorly organized, with extensive caseous-necrotic areas. In these animals, whereas $CD3^+$ T cells, $F4/80^+$ macrophages, FDC cells and DCs are barely detected (Fig. 8E, F), $7/4^+$ neutrophils persisted to dominate inflammatory infiltration (Fig. 8G). CCL19 chemokine appeared massively decreased in deficient mice, but its detection increased in lesions of control counterparts (Fig. 8H). $B220^+$ cells were not detected in IRF-$8^{-/-}$ lungs (Fig. S3).

Taken together, these findings suggest that the frequency of T cells and myeloid cells gradually declined throughout disease progression, even though their early recruitment occurs properly. Furthermore, the poorly organized granulomas were associated to a high recruitment and persistence of neutrophils.

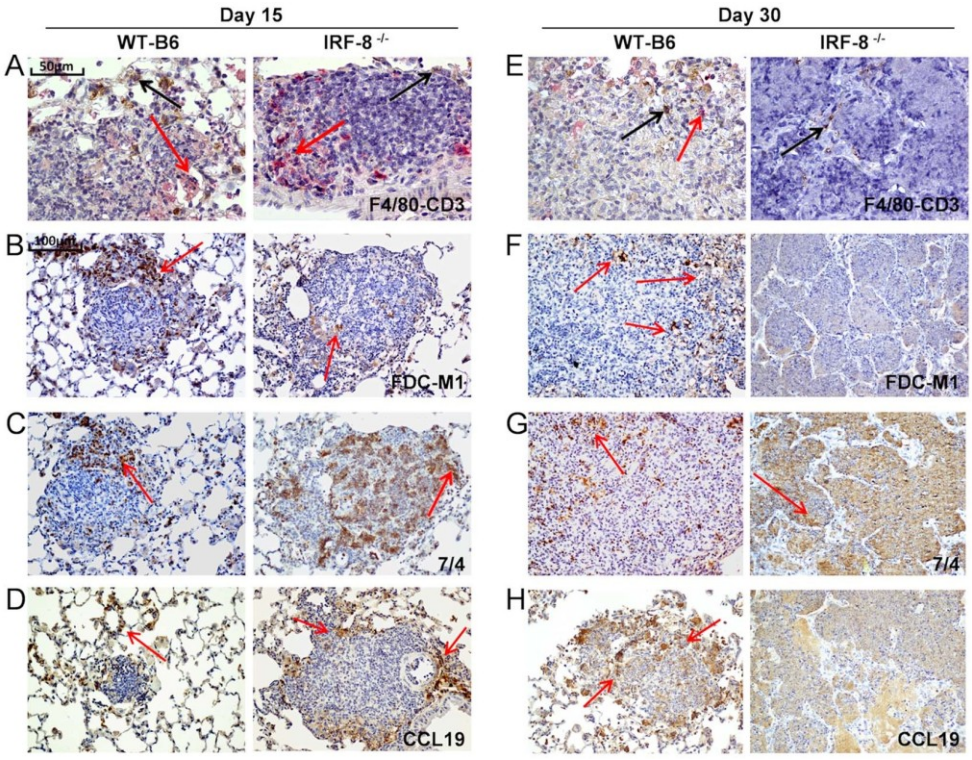

Figure 8. Immune infiltrates into granulomas of Mtb-infected IRF-8$^{-/-}$vs. WT-B6 mice. Immunohistochemistry of pulmonary sections, from both strains of mice at days 15 and 30 p.i., shows the distribution of immune cells within granulomatous lesions.
A, E) Co-staining with anti-F4/80 (brown) and anti-CD3 (red) mAbs, depicted by black and red arrows respectively. 400X magnification
B–D, F–H) Staining with the indicated mAbs (brown). Red arrows depict marker-positive cells. 200X magnification
Three independent experiments were performed.

2.6 Discussion and conclusion

The regulatory factor IRF-8 is very important for immune activity of DCs[215]. In addition, in humans and mice with a genetic mutation in IRF-8 gene show a primary immunodeficiency, confirming that this factor is involved in developing innate and adaptive immunity. This primary immunodeficiency compromises also the effective immune response during Mtb infection.

Focusing the importance of IRF-8 in TB, this factor was considered critical in regulation of host defence during the disease[216]. This aspect is supported by studies on BXH-2 mice, which were highly susceptible to Mtb infection[217]. These mice bear a mutation in IRF-8R394C allele and develop a systemic Mtb infection with disseminated and fatal TB.

Summarizing our results, in IRF-$8^{-/-}$ mice emerged three main aspects: 1. Uncontrolled growth and disorganized structure of granulomas with progressive loss of T lymphocytes; 2. Impaired development of LS; 3. Persistence of massive neutrophil infiltrate in LS and into granulomas.

Our results show that in KO mice the granulomas developed uncontrolled and disorganized, contrary of control animals. These findings confirmed the important of IRF-8 in the proper development of granuloma and pulmonary LS. Moreover, whereas control mice develop chronic infection, deficient mice succumbed to fatal acute TB within day 40 p.i. as a result of the high susceptibility to Mtb infection of these animals.

[215] Hambleton et al., "Mutations in IRF8 and Human Dendritic Cell Immunodeficiency."
[216] Fortin et al., "Host Genetics of Mycobacterial Diseases in Mice and Men: Forward Genetics Studies of BCG-Osis and Tuberculosis."
[217] Marquis et al., "Disseminated and Rapidly Fatal Tuberculosis in Mice Bearing a Defective Allele at IFN Regulatory Factor 8."

As demonstrated by Day and colleagues[218], secondary lymphoid organs are not necessary for developing the adaptive immunity during TB. In fact, protective immune response can be generated independently in Mtb infection and, in particular, local pulmonary priming of naïve T cells is granuloma-dependent[219]. The breakpoint in which occurs activation of adaptive immunity is considered at 15 day post-infection[220]. In this study the two mice strains diverged during TB infection at this stage. Several studies revealed that Mtb infection has been associated with the neogenesis of pulmonary LS[221,222]. Moreover, during Mtb infection granulomas can contain lymphoid areas that can be considered as neo-generated LS[223,224]. In lungs of both mice strains, occurred an early recruitment of immune cells, but, in LS and in granulomas of deficient mice, the immune-cells distribution appears different throughout Mtb infection, resulting from the unbalanced presence of immune cells in lungs in IRF-$8^{-/-}$ mice. The normal LS are characterized by a precise organization, in which the T zone and the B zone are proper separated. This feature is maintained in WT mice, whereas in IRF-$8^{-/-}$ animals LS were poorly defined and less organized with no separation between T and B zones, resulting in loss of immune infiltrate and LS disruption observed at late stage of disease. These different features were detected with efficient recruitment of immune cells in parenchyma[225].

Detection of CCL19 and CXCL12 in lungs gives another explanation of the impaired

[218] Day et al., "Secondary Lymphoid Organs Are Dispensable for the Development of T-Cell-Mediated Immunity during Tuberculosis."
[219] Ibid.
[220] Urdahl, Shafiani, and Ernst, "Initiation and Regulation of T-Cell Responses in Tuberculosis."
[221] Ulrichs et al., "Human Tuberculous Granulomas Induce Peripheral Lymphoid Follicle-like Structures to Orchestrate Local Host Defence in the Lung."
[222] Kahnert et al., "Mycobacterium Tuberculosis Triggers Formation of Lymphoid Structure in Murine Lungs."
[223] Tsai et al., "Characterization of the Tuberculous Granuloma in Murine and Human Lungs: Cellular Composition and Relative Tissue Oxygen Tension."
[224] Maglione, Xu, and John Chan, "B Cells Moderate Inflammatory Progression and Enhance Bacterial Containment upon Pulmonary Challenge with Mycobacterium Tuberculosis."
[225] Carragher, Rangel-Moreno, and Randall, "Ectopic Lymphoid Tissues and Local Immunity."

development of LS. In IRF-8$^{-/-}$ LS CCL19 was expressed at high levels, and it appeared correlated with the accumulation of immune infiltrates but not with the organization of these structures. Therefore, that the high levels of the chemokine could cause the pulmonary recruitment of immune infiltrates in the lungs, through a chemokine/lymphotoxin loop[226]. At the same time point, CXCL12 in WT mice appeared correlated with the recruitment and spatial localization of immune cells in the inflammatory infiltration. At day 30 p.i., a high expression of both chemokines was observed only in WT mice, correlated with maintenance of LS organization and immune infiltrates constituted by CD4$^+$ and CD8$^+$ T cells, macrophages, DC and FDC. These results confirm the non-overlapping roles of CCL19 and CXCL12 to promote LS organogenesis, confirming previous reports[227,228]. Moreover, the role of CXCL12 in driving the spatial organization of T and B zones in LS is confirmed by the high levels of this chemokine in WT mice and by its absence in IRF-8$^{-/-}$ animals.

It is known that neutrophils are immune cells, characterizing especially the acute phase of inflammation and they are also important in development of acute TB. Our results showed that, by 15 day p.i., a different balance of immune cells was found in lungs in two groups of animals, even though at that time point bacterial burden in lungs and spleens was similar in both mice strains. Focusing on the immune cell composing granulomas, both mice strains showed similar percentages of macrophages, DC, FDC, CD4$^+$ and CD8$^+$ T cells. This suggests an early and efficient recruitment of immune cells in deficient mice at early stage of disease. Nevertheless, IRF-8$^{-/-}$ mice displayed a massive neutrophil accumulation compared to WT mice, resulting in a feature of acute

[226] Foo and Phipps, "Regulation of Inducible BALT Formation and Contribution to Immunity and Pathology."
[227] Slight and Khader, "Chemokines Shape the Immune Responses to Tuberculosis."
[228] Luther et al., "Differing Activities of Homeostatic Chemokines CCL19, CCL21, and CXCL12 in Lymphocyte and Dendritic Cell Recruitment and Lymphoid Neogenesis."

inflammation[229]. On the other hand, the exuberant immune response observed in deficient mice the decrease of Treg suggests that such as inflammatory response is the predominant aspect and not protective immunity[230].

T cells are recruited 2-3 weeks p.i., starting adaptive immunity. Activation of T cells is driven by proper activation of DCs that become able to migrate in lymph nodes where naïve T cells priming occurs. The T cell priming play a pivotal role in developing adaptive immunity, and if this event falls specific immune response to Mtb infection is compromised, resulting in disease progression[231,232].

Conclusion

The influence of IRF-8 deficiency appears markedly at late stage of Mtb infection and did not express at early stage of disease. In fact, at 15 day p.i., an efficient recruitment of immune cells in granulomas of deficient mice has been observed. Lungs of these animals showed a failure in inflammatory response, displaying non-organized granulomas containing large caseous-necrotic center, resulting in loss of protective function of granuloma and following mycobacterial dissemination. As confirmed by the high level of that cytokine detected in IRF-$8^{-/-}$ animals, CCL19 supports the fast develop of granulomas, which on one hand is a protective structure but on the other represent a site where mycobacteria proliferate[233].

Presence and persistence of massive neutrophil infiltration in IRF-$8^{-/-}$ mice lungs, in both granulomas and LS represents an important finding to discuss. Recent studies have been focused on the increasing interest in neutrophils during Mtb infection and revealed

[229] Zhou, Dai, and Huang, "Neutrophils in Acute Lung Injury."
[230] Huynh, Joshi, and Brown, "A Delicate Dance: Host Response to Mycobacteria."
[231] Dorhoi, Reece, and Kaufmann, "For Better or for Worse: The Immune Response against Mycobacterium Tuberculosis Balances Pathology and Protection."
[232] Yoshikai, "Immunological Protection Against Mycobacterium Tuberculosis Infection."
[233] Lalita Ramakrishan, "Revisiting the Role of the Granuloma in Tuberculosis."

that immune activity of these cells were inefficient to control disease. Therefore, although their recruitment at early stage of infection should ensure protective immunity, the inefficiency in their activity may lead to detrimental immune responses[234,235]. In other words, an early infiltration of neutrophils can be correlated with protective immunity, whereas at late stage of Mtb infection their presence can assume a detrimental role[236]. In IRF-8$^{-/-}$ mice, however, the high number of neutrophils at early stage did not establish a protective immunity, damping the development of specific immunity against Mtb up to day 15 p.i. Neutrophils have an immunosuppressive function on T cells, as reported by Munder et al[237]. But it is also true that neutrophils can perform a protective role, secreting IL-12 and CXCL10 to attract T cells[238] and delivering activation signals to DCs[239].

