MUCOSAL IMMUNITY IN THE RESPIRATORY TRACT: 
THE ROLE OF IgA IN PROTECTION AGAINST 
INTRACELLULAR PATHOGENS 

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SUMMARY

The lungs and upper airways are mucosal surfaces that are common sites for infection with an enormous variety of inhaled pathogens. Therefore, induction of immune responses in the respiratory tract is crucial for protection against respiratory diseases.

One of the pathogens infecting the host via the respiratory tract is *Mycobacterium Tuberculosis*. The reported efficacy of the currently used Bacillus Calmette-Guérin (BCG) vaccine against tuberculosis is highly variable, ranging from 50% against pulmonary tuberculosis to 80% against disseminated tuberculosis. Recently, the current route of vaccination (intradermal) has been considered as a possible factor influencing the protective capacity of the BCG vaccine. In this regard, intradermal route most likely induces protective systemic responses while it fails to induce optimal responses in the lungs. Therefore, our working hypothesis is that vaccination should be directed towards the respiratory mucosal immunity in order to improve the degree of host protection in the lungs.

In this thesis we studied the effect of the route of immunization as well as of different mucosal adjuvants on the induction of mucosal immune responses against the mycobacterial surface antigen PstS-1. We found that, the intranasal (i.n.) route of immunization was a more favorable route inducing strong local immune responses, than intraperitoneal (i.p.) route. Indeed, i.n. route immunization, unlike the i.p. route, elicited strong IgA responses in the lungs accompanied by a major influx of CD4+ T cells and a significant local production of IFN-γ.

IgA, being the predominant Ig isotype at mucosal tissues, is considered a major effector molecule involved in defense mechanisms against viral and bacterial pathogens at these sites. Therefore, we investigated the possible role of IgA in the protection of the respiratory mucosa against mycobacterial infections, using mice deficient in IgA and in the polymeric Ig receptor. We show that, deficient mice are more susceptible to mycobacterial infections than wild type mice, thereby demonstrating a role for IgA in protection against mycobacteria. Importantly, our studies revealed a reduced production of protective factors, such as INF-γ and TNF-α, in the lungs of deficient mice that was associated with the higher susceptibility seen in these mice compared to wild-type mice. We also conducted challenge experiments against another respiratory pathogen, *Chlamydia pneumoniae*, using IgA deficient mice. Likewise to mycobacteria, our data support a role for IgA in the protection of the respiratory tract against *C. pneumoniae* infection.

Finally, we investigated the possible mechanisms explaining the reduced pro-inflammatory responses in IgA deficient mice. Our data indicated that IgA deficient mice present a defective response to stimulation with LPS or 19kDa which appears to be both, essentially due to suboptimal stimulation of macrophages and restricted to the lungs.
The Paradox of Life:
A bit beyond perception's reach
I sometimes believe I see
that Life is two locked boxes, each
containing the other's key.

Piet Hein, Danish mathematician,
physicist, philosopher.
This thesis is based on the following papers, which are referred to in the text by their corresponding roman numerals:


* These authors contributed equally
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<td>Ag85</td>
<td>Antigen 85 complex</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>APCs</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>M. bovis BCG</td>
<td>Mycobacterium bovis Bacillus Calmette-Guérin</td>
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<tr>
<td>CT</td>
<td>Cholera toxin</td>
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<tr>
<td>CD8-/-Igh6-</td>
<td>CD8+ T cell / B cell deficient</td>
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<tr>
<td>detPT</td>
<td>Non-toxic pertussis toxin</td>
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<tr>
<td>EB</td>
<td>Elementary body</td>
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<tr>
<td>FeR</td>
<td>Fc-receptor</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>HIV</td>
<td>Human Immunodeficiency virus</td>
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<td>HSP</td>
<td>Heat-Shock protein</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgA-</td>
<td>IgA deficient</td>
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<td>IgM-</td>
<td>B cell deficient</td>
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<tr>
<td>i.n.</td>
<td>Intranasal</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthetase</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MALT</td>
<td>Mucosal-associated lymphoid tissue</td>
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<td>M-cell</td>
<td>Microfold-cell</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MOMP</td>
<td>Major outer membrane protein</td>
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<tr>
<td>NALT</td>
<td>Nasal-associated lymphoid tissue</td>
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<td>pIgA</td>
<td>Polymeric immunoglobulin A</td>
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<td>pIgR</td>
<td>Polymeric immunoglobulin receptor</td>
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<td>pIgR-</td>
<td>pIgR deficient</td>
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<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
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<tr>
<td>RB</td>
<td>Reticulate body</td>
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<tr>
<td>RU 41.740</td>
<td>Purified glycoprotein extract from Klebsiella pneumoniae</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<td>slgA</td>
<td>Secretory immunoglobulin A</td>
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<td>slgM</td>
<td>Secretory immunoglobulin M</td>
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<tr>
<td>TAP</td>
<td>Transporters associated with antigen processing</td>
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<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>Th</td>
<td>Helper T cell</td>
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INTRODUCTION

THE IMMUNE SYSTEM

The immune system is able to generate an enormous variety of cells and molecules capable of recognizing and eliminating a large range of microorganisms (viruses, bacteria and parasites) and other potentially dangerous agents.

The immune response has been historically divided in two parts, one is phylogenetically the oldest and is called innate immunity, and the other is called adaptive or acquired immunity. The major difference between them is that the innate immunity involves a set of resistance mechanisms, such as phagocytosis, that is not specific to a particular pathogen, while the adaptive immunity displays a high degree of specificity as well as the remarkable property of “memory”. Normally, there is an adaptive immune response to an antigen within five to six days following the initial exposure to that antigen. In a second exposure to the same antigen the immune system exhibits “immunologic memory” by means of inducing an immune response that is quicker and stronger than the first, and often more effective in neutralizing and clearing the pathogen. Also, because of this property of the adaptive immunity, the immune system can confer life-long immunity to many infectious agents after initial encounter. Despite of these differences, the innate and adaptive immune responses are connected and interact with each other, and both are needed for an efficient immune protection.

The cells of the immune system that are responsible for the reaction and release of soluble molecules are: lymphocytes, such as B lymphocytes and T lymphocytes; phagocytic cells, such as dendritic cells, macrophages, neutrophils and eosinophils; and auxiliary cells, such as basophils and mast cells. The molecules released by these cells are: antibodies, cytokines (interleukins and interferons), chemokines, complement and different inflammatory mediators.

MUCOSAL IMMUNITY IN THE RESPIRATORY TRACT

Mucosal surfaces comprising the respiratory tract, the gastrointestinal tract, and the urogenital tract, represent the most important portal of entry for pathogens, especially
bacteria and viruses. Pathogens may either replicate and promote disease at the initial mucosal site or invade neighboring tissues and the blood stream, inducing disease at distant systemic localities (Kaul and Ogra, 1998).

During respiration, the airways are exposed to continuous challenge by an enormous load of airborne microorganisms and environmental antigens. Therefore, airway mucosal surfaces must employ robust non-specific as well as specific mechanisms to be protected from respiratory tract infections.

**Innate immunity**

The innate defenses of the airways are complex, consisting of several physical, cellular and antimicrobial components. Mechanical defenses prevent particulate antigens and microorganisms from entering the lungs. These mechanisms begin at the nose, which functions as a filter by capturing or trapping large particles in the nasal hair or fimbriae. The smaller particles that pass this filter are then inhaled and deposited in the lower airways, where mucins of the mucociliary blanket lining the airways surface act by trapping and removing them through ciliary movements (Rastogi *et al*., 2001). The particles or microorganisms that pass this barrier get then in contact with a range of soluble mediators present in the mucus, such as lysozyme, lactoferrin, collectin and defensins, produced by cells of the respiratory tract. The production of these molecules can lead directly to lysis of pathogens, or to destruction through opsonisation or the recruitment of inflammatory cells (Boyton and Openshaw, 2002). Additionally, another important mechanism of defense is the ingestion of microorganisms by phagocytic cells like macrophages and dendritic cells. Indeed, the phagocytic and microbicidal activities of these cells are essential for maintaining the lungs in a clean and sterile state.

**Adaptive immunity**

The immune system in the upper and lower respiratory tract consists of (Davis, 2001):

1- an epithelial compartment which includes the epithelial cells and underlying connective tissue containing the immunocompetent cells;
2- a mucosal-associated lymphoid tissue (MALT) involving the nose-associated lymphoid tissue (NALT), larynx-associated lymphoid tissue (LALT), and the bronchus-associated lymphoid tissue (BALT);

3- lymph nodes draining the respiratory tract.

The mucosal immune system can principally be divided into inductive sites, which are constituted by the MALT and where antigens sampled from mucosal surfaces are presented to naïve B- and T- lymphocytes, thereby initiating the immune response; and effector sites, where primed (memory-effector) lymphocytes after extravasation and differentiation exert their effector functions (Brandtzaeg and Pabst, 2004; Kiyono and Fukuyama, 2004) (Fig. 1).

The initiation of the antigen-specific immune responses occurs at special “gateways” which comprise microfold-cells (M cells) located in the epithelium overlaying the follicles of the MALT. These follicles contain all immunocompetent cells, such as B cells, T cells and antigen-presenting cells (APCs) that are required for the generation of an immune response (Brandtzaeg and Pabst, 2004).

The M cells are specialized in the luminal uptake and transport of antigens. Upon antigen transport from the luminal site, antigen presentation is required for the activation of T cells. APCs in the lungs are represented by submucosal and interstitial dendritic cells and alveolar macrophages. Alveolar macrophages constitute 85% of the cells in the alveoli, whereas dendritic cells account for not more than 1% of the cells in this lung compartment. In normal individuals, alveolar macrophages have been reported to be poor APCs compared to dendritic cells. Since alveolar macrophages are the most abundant cells at the alveoli, this property may protect the airways from undue inflammation under normal conditions. However, when encountering foreign particles or organisms, alveolar macrophages may influence the degree of activity or maturation of dendritic cells by releasing cytokines (Nicod et al., 2000). Dendritic cells localized at the epithelial border are believed to be the most potent APCs, promoting the development of naïve T cells and have been strongly associated with the initiation and potentiation of the immune response (Ogra, 2003). Dendritic cells capture the antigen, migrate to local draining lymphoid organs and after a process of maturation, select antigen-specific lymphocytes to which they
present the processed antigen, thereby initiating the adaptive immune responses (Fig. 1) (Banchereau and Steinman, 1998; Banchereau et al., 2000).

After being primed to become memory-effector cells, B- and T- lymphocytes migrate from the MALT and regional lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites. This process is directed by the local profile of vascular adhesion molecules and chemokines, especially the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Brandtzaeg and Pabst, 2004).

Figure 1. The mucosal immune system at the respiratory tract (Modified from Nature Reviews 2004).

