1. Introduction

1.1 Tuberculosis (TB)

Tuberculosis (TB) is a common and often deadly infectious disease caused by a unique infectious agent. Actually, one third of world's population is infected with TB bacillus, but only one tenth develops the pathology. According to last World Health Organization (WHO) estimates, there are eight millions of new cases of tuberculosis, annually, concentrated above all in developing countries: sub-Saharian Africa, Asiatic South-East and Latin America are the most hit areas. (see Fig.1). Incidence TB increment is strictly associated to infection with HIV, etiological agent of Acquired Immunodeficiency Syndrome (AIDS) (Corbett et al., 2002). This viral infection, seriously, alters immune system making HIV positive individuals more susceptible to opportunistic infections and to tuberculosis. The reciprocal interaction between these two pathogens influences outcome of two diseases. In this context while HIV infection makes TB individuals more susceptible to reactivation of latent forms of TB, this facilitates viral infection progression towards AIDS (Mancino et al., 1997). In industrialized countries TB incidence is, significantly, decreased within 1800 and 1940, before anti-tubercular drugs development due to improvement of life conditions.

Nevertheless, recent epidemiologic data (Smith et al., 2003) showed a reversal trend in occidental countries since 1985 because of immigration from high TB incidence countries and, in minor size, of HIV diffusion. Another factor that contributes to TB infection is appearance of multi-drug resistant MTB strains.

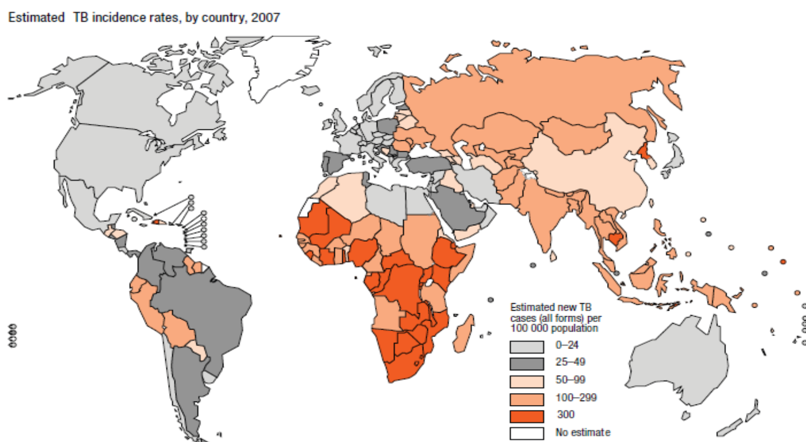


Fig. 1 Estimated TB incidence rates, by country, 2007 (WHO).

In most cases, TB is a curable disease, but restrict access to health care is an impediment for many people in developing countries. Infection by *Mycobacterium tuberculosis* is efficiently controlled by the immune system, as the vast majority of the billion infected humans contain infection, unless their immune system is compromised. The organism is a slow-growing acid-fast bacillus

transmitted primarily by the respiratory route, and although it may cause disease in most organs, pulmonary tuberculosis is the most common. The immune response mounted to the infection is generally successful in containing, although not eliminating, the pathogen. Active tuberculosis may result in a small percentage of infections, probably because of the lack initiation of an appropriate immune response. The clinical latency often extends for the lifetime of the individual. However, reactivation of the latent infection may occur in response to perturbations of the immune response, and active tuberculosis ensues (Chan et al., 2004). *M. tuberculosis* persists in macrophages within a granuloma in the organs of infected hosts. The granuloma consists of macrophages and giant cells, T cells, B cells and fibroblasts. In latent infections, the state of bacteria within the granuloma, or tubercle, is not known. The organism may be in a dormant non-replicating state, actively replicating but killed by the immune response, or metabolically altered with limited or infrequent replication cycles. Breakdown of immune responses designed to contain the infection may result in reactivation and replication of the bacilli, with necrosis and damage to the lung tissue. Thus, a constant battle between the host and mycobacterium is being triggered, and outcome depends on many factors. Clearly, the organism has developed mechanisms to evade its eradication by a strong cell-

mediated immune response. A more complete understanding of the role each component of the immune system plays in protection or exacerbation of tuberculosis, as well as of the bacterium's weapons to evade those components, will enhance development of preventive and therapeutic strategies against this enormously successful pathogen.