IRF-8 is required for the production of IL-12 and proper DCs maturation, which represent stages necessary in order to activate adaptive immunity[240]. Since IL-12 is also necessary to T cells for driving Treg cells expansion[241], the decreasing of these cells in deficient mice confirmed the indirect role in controlling adaptive immunity of IRF-8, through controlling several pivotal components for activation of adaptive immune response. Moreover, the marked decrease of CD4 and CD8 T cells and the disruption of LS are the key events that hamper the development and the amplification of a protective

234 Berry et al., "An Interferon-Inducible Neutrophil-Driven Blood Transcriptional Signature in Human Tuberculosis."
235 Lowe et al., "Neutrophils in Tuberculosis: Friend or Foe?"
236 Ibid.
237 Munder et al., "Suppression of T-Cell Functions by Human Granulocyte Arginase."
238 Mantovani et al., "Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity."
239 Behr, Schurr, and Philippe Gros, "TB: Screening for Responses to a Vile Visitor."
240 Gabriele and Ozato, "The Role of the Interferon Regulatory Factor (IRF) Family in Dendritic Cell Development and Function."
241 Chen et al., "IL-2 Simultaneously Expands Foxp3+ T Regulatory and T Effector Cells and Confers Resistance to Severe Tuberculosis (TB): Implicative Treg–T Effector Cooperation in Immunity to TB."

adaptive immunity.

Moreover, in this study IRF-8 is confirmed to play a pivotal role at late stage of disease, when in deficient mice was observed a poor outcome of disease, the massive recruitment of neutrophils and the loss of CD4 and CD8.

Mice bearing IRF-8 deficiency could represent an animal model of experimental acute TB. Recently it has been identified an IRF-8-dependent genetic control for human mycobacterial infection[242]. Lastly, this work highlights the importance to IRF-$8^{-/-}$ mouse model of acute TB as instrument for following comparative studies in order to analyse this mechanism underlying compromised immune reactions.

[242] Hambleton et al., "Mutations in IRF8 and Human Dendritic Cell Immunodeficiency."

CHAPTER 3

Histopathological and Biomolecular Evaluations in Cattle Tuberculin Skin Test Positive Slaughtered According to The Regional Eradication Program

2.1 Introduction

Bovine tuberculosis (bTB) is a chronic infectious disease of cattle, representing an unsolved animal health problem, especially for agricultural industries, because of economic burden in several countries, particularly where bTB is endemic[243,244]. The disease is caused by *Mycobacterium bovis*, bacteria belonging to the group called *Mycobacterium tuberculosis* complex, sharing almost 99,9% identity at DNA level[245]. Bovines are preferred host, but infection with *M. bovis* involves multiple species, including domestic avian and mammals. In fact, *M. bovis* infection has been reported in dogs, cats, horses, pigs and small ruminants[246,247]. Moreover, also wild species can harbour mycobacteria, and they play a pivotal role as source for reinfection of cattle. A recent study suggests that non-bovine species that develop *M. bovis* infection are capable to spread the disease, not only when they develop extensive pathology, but also when they do not show any tubercles at post-mortem examination[248]. Even humans can develop the disease, and even though the prevalence of the disease in human is low, the zoonotic aspect of bTB remains even more interesting issue, especially in those geographic areas in which the disease is endemic[249,250].

[243] Pollock et al., "Immune Responses in Bovine Tuberculosis."
[244] Schiller et al., "Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of Their Relevance for Disease Control and Eradication."
[245] Gordon et al., "Genomics of Mycobacterium Bovis."
[246] O'Reilly and Dabornt, "The Epidemiology of Mycobacterium Bovis Infections in Animals and Man: A Review."
[247] Broughan et al., "Mycobacterium Bovis Infections in Domesticated Non-Bovine Mammalian Species. Part 1: Review of Epidemiology and Laboratory Submissions in Great Britain 2004–2010."
[248] Delahay et al., "Bovine Tuberculosis Infection in Wild Mammals in the South-West Region of England: A Survey of Prevalence and a Semi-Quantitative Assessment of the Relative Risks to Cattle."
[249] O'Reilly and Dabornt, "The Epidemiology of Mycobacterium Bovis Infections in Animals and Man: A Review."
[250] Müller et al., "Zoonotic Mycobacterium Bovis– Induced Tuberculosis in Humans."

2.2 Epidemiologic importance of bTB in Europe, Italy and Sardinia and eradication programs

Since the foundation of European Union (EU), bTB is an important issue. So far, in EU, member states are classified into two categories: officially tuberculosis-free and not officially tuberculosis-free. The rank of "officially tuberculosis-free" it has been recognised in 11 member states, including Belgium, Czech Republic, Denmark, Germany, France, Luxembourg, Netherlands, Austria, Slovakia, Finland and Sweden, and some parts of Italy[251]. In accordance with European Commission legislation, 3 regions and 15 provinces were considered officially tuberculosis-free (European Council: 2007, Commission Decision 2007/174/CE of 20 March 2007 amending Decision 2003/467/EC).

All over the world, eradication programs for bTB are applied. In EU eradication programs are applied in all member states, especially in those with higher prevalence of disease, like United Kingdom. However, the standard control strategy and eradication programs are based on Tuberculin Skin Test (TST) and slaughter policy, associated with constant abattoir surveillance[252].

In Italy the on-going national eradication program is regulated by DM 15/12/1995. According to general guidelines and following the "test and slaughter" approach, eradication is composed by three phases[253]:

1. Identification of every single animal and their registration on national cattle anagraphic register;

2. Ante-mortem visit and execution of TST;

3. Post-mortem examination in slaughterhouse.

[251] F.J. Reviriego Gordejo and Vermeersch, "Towards Eradication of Bovine Tuberculosis in the European Union."
[252] Schiller et al., "Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of Their Relevance for Disease Control and Eradication."
[253] F.J. Reviriego Gordejo and Vermeersch, "Towards Eradication of Bovine Tuberculosis in the European Union."

Ante-mortem examination is required for any movement of cattle from the herd, in order to verify a good health status of the animals. According to OIE guidelines, the execution of TST in ante-mortem examination is required. Although several alternative diagnostic methods based on detection of early antigens of *M. bovis* are object of studies, the TST remains the only official diagnostic test to identify infected animals and it is based on Cell-mediated Immune response (CMI), according to OIE guidance. The test consists traditionally in an intradermal injection of Protein Purified Derivatives by culture of *M. bovis* (PPD-B) in the caudal fold of the tail or, more frequently, in the neck[254]. Then, 72 h after injection, reactors show a defined increase in skin thickness as evidence of delayed hypersensitivity, which is measured with callipers. Eventually, it is possible that animals sensitised by non-pathogenic environmental mycobacteria can react positively to PPD-B, then an additional comparative test is performed, using PPD derived by *M. avium*, to identify cattle infected with *M. bovis*[255].

The TST positive animals must be slaughtered separately from other healthy cattle, and the carcass is subjected to post-mortem exam for finding macroscopic lesion suggestive of tuberculosis, in order to lay off the meat.

However, TST positive cattle may not show any post-mortem findings, named non-visible-lesions (NVL). For instance, in EU countries with high prevalence of TB, especially in UK and Ireland, it was reported 50-80% of reactor cattle show no lesions of disease and failure to isolate *M. bovis*. NVL reactors can be: animals in the early stage of infection (granulomas are too small to be observed macroscopically); animals in latent stage of TB; animals that behave as false positive reactors (cattle exposed to

[254] Schiller et al., "Bovine Tuberculosis: Effect of the Tuberculin Skin Test on in Vitro Interferon Gamma Responses."

[255] Pollock, Welsh, and McNair, "Immune Responses in Bovine Tuberculosis: Towards New Strategies for the Diagnosis and Control of Disease."

environmental mycobacteria that cross-react with TST).[256].

[256] Rua-Domenech et al., "Ante Mortem Diagnosis of Tuberculosis in Cattle: A Review of the Tuberculin Tests, c-Interferon Assay and Other Ancillary Diagnostic Techniques."

2.3 Aim of the study

The TST is considered to be a good herd test, but a poor test for identifying individual infected animals[257]. In fact, even though cattle were TST positive and their carcass did not show any lesion suggestive of tuberculosis, cannot be excluded that internal organs or the meat of the animals are infected.

Although the TST is considered to be a good herd test, is a low-grade test for the identification of individual infected animals[258]. In fact, the carcass of the NVL reactors may not have lesions suggestive of tuberculosis, but it cannot be excluded that internal organs or the meat of the animals are infected.

The aims of the study is:

- Improving knowledge on pathogenesis of the disease, firstly, through characterizing the lesions suggestive of bTB, using a grading score.
- Analysing their distribution spending more attention on those districts considered "not common sites" of infection.
- Evaluating a possible correspondence with the age of animals.

Moreover, the detection of microscopic lesions and mycobacteria in the organs is also important for this purpose, also when macroscopic lesions are absent, especially because of the meat showing "non-visible lesion" can be laid off, as indicated in EU Regulation 854/2004. Since tubercles are characterised to be paucibacillary, in this study routine histochemical techniques were combined to molecular analysis, in order to improve detection of mycobacteria.

[257] Ibid., -.

[258] Rua-Domenech et al., "Ante Mortem Diagnosis of Tuberculosis in Cattle: A Review of the Tuberculin Tests, c-Interferon Assay and Other Ancillary Diagnostic Techniques."

2.4 Materials and methods

Samples collection

Sardinia is considered a free area for bTB in Italy and in this region outbreaks prevalence is very low. During these outbreaks, 118 samples are harvested from 22 TST positive animals in slaughterhouse. In details, have been collected samples of 104 lymph nodes, 4 lungs, 2 livers, 4 udders, and 4 tonsils.

Histopathology examination

Collected samples are fixed in 10% buffered formalin and paraffin embedded (FFPE), then 3 micron sections were deparaffinised in xylene, rehydrated with graded alcohol and stained in Haematoxylin Eosin (HE) following histological standard method:

- 1' Haematoxylin
- Rinse distilled water
- 30''Eosin
- Rinse distilled water
- Dehydration

Histological evaluation of sections was performed using Nikon Eclipse 80i light microscopy, according to classification suggested by Wangoo (2005)[259] and revisited by Palmer (2007)[260].

This classification consists in identification of four stages of maturation of the granuloma, by observing the types of inflammatory cells, the presence of AFB and other characteristic features (necrosis and mineralization) present in the granuloma. First stage (initial): granuloma is formed by epithelioid macrophages united in irregular clusters. Interspersed lymphocytes, a small number of neutrophils and Langhan's multinucleated cells were present. At this stage granuloma appear unencapsulated with

[259] Wangoo et al., "Advanced Granulomatous Lesions in Mycobacterium Bovis-Infected Cattle Are Associated with Increased Expression of Type I Procollagen, Gd (WC1+) T Cells and CD 68+ Cells."
[260] Palmer, Waters, and Thacker, "Lesion Development and Immunohistochemical Changes in Granulomas from Cattle Experimentally Infected with Mycobacterium Bovis."

no central necrosis. Second stage (solid): a thin fibrous capsule encapsulated clusters of epithelioid macrophages. Lymphocytes, neutrophils, Langhan's multinucleated giant cells and, sometimes, haemorrhage was present. Often central necrosis was seen. Third stage (necrotic); at this stage, the granuloma is completely encapsulated and characterized by central necrotic areas, often caseous and mineralized. Epithelioid macrophages surround the central necrosis with Langhan's multinucleated cells.