Among the T cells participating in the immune responses are CD4⁺, CD8⁺ and γδ⁺ T cells. The antigen-specific T cells are key effectors of immune functions, through the lysis of
infected cells or through the secretion of helper T 1 (Th1) or Th2 type of cytokines. The different ratios or polarization of these cytokines have the ability to modulate the immune response to infection. Additionally, activated CD4⁺ T cells help B cells to develop into immunoglobulin (Ig) A plasma cells (McGhee and Kiyono, 1999).

IgA in mucosal immunity

IgA is the primary Ig isotype induced at mucosal sites (Aittoniemi et al. 1999; Brandtzaeg, 1989) and is thought to mediate defense functions at these sites (Lamm, 1997; Mazanec et al., 1993). Polymeric IgA (pIgA) consists mainly of 2 or 4 IgA monomers polymerized through the J chain, which is added to the Ig molecules just before secretion by plasma cells (Johansen et al., 2000).

After secretion by plasma cells, mucosal IgA is transported from the baso-lateral epithelial compartment to the apical/luminal side (Fig. 2). Transport of IgA to the lumen is mediated by the polymeric Ig receptor (pIgR), which is expressed at the baso-lateral side of the epithelial cells that line mucosal surfaces (Mostov, 1994). During the transport, the pIgR is proteolytically cleaved and the extracellular portion of the molecule, the secretory component, is released in association with the pIgA, forming altogether the secretory IgA (sIgA) (Norderhaug et al., 1999).

![Figure 2](image.png)

**Figure 2.** Origin of sIgA, A) IgA transport across the epithelium B) Structure of sIgA
**Functions of IgA**

sIgA plays an important role in the mucosal immunity, preventing microorganisms and foreign proteins from penetrating the mucosal surfaces (Mestecky et al., 1999). It also neutralizes toxins and infectious organisms. sIgA has been proposed to act at different anatomical levels in relation to the mucosal epithelium. At the luminal site, pIgA encounters the antigen within the underlying tissue generating an immune complex, which is then processed along the excretory pathway and released into the secretions (Kaetzel et al., 1991; Stokes et al., 1975). In addition to this, during the transport through the lining epithelial cells, after the pIgR-mediated endocytosis, IgA is thought to be able to interact with intracellular pathogens such as viruses, blocking their replication, assembly and/or budding (Burns et al., 1996; Mazanec et al., 1992; Mazanec et al., 1995).

The identification and characterization of a leukocyte-Fc receptor for IgA (FcαR, CD89) in human neutrophils, eosinophils, and monocytes, has clearly demonstrated an active role for IgA in mucosal immunity. Indeed, a number of studies have now shown that, receptor-bound IgA and sIgA can trigger cellular functions such as degranulation and respiratory burst as efficiently, or better, than IgG. This is not surprising since the intracellular signaling via the FcαR is transduced through the same intracellular peptide used by IgG and IgE receptors, the γ chain (Morton et al., 1996).

**IgA deficiency**

The prevalence of IgA deficiency ranges from 1/223 to 1/1000 in community studies (Cunningham-Rundles, 2001).

Since sIgA plays an important role in mucosal immunity, it is still a mystery why most IgA deficient individuals remain healthy. In this regard, selective IgA deficiency is usually defined as a level less than 7 mg/dl of IgA in the serum, since this is the lowest level detectable in many commercial kits. Thus, a possible explanation might be that individuals diagnosed as IgA deficient may actually produce enough sIgA at mucosal sites to remain healthy. Indeed, some IgA deficient individuals have been reported to display normal numbers of IgA-bearing plasma cells in the intestine and produce normal levels of sIgA (Ammann and Hong, 1971). Additionally, the lack of disease in IgA deficient individuals...
can also be attributed to a compensatory increase in secretory IgM (sIgM) (Natvig et al., 1997). However, it is not clear if sIgM confers the same mucosal protection as sIgA. For instance, IgA deficient blood donors have been shown to harbor poliovirus for longer periods of time after oral vaccination than normal subjects (Savilahti et al., 1988).

Nevertheless, despite the fact that most IgA deficient individuals are not ill, IgA deficiency has been associated with a large number of specific disorders such as sinopulmonary infections, gastrointestinal diseases (giardiasis, celiac disease, nodular lymphoid hyperplasia), autoimmunity, and allergy (Ammann and Hong, 1971; Burks and Steele, 1986; Hammarström et al., 2000; Schaffer et al., 1991; Strober and Sneller, 1991).

**I.n. route of immunization**

The successful initiation of mucosal immune responses has been identified as dependent on factors such as, effective delivery of the antigen to the mucosal inductive sites and the use of improved antigen delivery systems for enhancement of the immune responses (Cripps et al., 2001).

Oral immunization is a commonly used route for induction of mucosal immune responses following antigen uptake at Peyer’s patches of the small intestine. However, this approach has its limitations as enzymatic and proteolytic degradation in the stomach can potentially compromise the immunogenicity of the ingested antigen. An additional problem associated with this route of immunization is the possibility of developing tolerance or immunological unresponsiveness to the ingested antigen. Consequently, other mucosal tissues such as the upper respiratory tract are being explored as alternative sites for delivering mucosal vaccines (reviewed in Sedgmen et al., 2004). In this regard, i.n. immunization has emerged as a very effective route for induction of both systemic and mucosal immunity (Gallichan and Rosenthal, 1995; Nugent et al., 1998; Renauld-Mongenie et al., 1996).

The i.n. route offers several advantages over other routes of immunization (Partidos, 2000):

1- the nose is easily accessible and highly vascularized;
2- the presence of numerous microvilli covering the nasal epithelium generates a large absorption surface;

3- after i.n. immunization, both mucosal and systemic immunity can be induced;

4- immune responses can be induced at distant mucosal sites owing to the dissemination of effector immune cells in the common mucosal immune system;

5- the nose can be used for the easy immunization of large population groups;

6- nasal immunization does not require needles and syringes, which are potential sources of infection.

**Delivery systems for i.n. immunizations**

Most protein antigens are poor immunogens when delivered mucosally, often resulting not in vaccine enhancement, but in immunological tolerance or unresponsiveness known as mucosally induced tolerance.

Adjuvants are often used to increase the immune responses to a vaccine antigen when co-administered with the antigen. Currently, alum (aluminium phosphate and aluminium hydroxide) and MF59 (a squalane o/w emulsion) are the only vaccine adjuvants approved for human use (Singh and O'Hagan, 1999). However, these two adjuvants are intended to induce and/or enhance the generation of protective immunity in the systemic immune compartment. Therefore, many studies have focused in the development of adjuvants to be used in the design of mucosal vaccines. Adjuvants studied include, toxin-based adjuvants such as cholera toxin (CT) and heat-labile toxin (LT) (Freytag and Clements, 1999), cytokines such as IL-12 (Boyaka et al., 1999) and IL-6 (Rincon et al., 1997), liposomes (Baca-Estrada et al., 2000), live attenuated vectors (Levine et al., 1996) and oil-based adjuvants such as Eurocine™ L3 (Haile et al., 2004; Hiroi et al., 2001).
TUBERCULOSIS

Tuberculosis (TB), together with acquired immune deficiency syndrome (AIDS) and malaria, remains today one of the leading infectious diseases. TB causes an estimated of 2 to 3 millions deaths per year (World Health Organization, 1999), and it was declared a global emergency by the World Health Organization (WHO) in 1993 (World Health Organization, 2002). The alarming increase in the incidence of TB during the last years, due to emergence of TB strains resistant to all major chemotherapeutic drugs (Snider and Castro, 1998) and to co-infection with human immunodeficiency virus (HIV) (Corbett et al., 2003; De Cock and Chaisson, 1999), has emphasized the need to develop immunological tools for TB control.

Establishment of mycobacterial infections

In 1882, Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of TB. *M. tuberculosis* is an obligate aerobe, generally characterized by a long replication time and a cell wall containing abundant lipids and waxes that provide hydrophobic characters, acid fast properties and intracellular survival (Gebbardt et al., 1996). There are five closely related mycobacteria grouped in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti* (van Soolingen et al., 1997; van Soolingen et al., 1998). Members of the *M. tuberculosis* complex can all cause disease in humans, although *M. tuberculosis* is the most prevalent. The natural reservoir of *M. tuberculosis* and *M. africanum* is limited to humans and that of *M. microti* is mainly limited to small rodents (Kremer et al., 1998). In contrast, *M. bovis* can cause disease in a wide range of wild and domestic animals as well as in humans (Brosch et al., 2002; Morris et al., 1994).

Transmission of TB in man usually occurs via airborne microscopic droplet nucleic (1-5 µm diameter) containing *M. tuberculosis*. The infectious droplets nucleic are inhaled and lodge in the pulmonary alveoli (Loudon and Roberts, 1967; Riley et al., 1995), where then the bacilli are phagocytosed by alveolar macrophages and remain in the phagosome of these cells (Armstrong and Hart, 1975). Following phagocytosis, *M. tuberculosis* replicates slowly but continuously, and is spread to the neighboring lung tissue and through lymphatic vessels to draining hilar lymph nodes (Frieden et al., 2003).
It is not fully understood how *M. tuberculosis* can survive and replicate intracellularly in macrophages, which are cells that have the microbicidal armory to destroy most pathogens. However, *M. tuberculosis* seems to have evolved mechanisms to survive most of the macrophage-effector functions. Some of these mechanisms involve the inhibition of the phagosome-lysosome fusion, where the bacilli have been found to retain a macrophage protein, called tryptophane aspartate-containing coat protein (TACO), on the surface of the phagosome preventing their delivery to the lysosome (Ferrari et al., 1999), and to use complement receptors 1 and 3 for cell entry, which do not trigger oxidative burst (Schlesinger et al., 1990; Wright and Silverstein, 1983). Other mechanisms of survival include degradation of reactive oxygen intermediates by catalase and superoxide dismutase produced by the bacilli, inhibition of apoptosis in infected macrophages (Fratazzi et al., 1999), and down-regulation of some modulators of the host immunity such as interleukin 12 (IL-12) (Hickman et al., 2002; Nau et al., 2002), major histocompatibility complex (MHC) class II (Noss et al., 2000), and interferon γ (IFN-γ), known to mediate activation of macrophages (Ting et al., 1999).