1.1.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a pathogenic bacterial species in the genus *Mycobacterium.* It is an obligate, aerobe and intracellular pathogen. Morphologically *M. tuberculosis* is a bacillus with length 2-4 μm and width 0,2-0,5 μm; alcohol-acid resistance is a characteristic of the *Mycobacterium* genus. In fact these bacteria are relatively impermeable to several basic colors, but upon staining are able to retain colors. The main constituent of delipidated cell walls treated with proteolytic enzymes is called "covalent skeleton" (Fig. 2): it consists of the peptidoglycan to which molecules of arabinogalactan are covalently linked. Free lipids represents about 25% of the weight of crude cell walls. Lipids compounds, as like as waxes and glycolipids are considered typical cell wall constituents. In particular in tubercular bacillus mycolic acids, that upon esterification with alcohols make wax part, are important in alcohol-

acid resistance. Among glycolipids called mycosides there is cord factor (trehalose-6,6-dimycolate), responsible of systemic toxicity, chemokines release from macrophages and an important capacity to induce a granulomatous response. Antigenic activity is also linked to protein and polysaccharide components, like tuberculin reaction induced by Purified Protein Derivative (PPD) and antibodies formation.

The sensitivity of mycobacteria to chemical agents is a typical trait: in fact, among disinfectants only formaldehyde, phenols and iodine are completely active, while acids and quaternary ammonium derivates are unable to kill bacilli. Moreover mycobacteria are resistant to antibiotics, normally active against other schizomycetes. Compounds able to contrast *M. tuberculosis* are: a. streptomycin, with bactericidal activity exerted on cell membrane and interfering with messenger RNA transcription; b. idrazide, rifampicin and other drugs like kanamycin, ethionamide, para-aminosalicylic acid and viomycin.

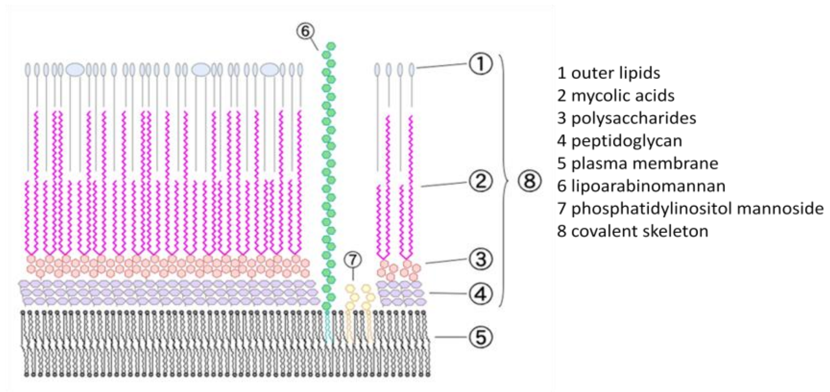


Fig 2. Representation of mycobacterial cell wall.

1.1.3 Immunopathology of tuberculosis

M. tuberculosis normally enters the host through the mucosal surfaces, usually via the lung, after inhalation of infectious droplets from an infected individual, and only occasionally via the gut after ingestion of infected material. Either way, the bacteria may be taken up by phagocytic cells that monitor these surfaces, and if not swiftly killed, may invade the host inside these cells. Some heavily *M. tuberculosis* exposed individuals show no signs of infection: no pathology, no symptoms and no apparent adaptive immune response. It is possible that in these cases, the innate immune response has

eliminated the pathogen at the earliest stage. More commonly, however, ingestion of the bacteria by an Antigen Presenting Cell (APC) rapidly induces an inflammatory response. Cytokines and chemokines release triggers the swift accumulation of a variety of immune cells and, with time, the formation of a granuloma, characterized by a relatively small number of infected phagocytes, surrounded by activated monocyte/macrophages and, further out, activated lymphocytes (Gonzales-Juarrero et al., 2001). If the infection is successfully contained at this stage, the granuloma shrinks and may eventually disappear, leaving a small scar or calcification and the patient's T cells become responsive to *M. tuberculosis*-derived antigens. If, conversely, the immune response does not successfully control the bacterial replication, the granulomas increase in size and cellularity. Eventually, cell death in the granuloma leads to necrosis. In this case, if the granuloma is close to the surface of the lung, the tissue destruction caused by necrosis may breach the mucosal surface and the granuloma contents leak into the lumen of the lung a process referred to as cavitation. This gives rise to the prototypic symptom of TB: a persistent cough with blood in the sputum. At this point, the patient is highly infectious, spreading the bacteria by aerosol. Tissue destruction in TB is not mediated by the activities of the bacteria alone, it is

primarily immunopathological in nature and the crucial point to understand is that an inflammatory immune response is critical for the survival of both the host and the bacteria. It thus appears that *M. tuberculosis* actively stimulates and then subverts this response.