The outer zone of granuloma contains macrophages admixed with lymphocytes and scattered neutrophils. Fourth stage (necrotic and mineralized): The granuloma appears thickly encapsulated, with large caseous-necrotic areas mostly mineralized. Epithelioid macrophages and Langhan's cells surround the necrosis with lymphocytes united in clusters near the capsule.

Table 1 - Summary of grading score used to classify granulomas[261]

Lesion Stages	Description
Stage I	Initial. Epithelioid macrophages with low numbers of lymphocytes and neutrophils. +/- Langhans cells No necrosis
Stage II	Solid. Epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes and +/- multinucleated giant cells. Minimal necrosis. AFB may be seen within macrophages and Langhans cells.
Stage III	Necrotic. Fibrous encapsulation is complete. Necrotic cores surrounded by epithelioid macrophages, multinucleated giant cells and lymphocytes.
Stage IV	Necrotic and mineralized. Irregular multicentric granulomas with multiple necrotic cores surrounded by a thick fibrous capsule. Necrotic cores admixed to foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surrounded necrotic areas, with moderate-to-marked infiltrates of lymphocytes. AFB present in moderate numbers, located within the necrotic core.

Acid fast staining

Three-micron sections were stained using Ziehl-Neelsen method with standard protocol:

- 15' Ziehl Fuchsine
- 5' Room Temperature
- 30'' Acid Alcol
- Rinse distilled water
- 1' Methylene Blue
- Rinse water
- Dehydration

By using a Nikon Eclipse 80i light microscopy, the slides were examined at 100X magnification. The samples were considered positive when at least 1 or more AFB were detected. A positive control was used.

[261] Wangoo et al., "Advanced Granulomatous Lesions in Mycobacterium Bovis-Infected Cattle Are Associated with Increased Expression of Type I Procollagen, Gd (WC1+) T Cells and CD 68+ Cells."

Molecular analysis

Ten sections (3mm) of each FFPE sample were collected in 1,5ml tubes. The DNA isolation was performed using QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's instructions.

Briefly:

- Deparaffinization in xylene,
- Lysis under denaturing conditions with proteinase K digestion,
- Incubation at 90°C to reverse formalin crosslinking.

The DNA bound to the membrane of the spin-columns, residual contaminants washed away, eluted in Buffer ATE and stored at –20°C until use. According to Eisenach et al. (1990), IS6110F 5'-CCTGCAGCGTAGGCGTCGG-3' and IS6110R 5'-GTCCAGCGCCGCTTCGG-3'were used as primers in order to identify bacteria belonging to tuberculosis complex.

2.5 Results

Macroscopic examination

Ten animals of 22 showed macroscopic tuberculous granulomatous lesions, which means that 23/118 samples (19,5%) displayed macroscopic lesions (tubercles) confirming active bTB in those animals. The most involved organs were lymph nodes belonging to retropharyngeal and mediastinal lymphocenters. In these organs yellowish, poorly encapsulated, confluent granulomatous nodule were founded, often containing central necrosis and mineralization. These macroscopic lesions corresponded to 4 stage of histological grading, typical old aging lesions.

Table 2 - List of sample collected with macroscopic, microscopic and molecular findings

ID Sample	Organ	Macroscopic lesion	Microscopic lesions (scored)	Ziehl Neelsen staining	PCR IS6110
R44/2011 A	Tonsils	+	FBG	-	-
R44/2011 B	Retropharyngeal LNDs	-	Absent	-	-
R44/2011 C	Mandibular LNDs	-	Absent	-	-
R44/2011 D	Tracheal LNDs (Right)	-	Absent	-	-
R44/2011 E	Tracheal LNDs (Left)	-	Absent	-	-
R44/2011 F	Mediastinal LNDs	-	Absent	-	-
R44/2011 G	Mesenteric LNDs	-	Absent	-	-
R44/2011 H	Hepatic LNDs	-	Absent	-	-
R44/2011 I	Sub-iliac LNDs	-	Absent	-	-
R44/2011 L	Udder LNDs	-	Absent	-	-
R44/2011 M	Udder	-	Absent	-	-
R44/2011 N	Lung	-	Absent	-	-
R202/2011 B-C	Retropharyngeal LNDs	-	Absent	-	-
R202/2011 A	Mandibular LNDs	-	RLH	-	-
R202/2011 D	Lung	+	II-III-IV	+	+
R202/2011 E-F	Liver	+	I-II-III-IV	+	+
R209/2011 A	Parotid LNDs	-	RLH	-	-
R209/2011 B	Retropharyngeal LNDs	-	RLH	-	-
R209/2011 C	Mandibular LNDs	-	RLH	-	-
R209/2011 D	Tracheal LNDs	+	III-IV	-	+
R209/2011 F	Hepatic LNDs	-	RLH	-	-

ID Sample	Organ	Macroscopic lesion	Microscopic lesions (scored)	Ziehl Neelsen staining	PCR IS6110
R209/2011 E	Lung	+	III-IV	-	-
R210/2011 A	Parotid LNDs	-	RLH	-	-
R210/2011 D	Tonsils	+	FBG	-	-
R210/2011 B	Retropharyngeal LNDs	-	RLH	-	-
R210/2011 C	Mandibular LNDs	-	Absent	-	-
R210/2011 E	Tracheal LNDs	-	RLH	-	+
R210/2011 G	Mediastinal LNDs	+	III-IV	+	-
R210/2011 H	Mesenteric LNDs	-	RLH	-	-
R210/2011 I	Udder LNDs	-	Absent	-	-
R210/2011 F	Lung	+	IV	-	-
R211/2011 A	Parotid LNDs	-	RLH	-	-
R211/2011 D	Tonsils	-	RLH	-	-
R211/2011 B	Retropharyngeal LNDs	-	RLH	-	-
R211/2011 C	Mandibular LNDs	-	Absent	-	-
R211/2011 E	Mediastinal LNDs	+	II-III-IV	+	-
R212/2011 A	Parotid LNDs	-	RLH	-	-
R212/2011 B	Retropharyngeal LNDs	-	RLH	-	-
R212/2011 C	Mandibular LNDs	-	RLH	-	-
R211/2011 F	Udder LNDs	+	III	-	-
R212/2011 D	Udder LNDs	-	RLH	-	-
R213/2011 A	Parotid LNDs	-	RLH	-	-
R213/2011 B	Retropharyngeal LNDs	-	RLH	-	-
R213/2011 C	Mandibular LNDs	-	RLH	-	-
R213/2011 D	Tracheal LNds (Right)	+	IV	-	-
R213/2011 E	Mediastinal LNDs	+	IV	+	-
R213/2011 F	Udder LNDs	-	Absent	-	-
R232/2011 B	Tonsils	-	Absent	-	-
R232/2011 A	Mandibular LNDs	+	IV	-	-
R232/2011 C	Mediastinal LNDs	+	II	+	-
R232/2011 E	Mesenteric LNDs	-	Absent	-	-
R232/2011 D	Hepatic LNDs	-	RLH	-	-
R232/2011 I	Sub-iliac LNDs	-	Absent	-	-
R232/2011 G	Udder LNDs	-	RLH	-	-
R232/2011 F	Udder	-	Absent	-	-
R232/2011 H	Uterine LND	-	Absent	-	-
R262/2011 C	Parotid LNDs	-	FH	-	-
R262/2011 B	Retropharyngeal LNDs	-	PH	-	-
R262/2011 A	Mandibular LNDs	-	PH	-	-
R262/2011 D	Mediastinal LNDs	-	Absent	-	-
R262/2011 E	Mesenteric LNDs	-	PH	-	+
R262/2011 F	Hepatic LNDs	-	PH	-	-
R263/2011 C	Parotid LNDs	+	IV	+	-

ID Sample	Organ	Macroscopic lesion	Microscopic lesions (scored)	Ziehl Neelsen staining	PCR IS6110
R263/2011 B	Retropharyngeal LNDs	-	Absent	-	-
R263/2011 A	Mandibular LNDs	-	Absent	-	-
R263/2011 D	Mediastinal LNDs	+	IV	+	-
R263/2011 E	Mesenteric LNDs	+	Absent	-	-
R263/2011 F	Hepatic LNDs	+	Absent	-	-
R264/2011 C	Parotid LNDs	+	IV	+	-
R264/2011 B	Retropharyngeal LNDs	-	Absent	-	-
R264/2011 A	Mandibular LNDs	+	IV	-	-
R264/2011 D	Mediastinal LNDs	+	IV	-	-
R264/2011 E	Mesenteric LNDs	+	I	+	-
R265/2011 B	Parotid LNDs	-	FH	+	-
R265/2011 A	Mandibular LNDs	+	I	-	-
R265/2011 C	Mediastinal LNDs	+	III-IV	-	-
R265/2011 E	Hepatic LNDs	-	Absent	-	-
R265/2011 H	Sub-iliac LNDs	-	Absent	+	+
R265/2011 D	Udder LNDs	-	Absent	-	-
R265/2011 F	Liver	+	III-IV	-	-
R265/2011 G	Uterine LNDs	+	III-IV	-	+
R266/2011 B	Parotid LNDs	-	Absent	-	-
R266/2011 A	Retropharyngeal LNDs	-	Absent	-	-
R266/2011 C	Mediastinal LNDs	+	III	-	+
R264/2011 F	Hepatic LNDs	-	Absent	-	-
R266/2011 D	Mesenteric LNDs	-	Absent	-	-
R266/2011 E	Hepatic LNDs	-	Absent	-	-
R266/2011 F	Udder LNDs	-	RLH	-	-
R266/2011 G	Udder	-	Absent	-	+
R159/2012 I491	Mesenteric LNDs	-	RLH	-	-
R159/2012 I492	Udder LNDs	-	Absent	-	-
R160/2012 I493	Udder LNDs	-	RLH	-	-
R161/2012 I494	Tracheal LNDs (right)	-	Absent	-	-
R161/2012 I495	Udder LNDs	-	Absent	-	+
R161/2012 I496	Udder LNDs	-	Absent	-	-
R162/2012 I498	Parotid LNDs	-	Absent	-	-
R162/2012 I499	Retropharyngeal LNDs	-	Absent	-	-
R162/2012 I497	Mandibular LNDs	-	Absent	-	-
R162/2012 I500	Tracheal LNDs (Right)	-	Absent	-	-
R162/2012 I501	Mesenteric LNDs	-	Absent	-	-
R163/2012 I503	Parotid LNDs	-	Absent	-	-
R163/2012 I504	Retropharyngeal LNDs	-	Absent	-	-
R163/2012 I502	Mandibular LNDs	-	Absent	-	-
R163/2012 I505	Mediastinal LNds (cranial)	-	Absent	-	-
R163/2012 I506	Udder	-	Absent	-	-

ID Sample	Organ	Macroscopic lesion	Microscopic lesions (scored)	Ziehl Neelsen staining	PCR IS6110
R164/2012 I507	Mandibular LNDs	-	Absent	-	-
R164/2012 I508	Mediastinal LNDs (Cranial)	-	Absent	-	-
R165/2012 I510	Parotid LNDs	-	Absent	-	-
R165/2012 I511	Retropharyngeal LNds	-	Absent	-	-
R165/2012 I509	Mandibular LNDs	-	Absent	-	-
R165/2012 I512	Udder LNDs	-	Absent	-	-
R166/2012 I514	Parotid LNDs	-	Absent	-	-
R166/2012 I515	Retropharyngeal LNDs	-	Absent	-	-
R166/2012 I513	Mandibular LNDs	-	Absent	-	-
R166/2012 I516	Udder LNDs	-	Absent	-	-
R167/2012 I517	Parotid LNDs	-	Absent	-	-
R167/2012 I518	Retropharyngeal LNDs	-	Absent	-	-
R167/2012 I519	Udder LNDs	-	Absent	-	-

FBG: Foreign body granuloma; RLH: Reactive lymph nodes hyperplasia; FH: Follicular hyperplasia; PH: Parafollicular hyperplasia.