After *M. tuberculosis* has entered the lungs, one of four potential fates might occur (Schluger and Rom, 1998):

1) the initial host response can be completely effective in the killing and elimination of the bacilli, such that these individuals have no chance to develop TB;

2) the bacilli can grow and multiply immediately after infection, causing clinical disease (primary TB);

3) the bacilli may become dormant and never cause disease at all, resulting in a latent infection that is manifested only as positive tuberculin skin test results;

4) the dormant bacilli can eventually begin to grow, due to factors like immunosupression, with resultant clinical disease (reactivation TB).
Pathogenesis of TB

Pulmonary TB

The most common clinical manifestation of TB is the pulmonary disease. After inhalation, the bacilli initiate small lesions in the lower respiratory tract. These lesions frequently heal to form tiny tubercle, which are too small to be seen by x-rays but may continue to harbor the bacilli indefinitely. In other cases, replication of the bacilli continues and the lesions expand and undergo caseation necrosis, which will destroy the normal tissue and leave the necrotic tissue in a semisolid, “cheesy” state. Caseation necrosis may eventually heal and become infiltrated with fibrous tissue and calcium deposits, or may continue to expand leaving cavities in the lungs (Gebbardt et al., 1996).

Extrapulmonary TB

Extrapulmonary TB is more common in children and in HIV-infected individuals (Shafer and Edlin, 1996). In extrapulmonary TB, the tubercle bacilli may spread through the bloodstream from the lesions in the lungs into other organs such as, bones and joints particularly the spine (Okuyama et al., 1996), kidneys and genital tract causing genitourinary TB (Gorse and Belshe, 1985), or the central nervous system causing TB meningitis (Thwaites et al., 2000). TB meningitis is fatal in almost all cases without treatment, therefore prompt identification and chemotherapy are crucial to prevent serious neurological sequels.

Another clinical manifestation of extrapulmonary TB is disseminated TB, which is defined as involvement of many organs simultaneously, and can occur as result of a primary progressive disease or reactivation of the latent infection (Hill et al., 1991).

Immune response to TB

Innate immune response

Recent immunological and genetic studies have corroborated the long-standing notion that, innate immunity is relevant in the host defense against M. tuberculosis.
The uptake of *M. tuberculosis* by alveolar macrophages represents the first step in the innate host defense against TB. This initial interaction is mediated by cellular receptors such as complement receptors, mannose receptors, surfactant receptors, and scavenger receptors (Chan *et al.*, 1992; Downing *et al.*, 1995; Flesch and Kaufmann, 1988; Gaynor *et al.*, 1995; Schlesinger *et al.*, 1993). Most recently, attention has focused on the role of toll-like receptors (TLRs) in mediating the uptake of mycobacteria by macrophages. Specifically, the role of TLR2 and TLR4 in sensing mycobacteria and promoting antimycobacterial responses has been demonstrated in several studies. *In vivo* studies using TLR2 or TLR4 deficient mice have shown that these mice are more susceptible to mycobacterial infection than wild-type mice (reviewed in Quesniaux *et al.*, 2004). Furthermore, *in vitro* studies using a human macrophage-like cell line have demonstrated that activation of TLRs by lipoproteins contained within the *M. tuberculosis* cell wall induces production of IL-12, an important pro-inflammatory cytokine in the host response against TB (Brightbill *et al.*, 1999). In addition, these studies showed that TLR-mediated IL-12 production also resulted in increased production of nitric oxide synthetase and nitric oxide, which are important for the intracellular killing of mycobacteria.

Thus, TLRs contribute to the innate immunity by detecting mycobacteria-associated molecular patterns and mediating the secretion of antimycobacterial effector molecules. However, TLRs can also influence the specific immunity by upregulation of immunomodulatory molecules supporting the development of pro-inflammatory responses (Schluger, 2001).

**Specific immune response**

The specific immune response to *M. tuberculosis* in the lungs is complex and involves multiple mechanisms. T cells are believed to be essential in the protective immune response against TB, and the interaction of T cells with macrophages is critical for the control of the infection. The production of inflammatory cytokines and chemokines, induced by ingestion of *M. tuberculosis* by alveolar macrophages (Means *et al.*, 1999), leads to the migration of monocyte-derived macrophages and dendritic cells to the site of infection. The dendritic cells that engulf mycobacteria, mature and migrate to regional lymph nodes (Bodnar *et al.*, 2001; Henderson *et al.*, 1997; Hertz *et al.*, 2001), where then T cells are primed against mycobacterial antigens. Primed T cells expand and migrate to
the site of infection in the lungs, presumably due to the upregulation of local adhesion molecules and chemokines. The migration of macrophages and T cells to the site of infection results in formation of a granuloma (Fig. 3), which also comprises other cells such as B cells, dendritic cells, endothelial cells, fibroblasts and probably stromal cells (Gonzalez-Juarrero et al., 2001).

The granuloma functions as an immune microenvironment to facilitate interactions between T cells and macrophages. In addition to providing a framework for these cells, granulomas serve to wall off mycobacteria from the rest of the lungs, limiting the dissemination of the infection. However, depending on the cellular composition and on the cytokine- and chemokine-secreting profile, granulomas can also be associated with pathology or at least lack of adequate containment of bacillary multiplication (Saunders and Cooper, 2000).

CD4+ T cells

CD4+ T cells play a central role in the immune response against *M. tuberculosis*. Peptide antigens from mycobacteria, degraded in the phagolysosomal compartment and complexed with the MHC class II molecules are recognized by CD4+ T cells, resulting in their activation (Davis and Bjorkman, 1988). The main function of CD4+ T cells in immunity to TB is thought to be the production of cytokines, specifically IFN-γ, which is critical for macrophage activation and the subsequent induction of microbicidal mechanisms (Flesch and Kaufmann, 1990). The critical role of IFN-γ in the control of mycobacterial infections has been demonstrated in animal models. Experimentally, mice deficient in IFN-γ or in IL-12, a critical cytokine in the induction of IFN-γ production, were highly susceptible to challenge with *M. tuberculosis* (Cooper et al., 1993; Cooper et al., 1997). In addition, studies in humans have shown that patients with IFN-γ receptor deficiency presented disseminated infection with *M. bovis* BCG and/or environmental mycobacteria, which resulted in the death of about half of the patients and required continuous antimycobacterial treatment in the survivors (reviewed in Casanova and Abel, 2002).

CD4+ T cells can also contribute to the control of acute mycobacterial infections through IFN-γ independent mechanisms. This has been demonstrated in a variety of experimental models using antibody depletion and mouse strains deficient in either CD4 or MHC class II
molecules (Caruso et al., 1999; Scanga et al., 2000). In mice deficient in CD4 or MHC class II molecules, the levels of IFN-γ were significantly diminished very early during infection, but later the IFN-γ production was similar to that seen in wild type mice. However, deficient mice were not rescued by this later production of IFN-γ, and succumbed to the infection. IFN-γ independent mechanisms of action of CD4+ T cells may also include a cytolytic function of these cells, as has been shown in murine models (Izzo and North, 1992) as well as in humans (Tan et al., 1997).

![Figure 3](image-url)

**Figure 3.** Structural organization of a granuloma (Adapted from the Current Opinion in Immunology 2001).
Further evidence of the importance of CD4\(^+\) T cells in the control of TB in humans is obtained from studies of the clinical course of co-infection with HIV. Depletion of CD4\(^+\) T cells during HIV infection dramatically increases the susceptibility to primary and reactivation TB (Havlir and Barnes, 1999; Jones et al., 1993).

**CD8\(^+\)** T cells

Despite the intraphagosomal location of *M. tuberculosis*, it is now recognized that CD8\(^+\) T cells, restricted either by MHC class I or CD1 molecules, participate in a successful antimycobacterial immune response. In contrast to the peptide epitopes presented by the MHC molecules, CD1 molecules present lipids or glycolipids to T cells (Porcelli and Modlin, 1999).

Experimentally, mice deficient in β2-microglobulin, a component of both MHC class I and non-classical MHC class 1b molecules, were found to be more susceptible to infection with *M. tuberculosis* than wild type mice (Flynn et al., 1992). Similarly, increased susceptibility to mycobacterial infections has been seen in mice deficient in transporters associated with antigen processing (TAP) molecules, which transport peptides from the cytosol to the endoplasmic reticulum for loading into MHC class I molecules (Behar et al., 1999; Sousa et al., 2000). In addition to these studies, vaccination of mice with DNA plasmids expressing mycobacterial antigens were also shown to induce antigen-specific CD8\(^+\) CTL, which conferred protection against challenge with *M. tuberculosis* (Smith and Dockrell, 2000). Despite all the experimental findings confirming the role of CD8\(^+\) T cells in the control of TB, it still remains unclear how phagosomically derived antigens interact with the MHC class I processing machinery.

CD8\(^+\) T cells appear to have two major functions in TB immunity, lysis of infected cells and production of cytokines, mainly IFN-γ. The relative contribution of these functions is unknown. It has been shown that CD8\(^+\) T cells from the lungs of infected mice are primed to produce IFN-γ, upon T cell receptor (TCR) interaction with *M. tuberculosis*-infected dendritic cells (Serbina and Flynn, 1999). However, unlike CD4\(^+\) T cells spontaneous ex vivo production of IFN-γ by CD8\(^+\) T cells is very low, suggesting that the production of this cytokine by CD8\(^+\) T cells in the lungs is limited (Serbina and Flynn, 1999). Evidence for a more direct role of CD8\(^+\) T cells come from studies showing lysis of infected human
macrophages and dendritic cells by CD1 and MHC class I restricted CD8\(^+\) T cells specific for *M. tuberculosis*, resulting in reduced numbers of intracellular bacteria (Cho *et al*., 2000; Stenger *et al*., 1997). The killing of the intracellular bacteria was shown to be perforin-dependent (Stenger *et al*., 1997). Perforin was required to form a pore, but the molecule responsible for the killing of the intracellular bacteria was granulysin, another cytotoxic granule protein (Stenger *et al*., 1998).

\(\gamma\delta^+\) T cells

A large amount of evidence from human and animal studies suggests that, \(\gamma\delta^+\) T cells play a significant role in the host response to TB (Boom, 1999). It is generally believed that these cells are involved in primary immune defense. Indeed, a recent study reported that \(\gamma\delta^+\) T cells accumulated in the lungs of BCG-infected mice three weeks earlier than antigen-specific \(\alpha\beta^+\) T cells, suggesting that \(\gamma\delta^+\) T cells in the lungs might help to control mycobacterial infection during the period between the innate and adaptive immunity. Additionally, results suggested that \(\gamma\delta^+\) T cells might also play an important regulatory role in the subsequent onset of \(\alpha\beta^+\) T cells (Dieli *et al*., 2003).

Experimentally, expansion of \(\gamma\delta^+\) T cells has been shown in mice exposed to mycobacterial antigens or live bacteria. In addition, *in vitro* studies have also shown expansion of human \(\gamma\delta^+\) T cells, specially the V\(\gamma\)9/V\(\delta\)2 TCR subset, by mycobacterial antigens and live mycobacteria (Boom *et al*., 1992; De Libero *et al*., 1991; Havlir *et al*., 1991; Ohmen *et al*., 1991; Panchamoorthy *et al*., 1991; Porcelli *et al*., 1992).