Histopathology findings: HE staining

Twenty-three samples (23/118, 19,5%) showed tuberculous lesions in different stages of maturation. Granulomas at stage IV were the most observed alone in 8 samples, including lung (n=1), tracheal LNDs (n=1), mediastinal LNDs (n=2), mandibular LNDs (n=2) and parotid LNDs (n=2). In other organs (n=9), this stage of granuloma was observed in co-presence of other grade of maturation. Except one uterine lymph node and two livers, the advanced IV stage lesions were detected in respiratory tract, precisely in lung (n=2), liver (n=2), tracheal LNDs (n=1) and mediastinal LNDs (n=3). This kind of lesions in liver and reproductive tract corresponded to a state generalized bTB observed in that animal. Moreover, early stage lesions (stage I) were observed in mesenteric (n=1) and in mandibular (n=1) lymph nodes.

ZN staining and Molecular analysis

ZN staining revealed AFB in 9,3% of samples (n=11/118). All of these lesions were paucibacillary, with few mycobacteria into the cytoplasm of macrophages and multinucleated giant cells.

Mycobacterial DNA was detected in 8,5% of samples (n=10/118): lung (n=1); liver (n=1), udder (n=1) and lymph nodes (n=7). Interestingly, macroscopic and microscopic lesions were absent in 4 of PCR-positive lymph nodes.

By adding PCR and ZN positive samples (n=19), 15% of samples (n=3/19) were positive to both test; 45% (n=9/19) only ZN positive; 37% (n=7/19) only PCR positive.

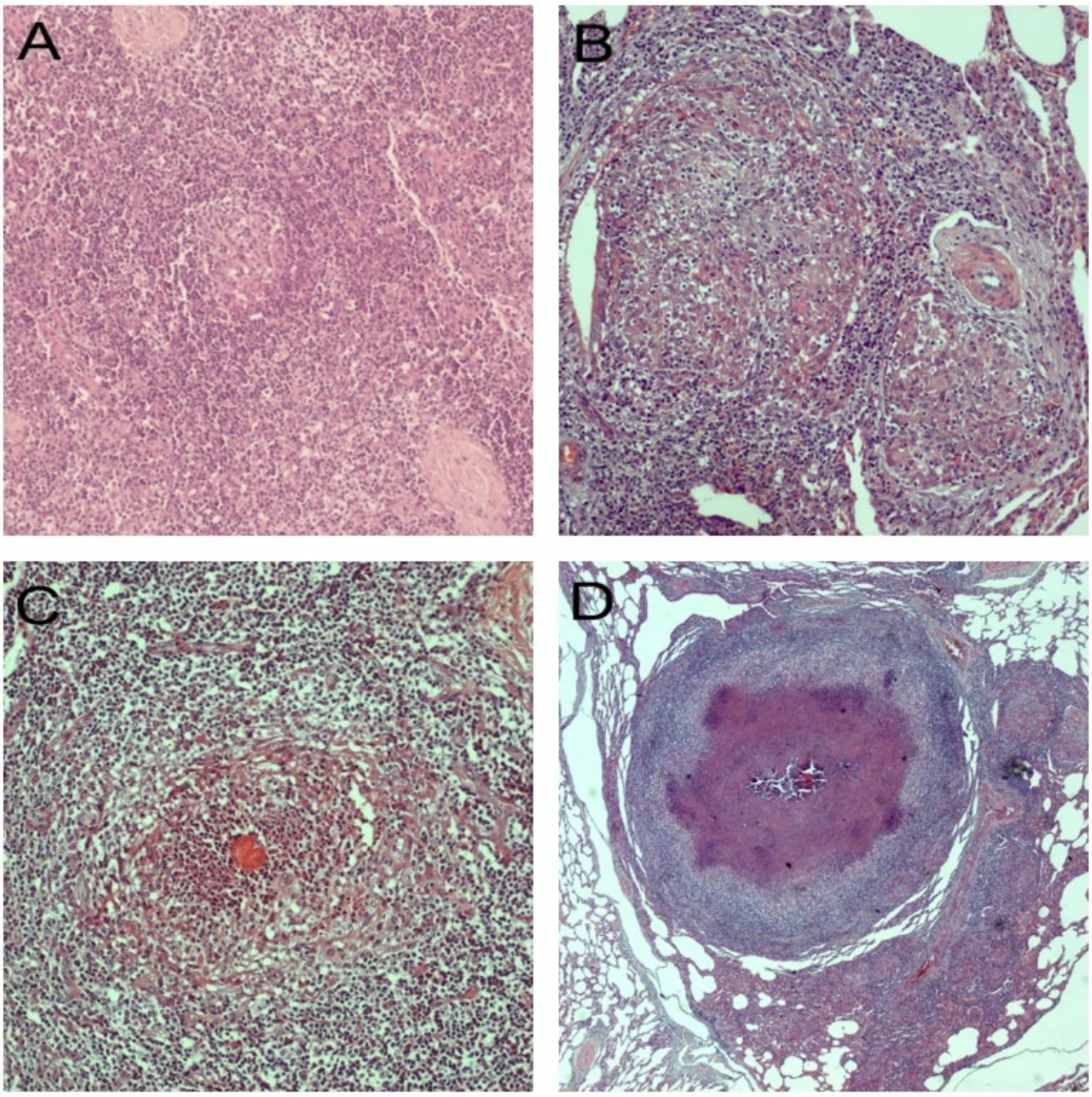

Figure 1 - The four stages of granuloma in lung and lymph nodes. A: Parotid Lymph node (R-265-A-2011), grade I. Unencapsuled clusters of epithelioid cells and macrophages. B: Lung (R-202-D-2011), grade II. Enlarged aggregates of mononuclear phagocytes (epithelioid cells and macrophages) without capsule and necrosis. C: Mandibular Lymph node (R-232-A-2011), grade III. Partially encapsulated with central minimal necrosis. D: Lung (R-202-D-2011), grade IV. Large, encapsulated with large central necrosis and mineralization.

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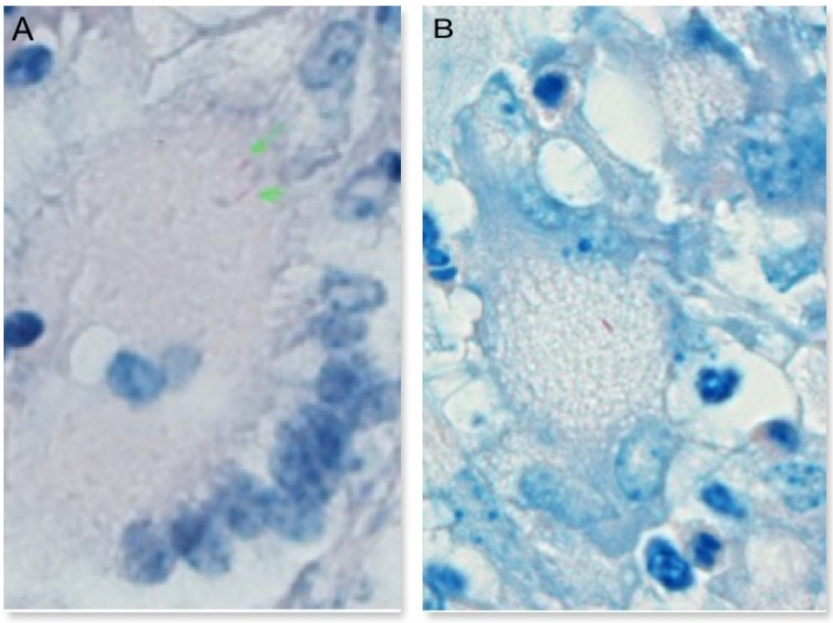

Figure 2 - Paucibacillary lesions, ZN staining. Few mycobacteria occupy the cytoplasm of foamy cells in lymph nodes. A: 100X magnification; B: 40X magnification.

2.6 Discussion and conclusions

The most involved organs were lymph nodes mediastinal and retropharyngeal. This finding confirmed that the aerogenous route is the main route transmission of infection, since the respiratory tract was the principal site of infection[262,263,264]. In particular, macroscopic findings of tuberculosis were seen in 23/118 samples (19,5%), including 12 lymph nodes and 3 lungs.

The three situations derived by PCR and ZN findings combination were very important to analyse. The first situation (positivity to both tests) was observed in upper respiratory tract and in thorax, classical site of primary complex in bTB. In addition, these lesions were classified as III-IV stages, resulting as old-aging granuloma. This information suggested that this type of lesion could be interpreted as an active phase of the disease in affected organs.

The second case, in which only ZN staining was positive, has been seen mainly in respiratory tract and in one mesenteric lymph node. The granulomas were classified as old aging lesions, corresponding to third and fourth stage. Components of mycobacterial wall were detected with ZN staining, whereas mycobacterial nucleic acid did not. Considering the characteristic paucibacilarity of Tb granulomas and the nature of FFPE samples used for PCR analysis, an explanation for this finding could be that, especially old-aging lesions can contain dead mycobacteria in necrotic area without nucleic acids.

The third situation is the most interesting. The samples positive to PCR test but not to ZN staining was detected in mammary, uterine and mesenteric lymph nodes, all of these

[262] Neill, Bryson, and Pollock, "Pathogenesis of Tuberculosis in Cattle."
[263] Menzies and Neill, "Cattle-to-Cattle Transmission of Bovine Tuberculosis."
[264] Domingo, Vidal, and Marco, "Pathology of Bovine Tuberculosis."

considered as atypical sites of *M. bovis* infection nor a common finding as lesions[265], distant to classical sites of primary complex, which is mainly detected in thorax of infected animals. This feature could be connected to a precise phase of bacterial life cycle called "dormant phase". Recent study revealed that mycobacteria belonging to *Mycobacterium tuberculosis* complex, during the dormant phase, could drive a down-regulation of several genes involved in biosynthesis of polysaccharides and also in those implicated in production of fatty acids, including mycolic acids. These are very important for a proper constitution of bacterial wall and cell membrane, resulting also necessary for a proper ZN staining of mycobacteria [266 , 267] . Another important consideration about dormant phase of mycobacteria is that, transporting this phase in the diagram of the pathogenesis of bTB, dormancy could correspond to latent phase of bTB[268]. In this study, animals, from which samples showing these features, could be in latent tuberculosis. In latency, infected animals can show positivity to TST and bearing macroscopic lesions visible at post-mortem examination. Moreover, it is possible that bovine in that phase do not develop any reaction to TST (then resulting as "not infected" in ante-mortem examination). Interestingly, in human TB, this phase of disease could evolve to the active phase through reactivation of dormant mycobacteria within granulomas and, subsequently, humans latent infected generate the condition for spreading bacteria and for giving birth to new outbreaks, even in areas where prevalence is low[269].

TST is based on detection of a pathway called Cell-mediate immunity, which develops

[265] Neill, Bryson, and Pollock, "Pathogenesis of Tuberculosis in Cattle."
[266] Cossu et al., "Expression Profiling of Mycobacterium Tuberculosis H37Rv and Mycobacterium Smegmatis in Acid-Nitrosative Multi-Stress Displays Defined Regulatory Networks."
[267] Gengenbacher and Kaufmann, "Mycobacterium Tuberculosis: Success through Dormancy."
[268] Pollock and Neill, "Mycobacterium Bovis Infection and Tuberculosis in Cattle."
[269] Esmail et al., "The Ongoing Challenge of Latent Tuberculosis."

in mycobacterial infections in presence of T-cells. CMI can be detected by 8 weeks from contact with infected animals, allowing an early diagnosis of disease through DHT skin testing[270]. It is known that cattle can fail to react to TST during early stage of disease and also when develop advanced or generalised TB (anergy). Moreover, animals could not have any reaction to TST during latent phase of bTB[271]. It could occur that such animals, even though they showed positivity to TST, do not display any macroscopic lesion at post-mortem examination, allowing the laying off the meat according to Regulation (EU) 854/2004.

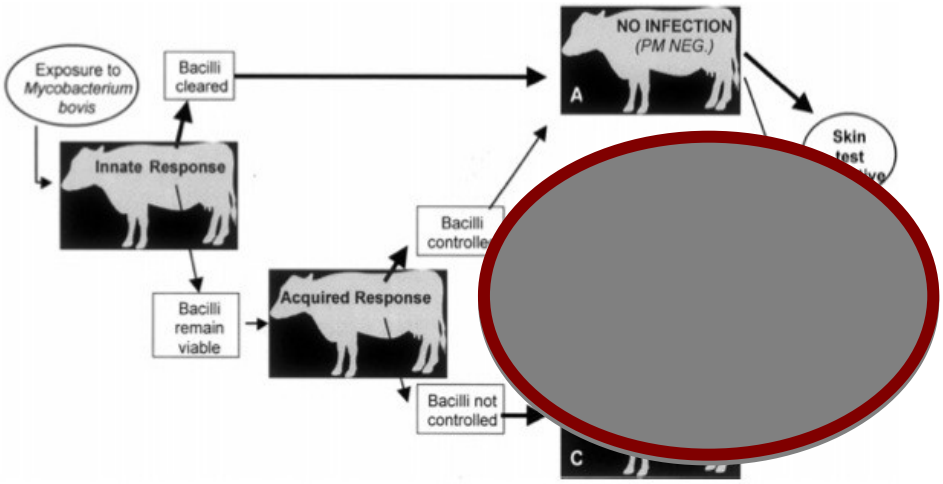

Fig. : Diagram representing the outcomes of bTB in infected cattle. Highlighted the latent phase. Both reactors and TST negative cattle can develop this stage. The thicker arrow indicates the most common pathway. PM POS/NEG represents the presence or not of macroscopic findings at post-mortem exam. Adapted from *Pollock and Neill,* 2002

In this study, 19,5% of samples showed tuberculous lesions at post-mortem examination (gross findings only). However, taken together macroscopic, microscopic and molecular data, positive samples reach 24,5%. This important gap of 5% highlight that the post-

[270] Pollock et al., "Immune Responses in Bovine Tuberculosis."
[271] Rua-Domenech et al., "Ante Mortem Diagnosis of Tuberculosis in Cattle: A Review of the Tuberculin Tests, c-Interferon Assay and Other Ancillary Diagnostic Techniques."

mortem examination based only on finding of macroscopic lesion is not sufficient to rule out the presence of microscopic lesions nor, even more important issue, the presence of mycobacteria.

Nevertheless, detection of mycobacteria, during particular phase of mycobacterial lifetime or during latency can hinder detection of mycobacteria, which can be difficult[272]. The low bacterial burden within lesions, often observed, is responsible of the characteristic paucibacillarity of the tuberculosis granulomas, and it can make the mycobacteria hard to find both in ZN and PCR. Latent tuberculosis is particularly studied in human TB, where it has been seen that people in latent phase of TB play a pivot role in shedding mycobacteria, causing a high epidemiological risk. In bTB could work similarly and latency would have the same pivotal role in TB epidemiology, in which reactivation of dormant bacteria within lesions allowing maintenance of bTB in endemic areas and its reappearance in areas considered free for this disease. It is interesting that, contrary of what happen for many human disease in which animal models can give many information on pathogenic mechanisms, the several studies on the pathology in humans, in particular about the latency, could make up for the lack of knowledge in bTB.

The major issue that affects the efficiency of any eradication program, including the bTB one, is to identify rapidly the infected animals. To do so, it is very important focusing the attention to improve the detection of mycobacteria, starting from improve knowledge in latent TB, human and bovine.

[272] Sakamoto, "The Pathology of Mycobacterium Tuberculosis Infection."

CONCLUSION CHAPTER

Since Robert Koch discovered *Mycobacterium tuberculosis*, the disease caused by these bacteria genres remains a great challenge for human and animal health all over the world, especially in undeveloped countries. This aspect represents not only a humanistic issue, but also it has severe repercussions for the control of disease in developed part of world. In fact, the global movement of people and animals are ever more increasing in actual contest of contemporary globalization, creating the basis that made tuberculosis one of the most important emerging infections worldwide.

In pathogenesis of tuberculosis, besides bacterial factors, the host response established immediately following infection plays a crucial role, and the progression of disease may depends on it. Further, above all, host immune response is important for the diagnosis of infection, in order to give a better treatment to patients (from human medicine perspective), or in order to improve eradication of disease (from veterinary medicine perspective). In addition, the outcome of disease derives from efficiency of host immune response. After infection, the established first type of immunity is called innate, and a proper growth, maturation and differentiation of immune cells involved in such response are necessary to activate adaptive immunity.

The host immune system condition dictates the rule in the delicate and complex relationship between innate and adaptive immune response, but some complications arise throughout tuberculosis pathogenesis. During the early phase of infection (primary infection), the obstacles created by host immune system to prevent mycobacterial growth generate an immune balance that leads infected animals to enter in a persistent stage of infection, the phase of latency. Throughout latent tuberculosis, mycobacteria "do not give up": they do not die, but nevertheless they apply different survival

strategies and enter into a particular phase of bacterial life cycle called dormancy. On one hand, these bacterial strategies, such as metabolic down-regulation, prevent the progression of disease; on the other hand, they make dormant bacterial very difficult to detect. The delicate balance between dormant bacteria and host immune response mat cause false negative at diagnostic screening tests, such a TST, resulting in a hard possibility to diagnose tuberculosis infection at latent phase.

In this thesis I choose to focus on two particular aspects in the contest of immunity during tuberculosis:

- The importance of a proper and efficient innate immune response at early stage of tuberculosis: IRF-8 deletion causes alterations in DCs activity and also in other cells, resulting in compromised adaptive immunity in KO mice
- Latency in bovine tuberculosis: how post-mortem examination failed in order to exclude the presence of mycobacteria within the carcass of infected animals

In the second chapter, IRF-$8^{-/-}$ mice develop inefficient acquired immunity on behalf of an enhanced unspecialized immunity, supported by neutrophil granulocytes. This kind of inflammatory exudate is completely inefficient to contrast mycobacterial growth and to prevent progression of disease. The result was large lesions with major damage that affect the organs, but also the premature death of sick animals. Moreover the acquired immunity observed in these KO animals, supported by lymphocytes, revealed to be poorly efficient, considering in a deficient recruitment of immune cells and in the low capability to form lymphoid specialized structures by these cells.

Subsequently, in the third chapter, I discussed the difficult diagnosis in the contest of bovine tuberculosis in order to eradication the disease. From sporadic outbreaks, it was investigated if mycobacteria were present in the carcass of slaughtered TST positive cattle. The post-mortem examination was insufficient to rule out the possibility to detect

mycobacteria into lymph nodes. The application of collateral diagnostic methods, such as histopathology and molecular analysis, has revealed how much underestimated is the possibility to find mycobacteria in carcass legally laid off for human consumption. At the same way, the importance of considering latency in application of bTB eradication programs remains still a challenging issue, whereas in human medicine many studies are already made for studying latent tuberculosis, for the purpose of an earlier diagnosis and a better patients care.

The use of experimental animal model of Mtb infection and the even more important economic repercussion of bovine tuberculosis give a great responsibility to Veterinary pathologists, which are largely involved in research focused on "problem-solving" of immunopathology of tuberculosis, still hard to understand.

Author's Declaration

I declare that this thesis is an original work.

The experimental work described in Chapter two was published in 2013 on PLoS ONE Journal, and it is the result of collaboration among Department of Veterinary Medicine (University of Sassari) with Department of Hemathology, Oncology, and Molecular Medicine (Istituto Superiore di Sanità, Rome, Italy) and Insitute of Microbiology (Catholic University of the Sacred Heart, Rome, Italy). Therefore, I declare that only experimental data resulting from histopathology and immunohistochemistry assays contained in this thesis are the result of my own work, carried out in the collaboration with Dr. Antonio Anfossi. The illustrations, captions and legend of illustrations that I used in chapter two are original images from the published paper indicated below.

Dr. Tiziana Cubeddu performed experimental data resulting from molecular analysis described in Chapter 3.

List of publication

Rocca S, Schiavoni G, Sali M, Anfossi AG, Abalsamo L, Palucci I, Mattei F, Sanchez M, ***Giagu A****, Antuofermo E, Fadda G, Belardelli, Delogu G, Gabriele L. (2013) Interferon Regulatory Factor 8-Deficiency Determines Massive Neutrophil Recruitment but T Cell Defect in Fast Growing Granulomas during Tuberculosis. PLoS ONE 8(5): e62751. doi:10.1371/journal.pone.0062751*

REFERENCES

Aleman M. "Neutrophil Apoptosis in the Context of Tuberculosis Infection." *Tuberculosis*, 2015, 1–5.

Aliberti J., O. Schulz, D.J. Pennington, H. Tsujimura, C. Reis e Sousa, K. Ozato, and A. Sher. "Essential Role for ICSBP in the in Vivo Development of Murine CD8+ Dendritic Cells." *BLOOD* 101, no. No. 1 (January 1, 2003): 305–10.

Alvarez A. H., C. Estrada-Chavez, and M.A. Flores-Valdez. "Molecular Findings and Approaches Spotlighting Mycobacterium Bovis Persistence in Cattle," 2009. doi:10.1051/vetres/2009005.

Becker, Amy M., Drew G. Michael, Ansuman T. Satpathy, Roger Sciammas, Harinder Singh, and Deepta Bhattacharya. "IRF-8 Extinguishes Neutrophil Production and Promotes Dendritic Cell Lineage Commitment in Both Myeloid and Lymphoid Mouse Progenitors." *BLOOD* 119, no. No. 9 (March 1, 2012).

Behr, M., E. Schurr, and P. Gros. "TB: Screening for Responses to a Vile Visitor." *Cell* 140 (March 5, 2010): 615–18. doi:10.1016/j.cell.2010.02.030.

Bekker, Linda-Gail, A. L. Moreira, A. Bergtold, S. Freeman, B. Ryffel, and G. Kaplan. "Immunopathologic Effects of Tumor Necrosis Factor Alpha in Murine Mycobacterial Infection Are Dose Dependent." *Infection and Immunity* 68, no. 12 (2000): 6954. doi:10.1128/IAI.68.12.6954-6961.2000.

Bellamy R.. "The Natural Resistance-Associated Macrophage Protein and Susceptibility to Intracellular Pathogens." *Microbes and Infection* 1 (1999): 23–27.

Berry, M.P.R., C.M. Graham, F.W. McNab, Z. Xu, S. A. A. Bloch, T. Oni, K.A. Wilkinson, et al. "An Interferon-Inducible Neutrophil-Driven Blood Transcriptional Signature in Human Tuberculosis." *Nature* 466, no. 19 (August 19, 2010): 973–79. doi:10.1038/nature09247.

Blanco, F.C., M.V. Bianco, V. Meikle, S. Garbaccio, L. Vagnoni, M. Forrellad, L.I. Klepp, A.A. Cataldi, and F. Bigi. "Increased IL-17 Expression Is Associated with Pathology in a Bovine Model of Tuberculosis." *Tuberculosis* 91 (2011): 57–63. doi:10.1016/j.tube.2010.11.007.

Blomgran, R., L. Desvignes, V. Briken, and J.D. Ernst. "Mycobacterium Tuberculosis Inhibits Neutrophil Apoptosis, Leading to Delayed Activation of Naive CD4 T Cells" 11, no. (1) (January 19, 2012): 81–90. doi:10.1016/j.chom.2011.11.012.

Bozzano, F., F. Marras, and A. De Maria. "Immunology of Tuberculosis" 6 (2014).

Briken, V, S.E. Ahlbrand, and S. Shah. “Mycobacterium Tuberculosis and the Host Cell Inflammasome: A Complex Relationship.” *Frontiers in Cellular and Infection Microbiology* 3, no. Article 62–1 (October 2013).