*M. tuberculosis*-reactive \(\gamma\delta^+\) T cells have been detected in the peripheral blood of tuberculin skin test (TST) positive and BCG-vaccinated individuals. These cells were found to be cytotoxic for monocytes pulsed with mycobacterial antigens and to secrete cytokines that may be involved in the granuloma formation (Cooper, 1993; Munk *et al*., 1990).

The role of \(\gamma\delta^+\) T cells in the granuloma formation in response to *M. tuberculosis* has been demonstrated in studies using mice with severe combined immunodeficiencies (SCID). In these studies, SCID mice did not form granulomas and rapidly succumbed to disease after BCG infection. However, these mice survived BCG inoculation, when engrafted with co-
isogenic lymph node cells depleted of CD4⁺ and CD8⁺ T cells, indicating that the remaining γδ⁺ T cells were responsible for this response (Izzo & North, 1992; North and Izzo, 1993).

Macrophages

Apart from their significant function in innate immunity, macrophages have been reported to play a pivotal role in the adaptive immune responses against mycobacteria by producing cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-1β (Fenton and Vermeulen, 1996). The importance of TNF-α has been extensively studied in knockout mice. In these studies, TNF-α knockout mice presented a profound susceptibility to aerogenic infection with *M. tuberculosis* characterized by a reduced macrophage differentiation and granuloma formation that resulted in mycobacterial overgrowth and rapid animal death (Saunders and Cooper, 2000). Additionally, TNF-α and IL-1β along with IFN-γ, produced by T cells, stimulate production of nitric oxide in macrophages. The production of nitric oxide and related reactive nitrogen intermediates by macrophages, is considered to be an effective host-defense mechanism against microbial intracellular pathogens like mycobacteria (Chan et al., 1992; Denis, 1991). In the murine model of TB, nitric oxide plays an essential role in the killing of *M. tuberculosis* by mononuclear phagocytes. For example, in the mouse strain with a genetic disruption for inducible nitric oxide synthetase (iNOS), infection with *M. tuberculosis* is associated with a significantly higher risk of dissemination and mortality. Although more controversial in humans, there is a growing body of evidence that nitric oxide produced by TB-infected macrophages has antimycobacterial effects against *M. tuberculosis*. The precise mechanism(s) by which nitric oxide and other reactive nitrogen species antagonize *M. tuberculosis* is not known, but may involve disruption of bacterial DNA, proteins, signaling, and/or induction of apoptosis of macrophages that harbor mycobacteria.

B cells

While the role of T cells in the protection against mycobacterial infections is well established, the role of B cells and antibodies is less understood. Studies conducted in mice lacking B cells have been controversial, making it difficult to define the role of these cells in antimycobacterial immunity. In this regard, it has been reported that B cells play no role...
at all (Johnson et al., 1997). On the other hand, other studies have suggested a role for B cells as APCs and in granuloma formation (Vordermeier et al., 1996), or a role in the regulation of chemokines and/or adhesion molecules expression leading to recruitment of neutrophils, macrophages and CD8⁺ T cells during early *M. tuberculosis* infection (Bosio et al., 2000). Moreover, attempts at passive vaccination with antibodies in man and mice have also produced contradictory results, having reported no effect (reviewed in Glatman-Freedman and Casadevall, 1998) or inhibition of bacilli dissemination (Pethe et al., 2001) and prolongation of survival in infected animals (Teitelbaum, 1998).

### Diagnosis

Tests for diagnosis of TB vary in sensitivity, specificity, speed and cost.

#### Microscopy

Microscopic examination of acid-fast bacilli in stained smears of clinical specimens is an easy, rapid and inexpensive diagnostic method. Also, because it gives a quantitative estimation of the number of bacilli being excreted, this method is of vital clinical and epidemiological importance in assessing the patient infectiousness. Two procedures are commonly used for acid-fast staining: the carbofuchsin procedure, which includes Ziehl-Neelsen and Kinyoun methods and the fluorochrome procedure, which uses auramine-O or auramine-rhodamine dyes. Smear microscopy has been shown to have low sensitivity, usually from 5,000 to 10,000 bacteria/ml are needed for positive results (American Thoracic Society and The Centers for Disease Control and Prevention, 2000). However, since cases with organisms in the smear are highly infectious, the identification of smear positive patients is of major importance.

#### Culture

Mycobacterial culture is the ultimate proof of a mycobacterial infection and is used as a reference method due to its high sensitivity (Walker, 2001). This method is able to detect as few as 10 bacteria/ml of clinical specimen and allows precise identification of mycobacterial species. Three different types of traditional culture media are available: the egg-based Lowenstein-Jensen medium, the agar based Middlebrook 7H10 or 7H11, and
the liquid Middlebrook 7H9 (American Thoracic Society and The Centers for Disease Control and Prevention, 2000). A disadvantage in the use of culture methods for diagnosis is the long period of time required by the bacilli to grow, ranging from two to four weeks in the case of solid culture media and from one to three weeks in the case of liquid media. Despite this, culture is required for definitive diagnosis and is essential for drug-susceptibility testing (Fadda and Sanguinetti, 1998).

**Tuberculin skin test**

A positive TST is a hallmark of primary infection with TB. There are two major techniques currently used for TST, the Mantoux test and the multi-puncture technique. The Mantoux test is the standard method used in many countries. The test involves intradermal injection of a purified protein derivative (PPD) solution, which raises an immediate wheal. The reaction is measured as mm of induration, after 48 to 72 h. The multi-puncture technique involves the inoculation of PPD using a multi-puncture device into the skin. The test is read after 5 to 7 days and results are based on the induration pattern surrounding the puncture site (Shingadia and Novelli, 2003).

The interpretation of the TST can be affected by several factors such as age, Bacillus Calmette-Guérin (BCG) vaccination status, exposure to environmental mycobacteria, and immunosuppression. In persons with reactive TST, major confounding factors are infection with mycobacteria other than *M. tuberculosis* and prior vaccination with BCG. False-negative TST reactions are a problem among debilitated persons and other immunocompromised hosts, particularly those with advanced HIV infection (American Thoracic Society, 1990). Although neither 100 % sensitive nor specific, the TST (Mantoux) remains the best method for detecting infection with *M. tuberculosis* because it is simple and cheap.

**Radiology**

Another method for diagnosis of TB is radiographic screening. The purpose of screening chest radiographs is to identify persons with active TB. Although radiography is often used in conjunction with the TST, this is the initial screening method selected when the TST results may be unreliable, when reading of the TST may be impractical, and/or when the

**Newer methods**

In 2001, the QuantiFERON®-TB test (manufactured by Cellestis Limited, Carnegie, Victoria, Australia) was approved by the Food and Drug Administration (FDA) as an aid for detecting latent *M. tuberculosis* infection (reviewed in Mazurek and Villarino, 2003). This test is an *in vitro* diagnostic aid that measures a component of cell-mediated immune reactivity to *M. tuberculosis*. The test is based on the quantification of IFN-γ released from sensitized lymphocytes in whole blood incubated overnight with PPD from *M. tuberculosis* and control antigens.

In addition to this *in vitro* test, molecular biological methods, such as PCR, are increasingly being applied. However, the widespread implementation of these methods is limited by high cost and potential for poor performance under field conditions (Frieden et al., 2003).

**BCG vaccine**

In 1908, Camille Guérin and Albert Calmette initiated their attempts to produce an anti-TB vaccine from a virulent bovine strain. In 1921, vaccination with BCG, an attenuated vaccine, was introduced (Sakula, 1983). The efficiency of the BCG vaccine has been questioned since its early use and therefore, a large number of trials have been carried out to determine its efficacy. In these studies it was found that, the BCG vaccine protected efficiently against leprosy (Fine and Rodrigues, 1990) as well as childhood manifestations of TB (disseminated TB) (Rodrigues et al., 1993). However, the protective efficacy against pulmonary TB was limited (Tuberculosis Research Centre (ICMR), Chennai, 1999).

Many hypotheses have been suggested to explain the low protective efficacy of BCG against pulmonary TB. These hypotheses include inappropriate treatment and storage of the vaccine, the use of different strains of BCG (Fine, 1995), and lack of an effective stimulation of the optimal blend of T cell populations and in particular that of the CD8+ T cells (Hess and Kaufmann, 1999). In addition to these hypotheses, the currently used
intradermal route of immunization has been suggested as another factor influencing the
capacity of BCG to induce optimal immunity in the lungs. In this regard, i.n. route of
immunization has recently been evaluated as a possible route for BCG delivery, in mouse
experimental models. Results from this study showed a high degree of protection against
challenge with *M. tuberculosis* in BALB/c mice, following i.n. BCG vaccination (Falero-
Diaz et al., 2000). In a similar model, i.n. vaccination with BCG conferred as good, if not
better protection than subcutaneous (s.c.) route, against challenge with virulent *M. bovis*
(Lyadova et al., 2001).

**Prospects for new vaccines**

Given the limitations of BCG in protection against adult pulmonary TB, there is a
considerable scope for improved vaccination strategies. Immunological research has a key
position in understanding the pathogenesis of TB, and thereby in developing novel designs
for effective prophylactic vaccination, immunodiagnostic tools and immunotherapeutic
agents. Two approaches have been considered for vaccine development. One involves the
replacement of BCG by a more potent vaccination inducing immune responses capable of
either complete elimination of the bacilli, or of reliable containment of persistent infection.
The second approach involves the post-exposure vaccination to boost immunity in
individuals whose natural immunity has already been primed by infection or BCG
vaccination (reviewed in Young and Stewart, 2002). Indeed, over the past decade research
efforts have been directed to evaluate potential vaccine candidates as well as alternative
routes of vaccine delivery, such as the i.n. route, in order to improve protection.

**New vaccine candidates**

A wide range of potential vaccine candidates have been generated and subjected to tests for
protective efficacy in experimental model of infection. New vaccine candidates include
live attenuated vaccines, subunit vaccines and DNA vaccines.

**Live attenuated vaccines**

Advances in the techniques required to genetically modify mycobacteria, as well as the
increase in the knowledge of the pathogenesis of the microorganism, have made possible to
delete genes encoding for potential virulence factors in *M. tuberculosis*, thereby enabling the generation of attenuated mutants. In addition to attenuated strains of *M. tuberculosis*, natural attenuated mycobacteria, such as *M. vaccae* and *M. microti*, are being studied as possible vaccine candidates (Nor and Musa, 2004). Another approach has been the improvement of the BCG immunogenicity by the addition of genes encoding cytokines, such as IFN-γ (Murray *et al.*, 1996) or mycobacterial proteins, such as the antigen 85 complex (Ag85) (Horwitz *et al.*, 2000).