Brosch, R., S.V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, et al. “A New Evolutionary Scenario for the Mycobacterium Tuberculosis Complex.” *PNAS* 99, no. No. 6 (March 19, 2002): 3684–89.

Broughan, J.M., S.H. Downs, T.R. Crawshaw, P.A. Upton, J. Brewer, and R.S. Clifton-Hadley. “Mycobacterium Bovis Infections in Domesticated Non-Bovine Mammalian Species. Part 1: Review of Epidemiology and Laboratory Submissions in Great Britain 2004–2010.” *The Veterinary Journal* 198 (2013): 339–45.

Canaday, D.H., R.J. Wilkinson, Q.Li, Clifford V. Harding, R.F. Silver, and W. H.Boom. “CD4+ and CD8+ T Cells Kill Intracellular Mycobacterium Tuberculosis by a Perforin and Fas/Fas Ligand-Independent Mechanism.” *The Journal of Immunology* 167 (2001): 2734–42. doi:10.4049/jimmunol.167.5.2734.

Carragher, D.M., J. Rangel-Moreno, and T.D. Randall. “Ectopic Lymphoid Tissues and Local Immunity” 20, no. (1) (February 2008): 26–42.

Cassidy, J.P., D.G. Bryson, M.M. Gutiérrez Cancela, F. Forster, J.M. Pollock, and S.D. Neill. “Lymphocyte Subtypes in Experimentally Induced Early-Stage Bovine Tuberculous Lesions” 124 (2001): 46–51. doi:10.1053/jcpa.2000.0427.

Cassidy, J.P., D.G. Bryson, J.M. Pollock, R.T. Evans, F.Forster, and S.D. Neill. “Early Lesion Formation in Cattle Experimentally Infected with Mycobacterium Hovis” 119 (1998): 27–44.

Chen, C.Y., D.Huang, S. Yao, L. Halliday, G. Zeng, R.C. Wang, and Z.W. Chen. “IL-2 Simultaneously Expands Foxp3+ T Regulatory and T Effector Cells and Confers Resistance to Severe Tuberculosis (TB): Implicative Treg–T Effector Cooperation in Immunity to TB” 188, no. 9 (May 1, 2012): 4278–88. doi:10.4049/jimmunol.1101291.

Chistiakov D.A. “Myeloid Dendritic Cells: Development, Functions, and Role in Atherosclerotic Inflammation.” *Immunobiology*, 2015.

Ciaramella, A., A. Cavone, M.B. Santucci, M. Amicosante, A. Martino, G. Auricchio, L.P. Pucillo, V. Colizzi, and M. Fraziano. “Proinflammatory Cytokines in the Course of Mycobacterium tuberculosis–Induced Apoptosis in Monocytes/Macrophages.” *The Journal of Infectious Diseases* 186 (2002): 1277–82.

Cooper, A.M.. “Cell Mediated Immune Responses in Tuberculosis” 27 (2009): 393–422. doi:10.1146/annurev.immunol.021908.132703.

Cossu, A., L. A. Sechi, E. Bandino, S. Z., and V. Rosu. "Expression Profiling of Mycobacterium Tuberculosis H37Rv and Mycobacterium Smegmatis in Acid-Nitrosative Multi-Stress Displays Defined Regulatory Networks." *Microbial Pathogenesis* 65 (2013): 89–96.

Dannenberg, A.M. Jr. "Roles of Cytotoxic Delayed-Tipe Hypersensivity and Macrophage-Activating Cell-Mediated Immunity in the Pathogenesis of Tuberculosis" 191 (1994): 461–73.

Day, T.A., M. Koch, G. Nouailles, M. Jacobsen, G. A. Kosmiadi, D. Miekley, S. Kuhlmann, et al. "Secondary Lymphoid Organs Are Dispensable for the Development of T-Cell-Mediated Immunity during Tuberculosis." *European Journal of Immunology* 40 (2010): 1663–73.

Delahay, R.J., G.C. Smith, A.M. Barlow, N. Walker, A. Harris, R.S. Clifton-Hadley, and C.L. Cheeseman. "Bovine Tuberculosis Infection in Wild Mammals in the South-West Region of England: A Survey of Prevalence and a Semi-Quantitative Assessment of the Relative Risks to Cattle." *The Veterinary Journal* 173 (2007): 278–301.

Deretic, V., and R.A. Fratti. "Mycobacterium Tuberculosis Phagosome." *Molecular Microbiology* 31, no. Number 6 (1999): 1603–9.

Dinarello, C.A. "Proinflammatory Cytokines." *CHEST* 118, no. Number 2 (August 2000).

Dinarello, C.A., and G. Fantuzzi. "Interleukin-18 and Host Defense against Infection" 187, no. Suppl 2–S375 (2003).

Domingo, M., E. Vidal, and A. Marco. "Pathology of Bovine Tuberculosis." *Research in Veterinary Science*, 2014. doi:10.1016/ j.rvsc.2014.03.017.

Dorhoi, A., S.T. Reece, and S.H.E. Kaufmann. "For Better or for Worse: The Immune Response against Mycobacterium Tuberculosis Balances Pathology and Protection." *Immunological Reviews* 240 (2011): 235–51.

El Shikh M.E.M., and Costantino Pitzalis. "Follicular Dendritic Cells in Health and Disease." *Frontiers in Immunology* 3, no. Article 292 (September 2012). doi:10.3389/fimmu.2012.00292.

Esmail, H., C.E. Barry, D.B. Young, and R.J. Wilkinson. "The Ongoing Challenge of Latent Tuberculosis" 369, no. 20130437 (n.d.).

Flynn J.L., and J. Chan. "Immunology of Tuberculosis" 19 (2001): 93–129.

Flynn, J.L., J. Chan, and P.L. Lin. "Macrophages and Control of Granulomatous Inflammation in Tuberculosis." *Mucosal Immunology* 3, no. Number 3 (May 2011).

Foo, S.Y., and S. Phipps. “Regulation of Inducible BALT Formation and Contribution to Immunity and Pathology.” *Mucosal Immunology* 3, no. No.6 (November 2010): 537–44.

Fortin, A., L. Abel, J.L. Casanova, and P. Gros. “Host Genetics of Mycobacterial Diseases in Mice and Men: Forward Genetics Studies of BCG-Osis and Tuberculosis.” *The Annual Review of Genomics and Human Genetics* 8 (May 10, 2007): 163–92. doi:10.1146/annurev.genom.8.080706.092315.

Gabriele, L. and K. Ozato. “The Role of the Interferon Regulatory Factor (IRF) Family in Dendritic Cell Development and Function.” *Cytokine & Growth Factor Reviews* 18 (2007): 503–10. doi:10.1016/j.cytogfr.2007.06.008.

Gadol, N., J.E. Johnson III, and R.H. Waldman. “Respiratory Tract Cell-Mediated Immunity: Comparison of Primary and Secondary Response.” *Infection and Immunity* 9, no. 5 (1974): 858.

Geissmann, F., M.G. Manz, S.J., M.H. Sieweke, M. Merad, and K. Ley. “Development of Monocytes, Macrophages, and Dendritic Cells.” *Science* 327 (February 5, 2010).

Gengenbacher, M., and S.H.E. Kaufmann. “Mycobacterium Tuberculosis: Success through Dormancy” 36, no. (3) (May 2012): 514–32. doi:2012 May ; 36(310.1111/j.1574-6976.2012.00331.x.

Giese, N.A., L. Gabriele, T.M. Doherty, D.M. Klinman, L. Tadesse-Heath, Christina Contursi, Suzanne L. Epstein, and Herbert C. Morse III. “Interferon (IFN) Consensus Sequence-Binding Protein, a Transcription Factor of the IFN Regulatory Factor Family, Regulates Immune Responses In Vivo through Control of Interleukin 12 Expression.” *The Journal of Experimental Medicine* 186, no. No.9 (2006): 1535–46.

Gordon, S., and A. Plűddemann. “Tissue Macrophage Heterogeneity: Issues and Prospects” 35 (2013): 533–40. doi:DOI 10.1007/s00281-013-0386-4.

Gordon, S.V., K. Eiglmeier, RT. Garnier, RR. Brosch, RJ. Parkhill, SB. Barrell, S.S.T. Cole, and RR.G. Hewinson. “Genomics of Mycobacterium Bovis.” *Tuberculosis* 81, no. 1/2 (2001): 157–63. doi:10.1054/tube.2000.0269.

Gracie, A., S.E. Robertson, and I.B. McInnes. “Interleukin-18.” *Journal of Leukocyte Biology* 73 (February 2003).

Guilliams, M., B.N. Lambrecht, and H. Hammad. “Division of Labor between Lung Dendritic Cells and Macrophages in the Defense against Pulmonary Infections.” *Nature* 6, no. Number 3 (May 2013): 464–73. doi:doi:10.1038/mi.2013.14.

Guirado, E., and L.S. Schlesinger. “Modeling the Mycobacterium Tuberculosis Granuloma – the Critical Battlefield in Host Immunity and Disease” Vol. 4, no. Article 98 (April 2013).

Guirado, E., L.S. Schlesinger, and Gilla Kaplan. “Macrophages in Tuberculosis: Friend or Foe” 35 (2013): 563–83.

Gutierrez, M. C., S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply, and V. Vincent. “Ancient Origin and Gene Mosaicism of the Progenitor of Mycobacterium Tuberculosis.” *PloS Pathogens* 1, no. 1 (September 2005).

Haiyan S.L., and S.S. Watowich. “Diversification of Dendritic Cell Subsets, Emerging Roles for STAT Proteins,” December 2013.

Hambleton, S., S. Salem, J. Bustamante, V. Bigley, S. Boisson-Dupuis, J. Avezedo, A. Fortin, et al. “Mutations in IRF8 and Human Dendritic Cell Immunodeficiency” 365, no. 2 (July 14, 2011): 127–38.

Hamza, T., J.B. Barnett, and B. Li. “Interleukin 12 a Key Immunoregulatory Cytokine in Infection Applications” 11 (2010): 789–806. doi:doi:10.3390/ijms11030789.

Hernandez-Cuellar, E., K. Tsuchiya, H. Hara, R. Fang, S. Sakai, I. Kawamura, S. Akira, and M. Mitsuyama. “Cutting Edge: Nitric Oxide Inhibits the NLRP3 Inflammasome.” *The Journal of Immunology* 189 (2012): 000–000. doi:10.4049/jimmunol.1202479.

Hett, E.C., and E.J. Rubin. “Bacterial Growth and Cell Division: A Mycobacterial Perspective.” *Microbiology and Molecular Biology Reviews* 72, no. No. 1 (n.d.): 126–56. doi:10.1128/MMBR.00028-07.

Holtschke, T., J. Löhler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, et al. “Immunodeficiency and Chronic Myelogenous Leukemia-like Syndrome in Mice with a Targeted Mutation of the ICSBP Gene.” *Cell* 87 (October 18, 1996): 307–17.

Houben, E.N.G., L. Nguyen, and J. Pieters. “Interaction of Pathogenic Mycobacteria with the Host Immune System.” *Current Opinion in Microbiology* 9 (2006): 76–85.

Hume, D.A. “Macrophages as APC and the Dendritic Cell Myth.” *The Journal of Immunology* 181 (2008): 5829–35.

Hume, D.A.. “The Mononuclear Phagocyte System.” *Current Opinion in Immunology* 18 (2006): 49–53. doi:DOI 10.1016/j.coi.2005.11.008.

Hunter, R. L. “Pathology of Post Primary Tuberculosis of the Lung: An Illustrated Critical Review.” *Tuberculosis* 91 (2011): 497–509. doi:10.1016/j.tube.2011.03.007.

Hu, W., and C. Pasare “Location, Location, Location: Tissue-Specific Regulation of Immune Responses.” *Journal of Leukocyte Biology* Volume 94 (September 2013).

Huynh, K.K., S.A. Joshi, and E.J. Brown. "A Delicate Dance: Host Response to Mycobacteria." *Current Opinion in Immunology* 23 (2011): 464–72.