Although encouraging results have been obtained in challenge experiments (Horwitz *et al.*, 2000; Smith *et al.*, 2001), a major consideration for the clinical use of live vaccines is safety, specifically when considering TB vaccination strategies for AIDS patients.

**Subunit vaccines**

Subunit vaccines are currently the most widely studied. This type of vaccine has been focused in particular on proteins present in filtrates prepared from *in vitro* cultures of *M. tuberculosis*, although non-secreted antigens have also been shown to induced protective responses in experimental studies (Coler *et al.*, 2001; Skeiky *et al.*, 2000).

The most extensively studied antigens are members of the Ag85 complex, a family of mycolyl transferases enzymes involved in cell wall biosynthesis and present in culture filtrates (Belisle *et al.*, 1997). The Ag85 has been reported to induce strong activation of T cells in several studies (Andersen *et al.*, 1995; Mustafa *et al.*, 1998).

Other antigens being studied are:

- early secreted antigenic target (ESAT-6), which has been reported to be absent from all BCG vaccine strains and to induce very strong T cell and antibody responses (reviewed Brodin *et al.*, 2004);

- heat-shock proteins (HSP), such as HSP-65 and HSP-70, found to induce a prominent immune response at both, the antibody and the T cell levels (reviewed in Silva, 1999);
- PstS-1 (38 kDa protein), a glycoprotein exposed on the surface of the bacillus and reported to be a powerful B and T cell antigen (Bothamley et al., 1992; Lefevre et al., 1997);

- 19 kDa protein, a lipoprotein found to induce the expression of IL-12 and iNOS in monocytes and dendritic cells through its binding to TLR2 (Brightbill et al., 1999; Thoma-Uszynski et al., 2000) and to promote neutrophil activation (Neufert et al., 2001).

A limiting factor of the subunit vaccines is the need of adjuvants for vaccine delivery. Currently research studies are focused on the choice of which adjuvant to use and whether immunomodulators, such as cytokines, should be used. Despite this drawback, subunit vaccines based on recombinant protein antigens are attractive because the techniques for production are established and this type of vaccine is expected to satisfy the regulatory requirements for use in humans more easily than the live vaccines.

**DNA vaccines**

Administration of naked DNA has the potential of eliciting both, cellular and humoral immunity against encoded antigens. Several mycobacterial antigens, including the PstS-1, HSP-65 and the Ag85 have been studied and found to induce protection in animal models (Bonato et al., 1998; Fonseca et al., 2001; Huygen et al., 1996). Although the results are promising, concerns about the safety of DNA vaccination have been raised, mainly regarding the possibility of DNA integration into the host genome affecting oncogenes or tumor suppressor genes and thereby inducing the development of cancer. However, the risk of integration has been reported to be low under a variety of experimental conditions (Manam et al., 2000; Martin et al., 1999).

**Experimental animal models in TB**

Discussions about the value of experimental animal models in TB research have a long-standing history. Experimental animal models are critical for delineating the general mechanisms underlying natural resistance, and acquisition of a protective immune response against TB. However, assessment of this information using experimental animals
should be conducted carefully since there are differences in the host defense mechanisms between experimental animals and humans.

Many experimental animal species such as mouse, guinea pig, and non-human primates, have been used for deciphering the mechanisms involved in TB. The mouse, without doubt, is a very sophisticated and cost-efficient model. The immune response of the mouse is very well understood, and reagents such as monoclonal antibodies against surface antigens and cytokines are available. More importantly, the genetic manipulation of this species is highly advanced. Transgene expression, gene knockout, gene knock-in have all become standard technologies, and a large variety of mouse mutants with defined immunodeficiencies are available to researchers studying the role of distinct cells and effector molecules in the in vivo setting of TB. Moreover, the recent elucidation of the murine genome promises to open a new area of research with enormous impact on our understanding of genetic disorders and also of host mechanisms in TB (Kaufmann, 2003).
**Chlamydia pneumoniae**

**Establishment of C. pneumoniae infections**

*C. pneumoniae* is another pathogen that enters the host via the respiratory tract. This Gram-negative intracellular bacterium was first discovered in 1986 as a respiratory pathogen by Grayston *et al.* (Grayston *et al.*, 1986; Grayston *et al.*, 1989; Grayston *et al.*, 1990).

The genus *Chlamydia* is currently divided into four species, *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and *C. pecorum* (Puolakkainen and Makela, 1999). All species from this genus share some common biological and immunological factors (reviewed in Hammerschlag, 2003):

- a unique developmental cycle with two morphologically and functionally distinct forms [the extracellular, metabolically inactive elementary body (EB) and the intracellular metabolically active reticulate body (RB)];

- a Gram-negative envelop without peptidoglycan ;

- a genus-specific lipopolysaccharide (LPS) antigen.

Infection with *C. pneumoniae* is initiated by attachment of the invasive EB to the epithelial cells lining the trachea and the nasopharynx (Fig. 4). In the lungs, the bacteria infect and replicate inside macrophages. Usually, the EB attaches to the cell surface of macrophages and is internalized by endocytosis. After invasion, the EB remains within the phagosome and inhibits the fusion of the phagosome to the lysosome. The EB instead promotes the association of the phagosome to exocytic vesicles, thereby avoiding intracellular killing (Moulder, 1991). In addition to this mechanism of survival, *C. pneumoniae* has also been reported to down-regulate expression of MHC class I molecules and to inhibit apoptosis in infected macrophages and epithelial cells. Once inside the phagosome, the EBs start to transform into RBs and during acute productive infection the RBs undergo division by binary fission. After approximately 36 hours the RBs differentiate into EBs, which are then released by cytolysis or by exocytosis, and infect new targets cells in the host.
Although, a *C. pneumoniae* infection induces immune responses in the host, sterile eradication is not achieved.

**Figure 4.** Developmental cycle of *C. pneumoniae*.

**Pathogenesis**

Infection with *C. pneumoniae* affects more than 50% of the human population worldwide causing pneumonia, sinusitis and bronchitis. Although, infections with *C. pneumoniae* are mild and subclinical, persistent or recurrent infection with this pathogen has been associated with chronic pulmonary conditions such as asthma (Hahn *et al.*, 1991) and also with extrapulmonary systemic disorders such as coronary heart disease (Linnanmaki *et al.*, 1993; Saikku *et al.*, 1988).
Data regarding histopathological findings in respiratory infection due to *C. pneumoniae* have been mostly obtained from studies in animals and non-human primates. In mice, i.n. infection with *C. pneumoniae* results in an inflammatory response, in the lungs, that is characterized by infiltrates of primarily polymorphonuclear leukocytes in the acute phase and mononuclear leukocytes in the chronic phase (Kuo, 1999). A characteristic feature is a patchy distribution of inflammatory infiltrates interspersed among areas of normal or relatively normal histology. After i.n. infection, the animals are usually not very ill and the infection resolves spontaneously, however the pathogen can disseminate to other sites of the body, probably through the circulation. Indeed, *C. pneumoniae* has been isolated in culture not only from the lungs, but also from the spleen (Kuo, 1999).

**Immune response to *C. pneumoniae***

The mouse model, which faithfully mimics important factors of human *C. pneumoniae* infection (Rottenberg *et al.*, 1999; Rottenberg *et al.*, 2000), has enabled to study immune mechanisms leading to protection against this pathogen. Yet, available information about protective immunity in *C. pneumoniae* infection is sparse.

Two stages have been described in reference to antichlamydial immune responses: 1) an early response requiring IFN-\(\gamma\) to limit the growth of the bacteria, and 2) a later adaptive immune response involving CD4\(^+\) and CD8\(^+\) T cells (Rottenberg *et al.*, 1999).

**Innate immune response**

The attachment of EBs to epithelial cells represents the first step of the innate immunity in chlamydial infections. *Chlamydia*-infected epithelial cells have been shown to secrete a variety of pro-inflammatory and immunoregulatory cytokines (Rasmussen *et al.*, 1997), such as granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-18. GM-CSF promotes the maturation of dendritic cells and IL-18 synergistically interacts with IL-12 to promote a Th1 cytokine differentiation pattern.

On the other hand, alveolar macrophages also play important role in the innate immunity against *C. pneumoniae*. These cells have been shown to elicit a marked inflammatory
response to the microorganism, which is characterized by production of TNF-α, IL-1β and IL-6 (Redecke et al., 1998).

Specific immune response

T cells

The mouse model has demonstrated the essential role of T cells in the protection against C. pneumoniae infections. Studies conducted in thymusless mice have shown that these mice are incapable of clearing chlamydial infection (Penttila et al., 1999). Specifically, a role for CD4+ and CD8+ T cells in resistance to chlamydial infections has been demonstrated in studies using mice depleted of CD4+ and CD8+ T cells by antibody administration, or mice deficient in CD4+, CD8+ T cell, β2-microglobulin, or β2-microglobulin/TAP1. In these studies deficient mice exhibited exacerbated infection compared to wild-type mice (Magee et al., 1995; Morrison et al., 1995; Perry et al., 1997; Rottenberg et al., 1999; Starnbach et al., 1994).

Both, CD4+ and CD8+ T cells participate in the immune response by producing IFN-γ in response to chlamydial infection. However, it has been proposed that CD8+ T cells are important in the early phase of the infection, whereas CD4+ T cells are involved in the later stage (Halme et al., 2000). IFN-γ appears to be essential in immune protection against chlamydial infections as demonstrated by the enhanced bacterial loads seen in IFN-γ or IFN-γ receptor deficient mice, or in mice treated with anti-IFN-γ antibodies compared to wild-type mice (Cotter et al., 1997; Ito and Lyons, 1999; Perry et al., 1997; Rottenberg et al., 1999). IFN-γ has been proposed to control chlamydial growth and to increase the accumulation of transcripts of enzymes which control high-output nitric oxide release, superoxide production, and catalysis of tryptophan (Rottenberg et al., 2000).

B cells

The involvement of humoral immunity in the outcome or eradication of the primary infection with C. pneumoniae has not been extensively studied. In this regard it is believed that, similarly to M. tuberculosis, C. pneumoniae as obligate intracellular bacteria may escape the humoral defense mechanisms. As a result of this assumption, the majority of the
studies in relation to \textit{C. pneumoniae} immunity have been focused in gathering information about the role of cellular immune responses and specifically of T cells.

\textbf{Diagnosis}

Laboratory diagnosis of \textit{C. pneumoniae} infection is based on isolation and culture of the microorganism, serology and/or detection of DNA by PCR.

\textbf{Culture}

\textit{C. pneumoniae} can be isolated from nasopharyngeal or throat swabs, sputa or pleural fluid from the patients. The nasopharynx appears to be the optimal site for isolation (Block \textit{et al.}, 1995). Upon isolation, the organism requires to be grown in tissue cultures. \textit{C. pneumoniae} grows readily in cell lines derived from respiratory tract tissue, specifically Hep-2 and HL cell lines (Roblin \textit{et al.}, 1992). After culturing the specimens for 72 hours, culture confirmation can be performed by staining with either a \textit{C. pneumoniae} species-specific or a \textit{Chlamydia} genus-specific fluorescein-conjugated monoclonal antibody.

\textbf{Serology}

Serological diagnosis in \textit{Chlamydia} is usually performed by using a micro-immunofluorescence test. This method is based on the microscopic detection of antibodies specific to a chlamydial antigen fixed onto glass slides as distinct dots. The assay is considered positive if a clinical specimen contains antibodies reacting with the antigen. This immunoreaction is then visualized with the use of fluorescein-conjugated secondary antibodies (Tuuminen \textit{et al.}, 2000). The micro-immunofluorescence test has proven to be a very specific and sensitive method; however the requirement of specialized fluorescent microscopy equipment and intact purified organisms as antigen for the test performance makes it inapplicable for use in a standard laboratory.

\textbf{DNA amplification methods (PCR)}

PCR appears to be the most promising technology in the development of a rapid method for detection of \textit{C. pneumoniae}. There are at least 19 in-house PCR assays for detection of this pathogen in clinical specimens reported in the literature (Boman \textit{et al.}, 1999).
However, none of these assays are standardized or have been adequately validated in comparison to culture methods, and they are still not commercially available or have the approval of the FDA. Major variations in these methods include the way of collecting and processing specimens, primer design, nucleic acid extraction, and amplification product detection and identification.

**Vaccine development**

So far, there is not effective vaccine against chlamydial infections. Early attempts to find a protective vaccine, using crude extracts of whole *C. trachomatis*, failed as a result of the induction of unwanted immune responses leading to pathology (Ward, 1995).

Despite the lack of a protective vaccine, *C. pneumoniae* infection can be treated with antibiotics such as tetracyclines, macrolides and quinolones that interfere with DNA or protein synthesis of the microorganism (Hammerschlag, 2003). However, sterile eradication of the bacteria is not achieved and some cells can remain persistently infected (Gieffers *et al.*, 2001). Therefore, the development of an effective vaccine to prevent or ameliorate acute and chronic infection with this pathogen is essential. Yet, vaccine development against *C. pneumoniae* has been hindered by the limited knowledge of the pathogen and of the immune mechanisms leading to protective or adverse immune responses. Currently, several antigens are being studied as potential vaccine candidates. Among these antigens, the most studied structures are the major outer membrane protein (MOMP), a cysteine-rich outer membrane protein (omp2), and the HSP-60. Although, a certain degree of protection has been seen in experiments using DNA vaccination (Penttila *et al.*, 2000; Svanholm *et al.*, 2000), none of them has been satisfactory enough to reach clinical developmental stage.
PRESENT STUDY

AIMS

With the premise that TB is an airborne disease, affecting primarily the lungs, the overall aim in this study was to target the mucosal immunity in the respiratory tract in order to induce optimal immune responses in the lungs and thereby achieve a better degree of host protection. In addition, we investigated the relevance of IgA in the respiratory mucosal immunity against intracellular pathogens such as, mycobacteria and *C. pneumoniae*.

Our specific objectives were:

- To evaluate the effects of the routes of immunization as well as of three different adjuvants in the induction of mucosal immune responses in the respiratory tract (paper I)

- To investigate the possible role of mucosal IgA in the protection of the respiratory tract against mycobacterial infections, using IgA deficient (IgA\(^{-/-}\)) mice (paper II) and pIgR deficient (pIgR\(^{-/-}\)) mice (paper III)

- To study the role of mucosal immunity and specifically of IgA in the protection of the respiratory tract against infection with *C. pneumoniae* (paper IV)
RESULTS AND DISCUSSION

Paper I

In this paper we have investigated the effect of the route of immunization and of three different mucosal adjuvants on the induction of mucosal immune responses against the mycobacterial surface antigen PstS-1. We found that, the i.n. route of immunization was a more favorable route inducing strong local immune responses, as compared to the i.p. route. Moreover, we showed that the overall Th cell development at mucosal inductive sites upon i.n. immunizations is influenced by the adjuvant used for immunizations.

Given the poor protective efficacy of the BCG vaccine against pulmonary TB and the fact that TB is transmitted as an airborne disease affecting primarily the lungs, for our studies we considered the route of immunization as one of the factors influencing the ability of BCG to induce protective immunity in the lungs. In this regard, preliminary experiments conducted in our laboratory showed that i.n. delivery of BCG elicited significantly higher IFN-γ production in the lungs compared to s.c. route (Fig. 5).

![Figure 5](image-url)

**Figure 5.** Production of IFN-γ in the lungs of mice inoculated with BCG, using i.n. or s.c. route. Groups of BALB/c mice were immunized three times with BCG or left untreated. Two weeks after the last immunization, mice were sacrificed and mononuclear cells were isolated from the lungs. Production of IFN-γ by lung
mononuclear cells was assayed by ELISPOT upon stimulation with PPD. Data are expressed as the mean cytokine-producing cells per $0.5 \times 10^5$ cells ± S.E.M. of five mice per group. **$p<0.01$ (Mann-Whitney U-test)

Taking this hypothesis into consideration, we first investigated the effect of the route of immunization on the immunogenicity of the mycobacterial PstS-1 antigen in the respiratory tract. For this purpose, mice were immunized by i.n. or i.p. routes or with a combination of both, using the PstS-1 antigen in combination with CT. Our results showed that the i.n. route of immunization elicited strong local immune responses in the respiratory tract in addition to systemic immune responses, whereas the i.p. route elicited only systemic responses. The local respiratory responses induced by i.n. immunizations were characterized by a significant expansion of CD4$^+$ T cells in the lungs and a mixed Th response, comprising cells producing IFN-γ, IL-4 and IL-5. Moreover, local responses were also characterized by the induction of strong IgA responses in the upper (NALTs and saliva) and lower (lungs and bronchoalveolar lavage, BAL) respiratory tract. The systemic immune responses evoked by i.n. immunizations were characterized by high levels of serum IgG1 and IgG2a. In contrast, the systemic responses elicited by i.p. immunizations were characterized by high levels of serum IgG1.

Noteworthy is to mention that, when combining i.n.-priming/i.p.-boosting, we found an increase in the production of IL-4 and IL-5 that was comparable to that seen when only i.n. immunizations were used, however no IFN-γ production was detected. These results indicate that the local production of IFN-γ in the lungs is only achieved after intense pulmonary stimulation by i.n. delivery of the antigen and that, in the absence of stimuli the IFN-γ producing cells might migrate to other tissues.

The induction of local immunity in the respiratory tract following i.n. immunizations is consistent with the idea that it is essential to target the MALT to initiate mucosal local immune responses (Kiyono et al., 1992). Yet, as indicated in our study, the type of Th cell responses evoked upon immunizations might be influenced by the route of antigen administration. In this regard, i.n. route of immunization appears to favor the induction of, local and systemic, Th1 and Th2 type of responses. In contrast, i.p. route of immunization mainly favors the induction of systemic Th2 type of responses. Moreover, as shown in other studies oral route of immunization appears to preferentially stimulate local and
systemic Th2 type of responses. Similarly, s.c. route has been shown to elicit preferentially Th2 type of responses (Mann et al., 2004; Stertman et al., 2004).

Since the adjuvant has been proposed to be a factor influencing the induction of mucosal immune responses, we further compared CT, a non-toxic pertussis toxin mutant (detPT) and a purified glycoprotein extract from *klebsiella pneumoniae* (RU 41.740) as mucosal adjuvants. CT is one of the most potential and commonly used adjuvants for induction of mucosal immune responses (Liang et al., 1989; Matsuo et al., 2000). Likewise CT, the detPT has been shown to be a potent mucosal adjuvant (Roberts et al., 1995). RU 41.740 is a potent immunomodulator and has been reported to augment responses in lymphocytes, macrophages and neutrophils (Bonde et al., 1986; Capsoni et al., 1991; Meroni et al., 1987). Our data showed that CT evoked the strongest mucosal responses, which were characterized by a major expansion of B and T cell populations and a significant increase in the IFN-γ production by mononuclear cells in the lungs. In contrast, detPT induced mainly an increase in the B cell population and in the production of IL-4 and IL-5, suggesting that this adjuvant favored the induction of Th2 type of responses. On the other hand, the RU 41.740 preferentially elicited an increase in the T cell population. However, the cytokine production evoked by this adjuvant was relatively modest compared to that induced by CT or detPT.

The mechanisms through which CT, an adjuvant known to preferentially stimulate Th2 type of responses, induces Th1 type of responses when delivered by i.n. route are not fully understood. However, it has been proposed that CT stimulates in the lungs the production of β-chemokines, such as MIP-1α and MIP-1β, which are reported to be chemotactic preferentially for Th1 cells (Jones et al., 2001).

Altogether, our data suggest that CT (delivered i.n.) might be a better adjuvant for the local induction of components of the immune system that have been implicated to play an important role in the protection against TB.

**Paper II**

In this paper we investigated the possible role of IgA in the protection against i.n. infection with *M. bovis* BCG, using IgA deficient mice. Induction of IgA responses in the
respiratory tract, being the site of infection in TB, might be a factor contributing to protective immunity against this disease. However, for many years the prevailing opinion has been that, antibodies have little or no role in protection against TB. Factors leading to this assumption include the difficulties in demonstrating a natural protective antibody response against *M. tuberculosis* and the belief that intracellular pathogens cannot be reached by antibodies (Glatman-Freedman and Casadevall, 1998). Recently, studies from several groups have challenged this traditional dogma and provided information on the ability of antibodies to affect the course of infection caused by intracellular pathogens (Arulanandam *et al.*, 2001; Edelson and Unanue, 2001; Hellwig *et al.*, 2001; Winslow *et al.*, 2000). With regard to TB, some studies have demonstrated that certain antibodies directed to surface-associated epitopes are capable of mediating a beneficial effect on survival of animals infected with mycobacteria (Chambers *et al.*, 2000; Teitelbaum *et al.*, 1998). Moreover, in a recent study passive i.n. administration of IgA monoclonal antibodies, specific for the α-crystallin homologue antigen, was found to reduce bacterial counts in the lungs of mice after aerosol challenge with *M. tuberculosis*. However, the achieved protection was evanescent (Williams *et al.*, 2004). In line with these studies, we showed that IgA−/− mice immunized with PstS-1 formulated with CT were more susceptible to i.n. infection with BCG than similarly treated wild-type (IgA+/+) mice, thereby suggesting a role for IgA in the protection against mycobacterial infection in the respiratory tract.