Jacobs, M., N. Brown, N. Allie, R. Gulert, and B. Ryffel. "Increased Resistance to Mycobacterial Infection in the Absence of Interleukin-10." *Immunology* 100 (2000): 494–501.

Johnson, L., G. Dean, S. Rhodes, G. Hewinson, M. Vordermeier, and A. Wangoo "Low-Dose Mycobacterium Bovis Infection in Cattle Results in Pathology Indistinguishable from that of High-Dose Infection." *Tuberculosis* 87 (2007): 71–76. doi:10.1016/j.tube.2006.04.002.

Kahnert, A., U.E. Höpken, M. Stein, S. Bandermann, M. Lipp, and S.H.E. Kaufmann. "Mycobacterium Tuberculosis Triggers Formation of Lymphoid Structure in Murine Lungs" 195 (January 1, 2007): 46–54.

Kashino, S.S., T. Vallerskog, G. Martens, J.L. Troudt, A. Keyser, J. Taylor, A. Izzo, H. Kornfeld, and A. Campos-Neto. "Initiation of Acquired Immunity in the Lungs of Mice Lacking Lymph Nodes after Infection with Aerosolized Mycobacterium Tuberculosis." *The American Journal of Pathology* 176, no. No.1 (n.d.): January 2010. doi:10.2353/ajpath.2010.090446.

Khader, S.A., and R. Gopal. "IL-17 in Protective Immunity to Intracellular Pathogens" 1:5 (n.d.): 423–26.

Korbel, D.S., B.E. Schneider, and E.U. Schaible. "Innate Immunity in Tuberculosis: Myths and Truth." *Microbes and Infection* 10 (2008): 995–1004.

Kozakiewicz, L., J. Phuah, J. Flynn, and J. Chan. "The Role of B Cells and Humoral Immunity in Mycobacterium Tuberculosis Infection." *Advances in Experimental Medicine and Biology* 783 (2013): 225–50. doi:10.1007/978-1-4614-6111-1_12.

Laskin D.L., B. Weinberger, and J.D. Laskin. "Functional Heterogeneity in Liver and Lung Macrophages." *Journal of Leukocyte Biology* 70 (August 2001).

Liebana E., A. Aranaz, F.E. Aldwell, J. McNair, S.D. Neill, A.J. Smyth, and J.M. Pollock. "Cellular Interactions in Bovine Tuberculosis: Release of Active Mycobacteria from Infected Macrophages by Antigen-Stimulated T Cells." *Immunology* 99 (2000): 23–29.

Lowe, D.M., P.S. Redford, R.J. Wilkinson, A. O'Garra, and A.R. Martineau. "Neutrophils in Tuberculosis: Friend or Foe?" *Trends in Immunology* 33, no. Number 1 (January 2012).

Lund, F.E., and T.D. Randall. "Effector and Regulatory B Cells: Modulators of CD4+ T Cell Immunity." *Nature Reviews | Immunology* 10 (March 12, 2010): 236–47. doi:10.1038/nri2729.

Lu, R.. "Interferon Regulatory Factor 4 and 8 in B-Cell Development." *Trends in Immunology* 29, no. No.10 (n.d.): 487–92.

Luther, S.A., A. Bidgol, D.C. Hargreaves, A. Schmidt, Y. Xu, J. Paniyadi, M. Matloubian, and J.G. Cyster. "Differing Activities of Homeostatic Chemokines CCL19, CCL21, and CXCL12 in Lymphocyte and Dendritic Cell Recruitment and Lymphoid Neogenesis." *The Journal of Immunology* 169 (2002): 424–33. doi:10.4049/jimmunol.169.1.424.

Lyakh, L., G. Trinchieri, L. Provezza, G. Carra, and F. Gerosa. "Regulation of Interleukin-12/interleukin-23 Production and the T- Helper 17 Response in Humans" 226 (December 2008): 112–31.

Maglione, P.J., J. Xu, and J. Chan. "B Cells Moderate Inflammatory Progression and Enhance Bacterial Containment upon Pulmonary Challenge with Mycobacterium Tuberculosis." *The Journal of Immunology* 178 (2007): 7222–34.

Mamane, Y., C. Heylbroeck, P. Génin, M. Algarté, M.J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin, and J. Hiscott. "Interferon Regulatory Factors: The next Generation." *Gene* 237 (1999): 1–14.

Manabe, Y.C., and W.R. Bishai. "Latent Mycobacterium Tuberculosis– Persistence, Patience, and Winning by Waiting." *Nature Medicine* 6, no. No. 12 (December 2000).

Mantovani, A., M.A. Cassatella, C. Costantini, and S. Jaillon. "Neutrophils in the Activation and Regulation of Innate and Adaptive Immunity." *Nature Reviews | Immunology* 11 (August 2011): 519–31. doi:10.1038/nri3024.

Marquis, J.F., R. LaCourse, L. Ryan, R.J. North, and P. Gros. "Disseminated and Rapidly Fatal Tuberculosis in Mice Bearing a Defective Allele at IFN Regulatory Factor 8." *The Journal of Immunology* 189 (2009): 3008–15.

Master, S. S., S.K. Rampini, A.S. Davis, C. Keller, S. Ehlers, B. Springer, G.S. Timmins, P. Sander, and V. Deretic. "Mycobacterium Tuberculosis Prevents Inflammasome Activation." *Cell Host & Microbe* 3 (April 2008): 224–32. doi:10.1016/j.chom.2008.03.003.

Mattei, F., G. Schiavoni, P. Borghi, M. Venditti, I. Canini, P. Sestili, I. Pietraforte, et al. "ICSBP/IRF-8 Differentially Regulates Antigen Uptake during Dendritic-Cell Development and Affects Antigen Presentation to CD4 T Cells." *BLOOD* 108, no. Number 2 (March 28, 2006): 609–17. doi:10.1182/blood-2005-11-4490.

McNair, J., M.D. Welsh, and J.M. Pollock. "The Immunology of Bovine Tuberculosis and Progression toward Improved Disease Control Strategies." *Vaccine* 25 (2007): 5504–11. doi:10.1016/j.vaccine.2007.02.037.

Menzies, F.D., and S.D. Neill. "Cattle-to-Cattle Transmission of Bovine Tuberculosis." *The Veterinary Journal* 160 (2000): 92–106. doi:10.1053/tvjl.2000.0482.

Mitchison, N.A. "T-cell–B-Cell Cooperation." *Nature Reviews | Immunology* 4, no. 308–312 (April 2004).

Moore, Amanda J., and Michele K. Anderson. "Dendritic Cell Development: A Choose-Your-Own-Adventure Story." *Advances in Hematology* 2013, no. Article ID 949513 (2013): 16 pages.

Mortaz E., I.M. Adcock, P. Tabarsi, M. Reza Masjedi, D. Mansouri, A.A. Velayati, and J.L. Casanova. "Interaction of Pattern Recognition Receptors with Mycobacterium Tuberculosis" 35 (2015): 1–10.

Müller, B., S. Dürr, S. Alonso, J. Hattendorf, C.J.M. Laisse, S.D.C. Parsons, P.D. van Helden, and Jakob Zinsstag. "Zoonotic Mycobacterium Bovis– Induced Tuberculosis in Humans." *Emerging Infectious Diseases* 19, no. 6 (June 2013). doi:http://dx.doi.org/10.3201/eid1906.120543.

Munder, M., H. Schneider, C. Luckner, T. Giese, C.D. Langhans, J.M. Fuentes, P. Kropf, et al. "Suppression of T-Cell Functions by Human Granulocyte Arginase." *BLOOD* 108, no. No.5 (September 1, 2006): 1627–34.

Neill, S.D., D.G. Bryson, and J.M. Pollock. "Pathogenesis of Tuberculosis in Cattle." *Tuberculosis* 81, no. 1/2 (2001): 79–86. doi:10.1054/tube.2000.0279.

Nguyen, H., J. Hiscott, and P. M. Pitha. "The Growing Family of Interferon Regulatory Factors." *Cytokine & Growth Factor Reviews* 8, no. No.4 (1997): 293–312.

North R.J., R. Lacourse, L. Ryan, and P. Gros. "Consequence of Nramp1 Deletion to Mycobacterium Tuberculosis Infection in Mice." *Infection and Immunity* 67, no. No. 11 (November 1999): 5811–14.

Okoye, I.S., and M.S. Wilson. "CD4+ T Helper 2 Cells – Microbial Triggers, Differentiation Requirements and Effector Functions." *Immunology* 134 (2011): 368–77.

O'Reilly, L.M., and C.J. Dabornt. "The Epidemiology of Mycobacterium Bovis Infections in Animals and Man: A Review." *Tubercle and Lung Disease* 76, no. Supplement 1 (1995): 1–46.

Palmer, M.V., W.R. Waters, and T.C. Thacker. "Lesion Development and Immunohistochemical Changes in Granulomas from Cattle Experimentally Infected with Mycobacterium Bovis" 44 (2007): 863. doi:10.1354/vp.44-6-863.

Parameswaran, N., and S. Patial. "Tumor Necrosis Factor-A Signaling in Macrophages" 20, no. (2) (March 2010): 87–103.

Parrish, N.M., J.D. Dick, and W.R. Bishai. “Mechanisms of Latency in Mycobacterium Tuberculosis.” *Trends in Immunology* 6, no. No. 3 (March 1998): 107–12.

Perié, L., and S.H. Naik. “Toward Defining a ‘lineage’ – The Case for Dendritic Cells,” 2015. http://dx.doi.org/10.1016/j.semcdb.2015.02.004.

Plantinga, M., H. Hammad, and B.N. Lambrecht. “Origin and Functional Specializations of DC Subsets in the Lung” 40 (2010): 2085–2130.

Pollock, J.M., J. McNair, M.D. Welsh, R.M. Girvin, H.E. Kennedy, D.P. Mackie, and S.D. Neill. “Immune Responses in Bovine Tuberculosis.” *Tuberculosis* 8, no. 1/2 (2001): 103–7.

Pollock, J.M., and S.D. Neill. “Mycobacterium Bovis Infection and Tuberculosis in Cattle.” *The Veterinary Journal* 163 (2002): 115–27. doi:10.1053/tvjl.2001.0655.

Pollock, J.M., J.D. Rodgers, M.D. Welsh, and J. McNair. “Pathogenesis of Bovine Tuberculosis: The Role of Experimental Models of Infection.” *Veterinary Microbiology* 112 (2006): 141–50. doi:10.1016/j.vetmic.2005.11.032.

Pollock, J.M., M.D. Welsh, and J. McNair. “Immune Responses in Bovine Tuberculosis: Towards New Strategies for the Diagnosis and Control of Disease.” *Veterinary Immunology and Immunopathology* 108 (2005): 37–43.

Pozzi L.M., J.W. Maciaszek, and K.L. Rock. “Both Dendritic Cells and Macrophages Can Stimulate Naive CD8 T Cells In Vivo to Proliferate, Develop Effector Function, and Differentiate into Memory Cells.” *The Journal of Immunology*, 2005.

Prezzemolo, T., G. Guggino, M.P. La Manna, D. Di Liberto, F. Dieli, and N. Caccamo. “Functional Signatures of Human CD4 and CD8T Cell Responses to Mycobacterium Tuberculosis.” *Frontiers in Immunology* 5, no. Article 180 (April 22, 2014).

Rajarama, M.V.S., B. Nia, C.E. Dodda, and L.S. Schlesingera. “Macrophage Immunoregulatory Pathways in Tuberculosis.” *Seminars in Immunology* 26 (2014): 471–85.

Ramakrishan L.. “Revisiting the Role of the Granuloma in Tuberculosis.” *Nature Reviews | Immunology* 12 (May 2012): 352–66. doi:10.1038/nri3211.

Redford, P.S., P.J. Murray, and A. O’Garra. “The Role of IL-10 in Immune Regulation during M. Tuberculosis Infection.” *Mucosal Immunology* 4, no. Number 3 (May 2011).