In order to understand the immune mechanisms leading to a better protection against BCG infection in IgA+/+ mice, the immune responses induced after i.n. immunizations with the PstS-1 formulated with CT were characterized in both, IgA+/+ and IgA−/− mice. As expected, IgA−/− mice had no detectable IgA either in the saliva or in the BAL. However, these mice displayed higher levels of total and antigen-specific IgM than the IgA+/+ mice. More importantly, it was found that immunized IgA−/− mice exhibited impaired T cell responses upon *in vitro* stimulation with mycobacterial related antigens, as indicated by the significant decreased IFN-γ production observed in the lungs of these mice compared to IgA+/+ mice. In addition, deficient mice displayed a significantly reduced TNF-α production in the lungs. Furthermore, analysis of the cytokine responses in immunized mice at week 4 after infection also revealed a significant reduced production of IFN-γ and TNF-α in the lungs of IgA−/− mice compared to IgA+/+ littermate mice.
Although impaired T cell responses have been reported in IgA- (Arulanandam et al., 2001), (Zhang et al., 2002) and in B cell- deficient mice (Vordermeier et al., 1996), the mechanisms explaining this event are not fully understood. It has been recently proposed that IgA deficient mice may present a defect in APC functions, leading to impaired Th cell priming (Arulanandam et al., 2001). However, the mechanisms explaining our finding may also involve signaling through Fc-receptors for IgA. Despite extensive studies of the FcαR (CD89) have been conducted in man, little is known about the structure and function of FcαRs in mice. Using the human FcαR probe, two cDNAs named PIR-A and PIR-B have been isolated from a mouse splenic library; their transcripts were detected in mouse lymphoid cells and predicted on the basis of their sequence to be cellular receptors (Kubagawa et al., 1997). More recently, six mouse genes of a diverse family of FcR homologous have been identified (Davis et al., 2002). While these receptors and a common Fcα/µR (Sakamoto et al., 2001) are expressed in B cells and macrophages, previous studies have reported FcαRs expression on activated mouse T lymphocytes (Sandor et al., 1992). In addition to this, it has recently been found that monomeric and polymeric IgA stimulated TNF-α production and apoptosis in mouse macrophage cell lines (Reljic et al., 2004). Thus, deficiency in IgA, the major isotype present at mucosal sites, may also lead to a significant reduced or inadequate activation of macrophages that account for more than 85 % of the immune cells in the lungs (Lawn et al., 2002).

Taken together, our results imply a role for IgA in the protection of the respiratory tract against TB, by blocking the entrance of the pathogen to the lungs and/or modulating the local pro-inflammatory responses. The fact that the IgA−/− mice either immunized with PstS-1 antigen or exposed to CT alone (although in a lesser degree), exhibited higher bacterial loads than the IgA+/+ littermate mice support that the mechanism of action of IgA in our model might involve both, antigenic specificity and signals through the FcαR or a homologous receptor.

**Paper III**

In parallel to our previous study, we also investigated the role of the pIgR and of actively secreted antibodies, specifically sIgA, in the protection against mycobacterial infections. Being the first line of defense at mucosal sites, sIgA trapped within mucus serves as immunological external barrier through immune exclusion. Moreover, during its transport
towards the lumen pIgA is believed to neutralize pathogens within epithelial cells. On the other hand, the secretory component is also believed to play an important function in the protection of the epithelial barrier. Indeed, beside its role in protecting pIgA from proteolytic degradation, the secretory component has also been proposed to function as a non-specific microbial scavenger preventing epithelial cell-pathogen interactions (Phalipon and Corthesy, 2003).

The availability of pIgR<sup>−/−</sup> mice has offered the unique opportunity to explore the relative contribution of secretory antibodies, being primarily sIgA, versus systemic immunity in the protection against mycobacteria. Hence, in our study the pIgR<sup>−/−</sup> mice, which cannot actively transport IgA or IgM into the lumen, were used in challenge experiments with <i>M. bovis</i> BCG or with a virulent strain of <i>M. tuberculosis</i>. Our results showed that actively secreted IgA plays a role against mycobacterial infections. Importantly, a role for sIgA in the modulation of mycobacteria-induced pro-inflammatory immune responses, at the early stage of the infection, was suggested by the reduced expression of factors such as TNF-α and iNOS in pIgR<sup>−/−</sup> mice compared to wild-type mice.

We initially compared the antibody responses between wild-type and pIgR<sup>−/−</sup> mice, induced upon i.n. immunizations with the PstS-1 antigen formulated with CT. As expected, no antigen-specific IgA antibodies were detected in the saliva. However, high levels of antigen-specific IgA antibodies were found in the BAL. The presence of antigen-specific IgA in the BAL of deficient mice might be due to some mechanism of passive transport or leakage, which has been indeed reported in earlier studies (Johansen <i>et al.</i>, 1999). Nevertheless, this result suggests differences in the mechanisms of IgA transport to the lumen between the upper and lower respiratory tract. Most likely, the IgA transport across the epithelium in the upper respiratory tract is mainly dependent on pIgR-mediated mechanisms while, in the lower respiratory tract IgA transport involves both, pIgR-mediated and passive-diffusional mechanisms.

Noteworthy is to mention that, the secretory component has been reported to contribute to the pIgA stability and anchoring to the mucus (Phalipon and Corthesy, 2003). Hence, despite the presence of antigen-specific IgA antibodies in the BAL of the pIgR<sup>−/−</sup> mice, the fact that these IgA molecules lack the secretory component probably interfere with the optimal performance of the IgA in the mucosal immunity.
When the susceptibility of the plgR−/− mice to i.n. infection with *M. bovis* BCG was examined, our data clearly showed that the plgR−/− mice displayed higher bacterial loads than wild-type mice. Importantly, cytokine analysis at week-4 post-infection showed a significant reduction in the production of IFN-γ and TNF-α by lung mononuclear cells. As stated before, IFN-γ and TNF-α play a significant role in protective immunity against mycobacterial infections (Chan *et al.*, 1992; Denis, 1991). Consequently, the impaired mycobacteria-induced pro-inflammatory immune responses seen in plgR−/− mice were in line with the higher bacterial loads found in the lungs of these mice.

We further studied the role of sIgA in the natural protection against aerosol infection with *M. tuberculosis*. Our results showed that the plgR−/− mice were significantly more susceptible than wild-type mice at the early phase of infection (week-3 post-infection). However, no major differences were seen between both mouse strains at the late phase (week-8 post-infection). The impaired control of *M. tuberculosis* growth in the lungs of plgR−/− mice was associated to a substantial reduced expression of IFN-γ, TNF-α and iNOS in these mice compared to wild-type mice. Interestingly, deficient mice also displayed reduced expression of the chemokine RANTES, which is involved in the attraction of monocytes and lymphocytes to the site of infection as well as in promoting Th1 type of responses (Chensue *et al.*, 1999; Dairaghi *et al.*, 1998). Additionally, histological analysis of the lungs was performed to investigate the nature of granuloma infiltrations. At week-3 post-infection, the histological appearance of granulomas in wild-type mice was characterized by clustering of lymphocytes, macrophages and neutrophils and minimal necrosis and karyorrhexis. In contrast, granulomas in the plgR−/− mice were characterized by high infiltration of neutrophils, but reduced numbers of lymphocytes and macrophages, and higher necrosis and karyorrhexis. At week-8 post-infection no major differences in the cellular composition of the granulomas was detected between the two mouse strains. The higher infiltration of neutrophils seen in plgR−/− mice could be explained by the lack the secretory component in the mucosal secretions of these mice. The secretory component has been proposed to bind IL-8 forming an inactive complex, which results in the inhibition of IL-8 mediated recruitment of neutrophils to the airways (Marshall *et al.*, 2001). Hence, in the absence of secretory component, IL-8 can exert its activity as a neutrophil chemoattractant.
In a broader perspective we could hypothesize that, upon infection the pIgR−/− mice fail to up-regulate expression of RANTES and consequently, to efficiently induce the attraction of monocytes and lymphocytes to the lungs. As a result, there is a delay in the induction of immune responses at the site of infection allowing the growth of the bacilli. By week-3 post-infection, the higher bacterial loads existing in the lungs of deficient mice begin then to trigger higher granulomatous infiltration. This infiltration results in the later effective control of the bacilli growth as indicated by the low bacterial loads present in the lungs of pIgR−/− mice at week-8 post-infection.

Taken together, our results suggest a role for sIgA in the modulation of mycobacteria-induced pro-inflammatory immune responses and consequently in the protection against TB in the early phase of the infection.

**Paper IV**

In this paper we aimed to investigate the role of IgA in the protection against another pathogen causing respiratory tract infections, namely *C. pneumoniae*. This pathogen shares several characteristics with *M. tuberculosis*. It enters the body via the respiratory tract and targets alveolar macrophages as its preferred habitat. Likewise *M. tuberculosis*, mechanisms of survival inside macrophages reported for *C. pneumoniae* involve the inhibition of the phagosome-lysosome fusion, inhibition of apoptosis and downregulation of MHC molecules in the infected cells.

We first compared the capacity of two *C. pneumoniae* antigens namely, MOMP and the HSP-60, to induce protective local immune responses against infection with this pathogen. The protective capacity of these antigens was evaluated when given as DNA or protein antigen, and when administered by i.n. or i.p. route. MOMP is an immunodominant antigen in *C. trachomatis* that induces production of neutralizing antibodies (Su and Caldwell, 1991). Furthermore, HSPs seem to play an important role in the immunopathogenesis of chlamydial infections (Eckert *et al.*, 1997). Additionally, HSPs appear to have immunoregulatory properties, by activation of the innate immune system (Srivastava *et al.*, 1998). Our results showed that i.n. immunizations with both antigens delivered as DNA were protective against i.n. challenge with the bacteria. Protective immunity conferred by DNA immunizations was probably due to the induction of local T
cell-mediated immune responses, which are known to play a central role in protection against chlamydial infections (Magee et al., 1995; Perry et al., 1997; Rothfuchs et al., 2004).

Comparison between the respective protective capacity of MOMP and HSP-60, when both were delivered as protein antigens showed that i.n. immunizations with MOMP conferred some degree of protection against *C. pneumoniae* infection. In contrast, i.n. immunizations with HSP-60 did not protect the mice against infection by the bacteria. Furthermore, i.p. immunizations with either antigen did not protect mice against *C. pneumoniae* infection. The lack of protection observed in the mice immunized by i.p. route might be due to the poor efficiency of this route of immunization to elicit immune responses in the respiratory tract. Indeed, analysis of the antibody responses revealed that i.p. immunizations with the protein antigens evoked only high specific systemic IgG responses, while i.n. immunizations elicited strong local specific IgA responses in the respiratory tract in addition to systemic specific IgG responses. In line with our previous studies, these results suggest that induction of mucosal immune responses and specifically of mucosal IgA in the respiratory tract by i.n. immunizations, could be important to achieve local protection against *C. pneumoniae* infections. Although in the case of the HSP-60 given as protein, i.n. immunizations did not protect against *C. pneumoniae* infection, we hypothesize that the internal location of this antigen in the bacteria might have interfered with the function of the antibodies. Nevertheless, it was important to confirm whether the protection conferred by i.n. immunizations with MOMP was related to the strong local IgA responses displayed in this group of mice.