Reviriego Gordejo F.J., and J.P. Vermeersch. “Towards Eradication of Bovine Tuberculosis in the European Union.” *Veterinary Microbiology* 112 (2006): 101–9. doi:10.1016/j.vetmic.2005.11.034.

Roach, D.R., A.G.D. Bean, C. Demangel, M.P. France, H. Briscoe, and W.J. Britton. "TNF Regulates Chemokine Induction Essential for Cell Recruitment, Granuloma Formation, and Clearance of Mycobacterial Infection." *The Journal of Immunology* 168 (2002): 4620–27. doi:10.4049/jimmunol.168.9.4620.

Robinson, R.T., I.M. Orme, and A.M. Cooper. "The Onset of Adaptive Immunity in the Mouse Model of Tuberculosis and the Factors That Compromise Its Expression." *Immunological Reviews* 264 (2015): 46–59.

Rodríguez-Pinto, D.. "B Cells as Antigen Presenting Cells." *Cellular Immunology* 238 (2005): 67–75. doi:10.1016/j.cellimm.2006.02.005.

Rodríguez-Pinto D., and J. Moreno. "B Cells Can Prime Naive CD4+ T Cells in Vivo in the Absence of Other Professional Antigen-Presenting Cells in a CD154-CD40-Dependent Manner." *European Journal of Immunology* 35 (2005): 1097–1105. doi:10.1002/eji.200425732.

Rua-Domenech, R. de la, A.T. Goodchild, H.M. Vordermeier, R.G. Hewinson, K.H. Christiansen, and R.S. Clifton-Hadley. "Ante Mortem Diagnosis of Tuberculosis in Cattle: A Review of the Tuberculin Tests, c-Interferon Assay and Other Ancillary Diagnostic Techniques." *Research in Veterinary Science* 81, no. 190–210 (2006).

Russell, D.G. "Mycobacterium tuberculosis: dere today, and here tomorrow." *Nature Reviews | Molecular Cell Biology* 2 (August 2001).

Sakamoto, K. "The Pathology of Mycobacterium Tuberculosis Infection." *Veterinary Pathology* 49, no. (3) (January 18, 2012): 423–39. doi:10.1177/0300985811429313.

Saraiva, M., and A. O'Garra. "The Regulation of IL-10 Production by Immune Cells." *Nature Reviews | Immunology* 10 (March 2010): 170–81. doi:10.1038/nri2711.

Schiavoni, G., F. Mattei, P. Borghi, P. Sestili, M. Venditti, H.C. Morse III, F. Belardelli, and L. Gabriele. "ICSBP Is Critically Involved in the Normal Development and Trafficking of Langerhans Cells and Dermal Dendritic Cells." *BLOOD Immunobiology* 103, no. No. 6 (March 15, 2004): 2221–28.

Schiavoni, G., F. Mattei, P. Sestili, P. Borghi, M. Venditti, H.C. Morse III, F. Belardelli, and L. Gabriele. "ICSBP Is Essential for the Development of Mouse Type I Interferon-Producing Cells and for the Generation and Activation of CD8α+ Dendritic Cells." *The Journal of Experimental Medicine* 196, no. No.11 (December 2, 2002): 1415–25.

Schiller, I., B. Oesch, H.M. Vordermeier, M.V. Palmer, B.N. Harris, K.A. Orloski, B.M. Buddle, T.C. Thacker, K.P. Lyashchenko, and W.R. Waters. "Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of Their Relevance for Disease Control and Eradication." *Transboundary*

and Emerging Diseases. 57 (2010): 205–20. doi:10.1111/j.1865-1682.2010.01148.x.

Schiller, I., H.M. Vordermeier, W.R. Waters, A.O. Whelan, M. Coad, E. Gormley, B.M. Buddle, et al. "Bovine Tuberculosis: Effect of the Tuberculin Skin Test on in Vitro Interferon Gamma Responses." *Veterinary Immunology and Immunopathology* 136 (2010): 1–11. doi:10.1016/j.vetimm.2010.02.007.

Schlitzer, A., and F. Ginhoux. "Organization of the Mouse and Human DC Network." *Current Opinion in Immunology* 26 (2014): 90–99.

Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kiedorf, M. Prinz, et al. "A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells." *Science* 336 (April 6, 2012).

Sharma, S., and M. Bos. "Role of Cytokines in Immune Response to Pulmonary Tuberculosis." *ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY* 19 (2001): 213–19.

Shortman, K., and W.R. Heath. "The CD8+ Dendritic Cell Subset." *Immunological Reviews* 234 (2010): 18–31.

Slight, S.R., and S.A. Khader. "Chemokines Shape the Immune Responses to Tuberculosis." *Cytokine Growth Factor Rev,* 24, no. 2 (April 2013): 105–13. doi:10.1016/j.cytogfr.2012.10.002.

Smyth, A.J., M.D. Welsh, R.M. Girvin, and J.M. Pollock. "In Vitro Responsiveness of γδT Cells from Mycobacterium Bovis-Infected Cattle to Mycobacterial Antigens: Predominant Involvement of WC1+ Cells." *Infection and Immunity* 69, no. No. 1 (January 2001): 89–96. doi:10.1128/IAI.69.1.89–96.2001.

Tailor, P., T. Tamura, H.J. Kong, T. Kubota, M. Kubota, P. Borghi, L. Gabriele, and K. Ozato. "Type I Interferon Induction in Dendritic Cells Requires IRF-8 That Effects the Feedback Phase of Transcription." *Immunity* 27, no. 2 (August 27): 228–39. doi:10.1016/j.immuni.2007.06.009.

Takeuchi, S., and M. Furue. "Dendritic Cells—Ontogeny" Vol 56, no. No3 (2007).

Tamura, T., D. Kurotaki, and S. Koizumi. "Regulation of Myelopoiesis by the Transcription Factor IRF8." *Progress in Hematology*, March 7, 2015. doi:10.1007/s12185-015-1761-9.

Torrado, E., and A.M. Cooper. "IL-17 and Th17 Cells in Tuberculosis." *Cytokine & Growth Factor Reviews* 21 (2010): 455–62.

Trinchieri G.. "Interleukin-12: A Proinflammatory Cytokine with Immunoregulatory Functions That Bridge Innate Resistance and Antigen-Specific Adaptive Immunity," 1995, 251–76.

Tsai, M.C., S. Chakravarty, G. Zhu, J. Xu, K. Tanaka, C. Koch, J. Tufariello, J. Flynn, and J. Chan. "Characterization of the Tuberculous Granuloma in Murine and Human Lungs: Cellular Composition and Relative Tissue Oxygen Tension." *Cellular Microbiology* 8, no. (2) (September 16, 2006): 218–32. doi:10.1111/j.1462-5822.2005.00612.x.

Tsujimura, H., T. Tamura, and K. Ozato. "Cutting Edge: IFN Consensus Sequence Binding Protein/IFN Regulatory Factor 8 Drives the Development of Type I IFN-Producing Plasmacytoid Dendritic Cells." *The Journal of Immunology* 170 (2003): 1131–35. doi:10.4049/jimmunol.170.3.1131.

Turcotte, K., S. Gauthier, D. Malo, M. Tam, M.M. Stevenson, and P. Gros. "Icsbp1/IRF-8 Is Required for Innate and Adaptive Immune Responses against Intracellular Pathogens." *The Journal of Immunology* 179 (2007): 2467–76. doi:10.4049/jimmunol.179.4.2467.

Ulrichs, T., G.A. Kosmiadi, V. Trusov, S. Jörg, L. Pradl, M. Titukhina, V. Mishenko, N. Gushina, and S.H.E. Kaufmann. "Human Tuberculous Granulomas Induce Peripheral Lymphoid Follicle-like Structures to Orchestrate Local Host Defence in the Lung." *The Journal of Pathology* 204 (2004): 217–28. doi:10.1002/path.1628.

Underhill, D.M., A. Ozinsky, K.D. Smith, and A. Aderem. "Toll-like Receptor-2 Mediates Mycobacteria-Induced Proinflammatory Signaling in Macrophages" vol. 96, no. no. 25 (December 7, 1999): 14459–63.

Urdahl, K.B., S. Shafiani, and J.D. Ernst. "Initiation and Regulation of T-Cell Responses in Tuberculosis." *Mucosal Immunology* 4, no. Number 3 (May 2011).

Van Crevel R., T.H.M. Ottenhoff, and Jos W. M. van der Meer. "Innate Immunity to Mycobacterium Tuberculosis" 15, no. Number 2 (2002): :294. doi:10.1128/CMR.15.2.294-309.2002.

Van Rhijn I., J. Godfroid, A. Michel, and V. Rutten. "Bovine Tuberculosis as a Model for Human Tuberculosis: Advantages over Small Animal Models." *Microbes and Infection* 10 (2008): 711–15. doi:10.1016/j.micinf.2008.04.005.

Villarreal-Ramos, B., M. McAulay, V. Chance, M. Martin, J. Morgan, and C.J. Howard. "Investigation of the Role of CD8+ T Cells in Bovine Tuberculosis In Vivo." *Infection and Immunity* 71, no. No. 8 (August 2003): 4297–2303. doi:10.1128/IAI.71.8.4297–4303.2003.

Vordermeier, H.M., M.A. Chambers, P.J. Cockle, A.O. Whelan, J. Simmons, and R.G. Hewinson. "Correlation of ESAT-6-Specific Gamma Interferon Production with Pathology in Cattle Following Mycobacterium Bovis BCG Vaccination against Experimental Bovine Tuberculosis." *Infection and Immunity* 70, no. No. 6 (June 2002): 3026–32. doi:10.1128/IAI.70.6.3026–3032.2002.

Vurmanovic-Stejic, M., M.J. Thomas, A. Noble, and D.M. Kemeny. "Specificity, Restriction and Effector Mechanisms of Immunoregulatory CD8 T Cells." *Immunology* 102 (2001): 115–22.

Wang, H., and H.C. Morse III. "IRF8 Regulates Myeloid and B Lymphoid Lineage Diversifcation" 43 (2009): 109–17. doi:10.1007/s12026-008-8055-8.

Wangoo, A., L. Johnson, J. Gough, R. Ackbar, S. Inglut, D. Hicks, Y. Spencer, G. Hewinson, and M. Vordermeier. "Advanced Granulomatous Lesions in Mycobacterium Bovis-Infected Cattle Are Associated with Increased Expression of Type I Procollagen, Gd (WC1+) T Cells and CD 68+ Cells" 133 (2005): 223–34.

Winslow, G.M., A. Cooper, W. Reiley, M. Chatterjee, and D.L. Woodland. "Early T-Cell Responses in Tuberculosis Immunity." *Immunological Reviews* 225 (2008): 284–99.

Witchell, J., S.V.P.K. Maddipatla, A. Wangoo, M. Vordermeierc, and M. Goya. "Time Dependent Expression of Cytokines in Mycobacterium Bovis Infected Cattle Lymph Nodes." *Veterinary Immunology and Immunopathology* 138 (2010): 79–84. doi:10.1016/j.vetimm.2010.07.004.

Wolf, A.J., L. Desvignes, B. Linas, N. Banaiee, T. Tamura, K. Takatsu, and J.D. Ernst. "Initiation of the Adaptive Immune Response to Mycobacterium Tuberculosis Depends on Antigen Production in the Local Lymph Node, Not the Lungs." *The Journal of Experimental Medicine* 205, no. No.1 (January 21, 2008): 105–15.

Yoshikai, Y. "Immunological Protection Against Mycobacterium Tuberculosis Infection." *Critical Reviews in Immunology* 26, no. (6) (2006): 515–26.

Zhou, X., Q. Dai, and X. Huang. "Neutrophils in Acute Lung Injury." *Frontiers in Bioscience* 17 (June 1, 2012): 2278–83.

Zhu, J., and W.E. Paul. "CD4 T Cells: Fates, Functions, and Faults." *BLOOD* 112, no. Number 5 (September 2008).