Thus, to further investigate if mucosal IgA could play a role in protection against *C. pneumoniae* infections, we conducted challenge experiments using IgA−/− mice i.n. immunized with the external antigen MOMP. Our data revealed that IgA−/− mice were less protected against i.n. infection with *C. pneumoniae* than wild-type littermate mice, thereby suggesting that local IgA antibodies specific to the external antigen MOMP in fact might contribute to the protection against i.n. challenge with *C. pneumoniae*.

Moreover the role of B cells in the natural immunity against *C. pneumoniae* infections was investigated in naïve mice deficient in either, B cells (Igh6−/−) or in both CD8+ T cells and B cells (CD8−/−/Igh6−/−). Our data showed that, B cell deficient mice exhibited only slightly
higher numbers of IFU compared to wild-type mice. However, when lack of B cells and CD8\(^+\) T cells was combined, double knockout mice displayed significantly higher susceptibility than CD8\(^+\) T cell knockout mice to \textit{C. pneumoniae} infection, as compared to wild-type mice, thereby supporting a role for B cells in antichlamydial immunity. The mechanisms by which B cells might contribute to natural protection against \textit{C. pneumoniae} could involve APC function and/or regulation chemokines and adhesion molecules expression leading to recruitment of macrophages and neutrophils, which has been previously reported in B cell deficient mice infected with \textit{M. tuberculosis} (Bosio \textit{et al.}, 2000; Vordermeier \textit{et al.}, 1996).

Despite a central role for cellular immunity in the protection against \textit{C. pneumoniae} infection was supported by the significantly higher degree of host protection observed in mice immunized with the HSP-60 DNA antigen. In this paper we have shown that mucosal IgA might contribute to protection of the lungs against \textit{C. pneumoniae} as indicated by both, the partial protection conferred by i.n. immunizations with the external protein antigen MOMP and the higher susceptibility of similarly immunized IgA\(^-\) mice to i.n. challenge with this pathogen as compared to wild-type mice. The lack of protection seen in mice immunized by i.n. route with the HSP-60 given as protein implies that the mechanism of action of the IgA in our model probably involved antigenic specificity and neutralization the pathogen and/or inhibition its entrance into the lungs. Indeed, a protective effect of neutralizing antibodies has been reported in previous studies for other \textit{Chlamydiae} species such as \textit{C. trachomatis} (Pal \textit{et al.}, 1997; Whittum-Hudson \textit{et al.}, 1996) and \textit{C. psittaci} (de Sa \textit{et al.}, 1995). Additionally, a role of B cells in the natural immune protection was supported by the higher susceptibility of CD8\(^-\)/Igh6\(^-\) mice compared to CD8\(^+\) T cells deficient mice.

\textit{In vitro} studies in relation to the mechanisms of action of IgA (in progress)

In view of the results showing impaired cytokine production in IgA\(^-\) mice upon immunization or BCG infection, we next attempted to understand the mechanisms by which lack of IgA might have affected the pulmonary pro-inflammatory response. For this purpose, cytokine production by lung mononuclear cells and spleen cells isolated from IgA\(^-\) and IgA\(^+\) mice was assayed after \textit{in vitro} stimulation with, Con A, LPS and 19kDa antigen from \textit{M. tuberculosis}. Con A is a potent polyclonal T cell activator through its
cross-linking with glycoproteins that are present on the surface of these cells. LPS is a potent stimulator of B cells and macrophages, through its interaction with TLR4 molecules present on the surface of both cells. The mycobacterial lipoprotein 19kDa activates macrophages and neutrophils, through its interaction with TLR2 molecules present on the surface of these cells (Brightbill et al., 1999; Thoma-Uszynski et al., 2000).

The assessment of the cytokine production in lung mononuclear cells showed no major differences in the IFN-γ production between IgA^-/- and IgA^+/+ mice, upon co-culture with any of the stimuli (Fig. 6). However, when the levels of TNF-α were measured, a significant reduction in the production of this cytokine was seen in IgA^-/- mice, upon stimulation with LPS and 19kDa, compared to that seen in IgA^+/+ mice. Furthermore, cytokine analysis of spleen cells showed no significant differences in the production of IFN-γ or TNF-α, between IgA^-/- and IgA^+/+ mice upon activation using any of the stimuli.

**Figure 6.** Cytokine production in IgA^-/- and IgA^+/+ mice upon stimulation with Con A, LPS and 19kDa. Lung mononuclear and spleen cells isolated from naïve IgA^-/- and IgA^+/+ were cultured in the presence of Con A, LPS and 19kDa. After 48h supernatants were collected and the amounts of TNF-α and IFN-γ were measured using ELISA. *p<0.05 (Mann-Whitney U-test)
From these results a few implications could be depicted: 1) T cells appear to be similarly activated in both, IgA⁻/⁻ and IgA⁺⁺/⁺⁺ mice, as indicated by the equivalent production of IFN-γ by lung mononuclear cells in these mouse strains; 2) IgA⁻/⁻ mice appear to have a diminished or weakened activation in either B cells or macrophages, as suggested by the impaired TNF-α production in these mice after stimulation with LPS or 19kDa; 3) The defect in the activation of B cells and/or macrophages found in IgA⁻/⁻ mice is apparently restricted to mucosal sites, in our case the lungs.

To further investigate these implications, lung mononuclear and spleen cells from IgA⁻/⁻ and IgA⁺⁺/⁺⁺ mice were subjected to flow cytometry analysis upon stimulation with LPS or the 19kDa. When analyzing the LPS-stimulated lung mononuclear cells, results showed no disparities in the proportion of T cells between IgA⁻/⁻ and IgA⁺⁺/⁺⁺ (Fig. 7A). Similarly, no major differences in the proportion of B cells were seen between both mouse trains (Fig. 7B). However, an increase in the proportion of macrophages, with a marked additional population was detected in the lung cells of IgA⁺⁺/⁺⁺, but not in lung cells of IgA⁻/⁻ mice (Fig. 7C). In addition, IgA⁺⁺/⁺⁺ mice displayed a higher proportion of cells expressing the activation surface markers, MHC class II and CD40 (Fig. 7D, E respectively). The MHC class II and the CD40 are activation markers that can be expressed on both, B cells and macrophages. Therefore, a double-staining of the cells was performed to assess possible differences in the proportion of B cells expressing either CD40 or MHC class II, between IgA⁻/⁻ and IgA⁺⁺/⁺⁺ mice. Our results showed no differences in the proportion of B cells expressing these activation markers between wild type and deficient mice (Fig 8).

When examining the LPS-stimulated spleen cells, our data showed no major differences in the proportion of T and B cells or in the proportion of cells expressing MHC class II or CD40 activation markers, between wild type and deficient mice (data not shown). Noteworthy is to mention that in contrast to the lung cells, spleen cells had no detectable positive population of macrophages reflecting the low percentage of macrophages in this organ. Furthermore, the analysis of lung mononuclear and spleen cells upon stimulation with the 19-kDa showed similar results as with the LPS stimulation (data not shown).
Figure 7. Flow cytometry analysis of lung mononuclear cells from IgA+/+ and IgA−/− mice upon stimulation with LPS. LPS-stimulated lung mononuclear cells were collected after 48h and stained with antibodies specific for A) CD3, B) B220, C) F4/80, D) H-2b and E) CD40 surface markers.
Figure 8. Analysis of the expression of stimulatory markers in B cells from IgA^{+/-} and IgA^{-/-} mice upon stimulation with LPS. LPS-stimulated lung mononuclear cells were collected after 48h. Two-color flow cytometry analysis was performed in mononuclear cells previously stained with pair of antibodies as follows A) anti-B220-FITC and anti-H-2b-PE B) anti-B220-FITC and anti-CD40-PE. Double-positive stained cells are situated in the upper right quadrants of each dot plot.

The flow cytometry results corroborated that IgA^{-/-} mice present indeed a defective response to stimulation with LPS or 19kDa which appears to be both, due to poor stimulation of macrophages and restricted to the lungs. Further experiments using lung macrophage-enriched cultures and bone-marrow derived macrophages isolated from both mouse strains have also supported this observation (Fig. 8). In these experiments, LPS-stimulated lung macrophage-enriched cultures from IgA^{-/-} mice exhibited a significantly reduced nitric oxide production compared to IgA^{+/-} mice. Moreover, no differences were seen between the two mouse strains, in similarly stimulated bone-marrow derived macrophages. As discussed before, mechanisms explaining the defective stimulation of pro-inflammatory responses in the lungs of IgA^{-/-} mice might involve impaired APC function or impaired signaling through the FcαR or a homologous receptor, which may lead to unsuccessful activation of pulmonary macrophages. In any case, poor activation of macrophages might result in a suboptimal stimulation of production of cytokines and chemokines which are required for attraction or migration of immune cells to the lungs.
**Figure 8.** Production of nitric oxide by macrophages from IgA⁻/⁻ and IgA⁺/⁺ mice upon stimulation with LPS and 19kDa. Lung macrophages-enriched cultures and bone-marrow derived macrophages isolated from naïve IgA⁻/⁻ and IgA⁺/⁺ were stimulated with LPS and 19kDa. After 48h supernatants were collected and the amounts of nitric oxide were measured. *p<0.05 (Mann-Whitney U-test)
CONCLUDING REMARKS

The fact that, antibodies can favorably affect the course of infection by intracellular pathogens, if present at the moment and at the site of infection, opens new possibilities for vaccine development against respiratory tract infections. However, elucidation of the condition for optimal antibody function against these infections, such as antibody specificity, is essential.

The presence of antibodies at the site of infection is of particular importance. In this regard, IgA has the potential advantage of being the most abundant Ig produced at mucosal sites. The data in this thesis support a role for mucosal IgA in the protection of the respiratory tract against pathogens such as *M. tuberculosis* and *C. pneumoniae*. Moreover, our results suggest that the mechanisms by which IgA exerts its protective effects most likely involve both, antigenic specificity of the antibodies in the secretions resulting in the inhibition of the pathogen entrance to the lungs and/or stimulation of macrophages in the lungs resulting in optimal induction of local immunity.

The progress made during the past years regarding the role of antibodies in protection against intracellular pathogens is encouraging. The challenge for the coming years will be to thoroughly dissect the mechanisms of action of antibodies in protective immunity against pathogens and to develop vaccine candidates that will work by inducing protective antibody responses.
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