The outer surface of *M. tuberculosis* contains a number of molecules that bind to the host's pathogen-associated molecular pattern (PAMP) receptors, such as the Toll-like Receptor (TLR) family (Kort et al., 2005). Thus, although engagement of PAMP receptors appears to be a crucial initial step for anti-mycobacterial immune responses (Quensniaux et al., 2004; Stenger et al., 2002), all clinical strains of *M. tuberculosis* express a number of molecules (both expressed on the bacteria's surface and secreted) that trigger these pathways. Interestingly, the majority of these molecules do not seem to be crucial to mycobacterial viability and, given that this pathogen has a long co-evolutionary history with human beings (Brosch et al., 2001; de Jonge et al., 2005), it suggests that their conservation serves another important function. The simplest explanation is that *M. tuberculosis* depends on the Immunopathology that promotes cavitations for spread to new hosts. A failure to stimulate inflammatory immune responses is therefore an evolutionary dead end for the bacteria. At the same time, the same immune responses are essential for the host to control bacterial replication. This balance

is clearly illustrated by the course of TB in HIV-infected individuals, whose immune deficiency renders them simultaneously more susceptible to fatal bacteremia, and less infectious than normal, because they cavitate less frequently than people with an intact immune response (Aaron et al., 2005). Thus, because it cannot evade the induction of cell-mediated immunity, *M. tuberculosis* has evolved to survive it, and, even if the initial infection is successfully controlled, many infected individuals develop a latent infection that may persist for decades (Andersen et al., 2007; Manabe et al., 2000; Morrison et al., 2008; Stewart et al., 2003).

1.2. Innate immune response

1.2.1 Interaction with macrophages and innate immune response

A major component of *M. tuberculosis'*s success as a pathogen is represented by its ability to survive within host cells, especially immune cells such as macrophages. These cells are in charge both to kill bacteria directly by phagocytosis, and to prime the adaptive immune response by antigen presentation.

M. tuberculosis interferes with the process of macrophage activation and phagocytosis at virtually every stage (Fig.3). This interference starts immediately on contact between the bacteria and the cell's

receptors. Mannose derivatives on the bacillus' surface, from pathogenic (but not from non-pathogenic) mycobacteria, inhibit the phagocytosis by activated macrophages (Stokes et al., 2004) and therefore potentially allow the pathogen to target cell types more susceptible to infection. It is known that Lipoarabinomannan (LAM), a major cell wall component of *M. tuberculosis*, may bind to the DC-SIGN molecule, expressed on the surface of Dendritic cells. DC-SIGN is crucial to Dendritic cells maturation, and LAM binding inhibits this process, decreases IL-12 production and induces Dendritic cells (DCs) to secrete IL-10 (Appelmeck et al., 2003; van Kooyk et al., 2003), which inhibits antigen presentation, expression of major histocompatibility complex (MHC) molecules and expression of co-stimulatory receptors. Accordingly, recent studies have found that expression of IL-10 is significantly elevated in TB patients with active disease (Jang et al., 2008; Olobo et al., 2001; Redpath et al., 2001). In addition, the cell wall of *M. tuberculosis* includes many long-chain fatty acids (Korf et al., 2005; Quesniaux et al., 2004; Brennan et al., 2003; Briken et al., 2004) that strongly stimulate host inflammatory responses, leading to granuloma formation (Sugawara et al., 2002), up-regulation of antigen presentation and subsequent NK and T-cell responses (Hunter et al., 2006; Rill et al., 2001). If this immunological process was allowed to

develop as described above, the infection would be rapidly eliminated. However, some of those lipoproteins apparently modulate this process to the pathogen's advantage. The 19 kDa lipoprotein of *M. tuberculosis* interacts with host APCs via TLR1/2 (Sugawara et al., 2003; Takeda et al., 2002), but instead of activating protective immunity, this leads to inhibition of cytokine production, reducing the expression of over one third of the interferon (IFN)-γ-activated genes (Pai et al., 2004), and reduced antigen-processing and MHC II expression (Pai et al., 2004; Fortune et al., 2004; Nos et al., 2001). This lipoprotein appears to be a virulence factor (Henao-Tamayo et al., 2008) that reduces overall immunity to the bacterium in mice (Yeremeev et al., 2000). ESAT-6 protein has a similar effect, also operating through TLR-2 (Pathak et al., 2007). These two mentioned MTB proteins and similar molecules may contribute to the virulence of epidemic Beijing strains of *M. tuberculosis* in humans by inducing higher levels of IL-4 and IL-13 than non-epidemic strains (Manca et al., 2004; Reed et al., 2004). TLR2/4 ligation was once considered crucial to the inflammatory response to mycobacteria (Jo et al., 2007; Salgame et al., 2007), but now it appears more like interfering in IFN-γ-signaling via TLR signaling and a potential virulence mechanism (Reiling et al., 2008). It has even been suggested that by turning the expression of proteins on or

off, such as the 19 kDa decoy molecule, *M. tuberculosis* may evade immune surveillance during the latent phase of infection (Pai et al., 2004; Noss et al., 2001; Fulton et al., 2004), still allowing the initiation of inflammatory immune responses leading to tissue destruction and cavitations during the acute infection or reactivation.

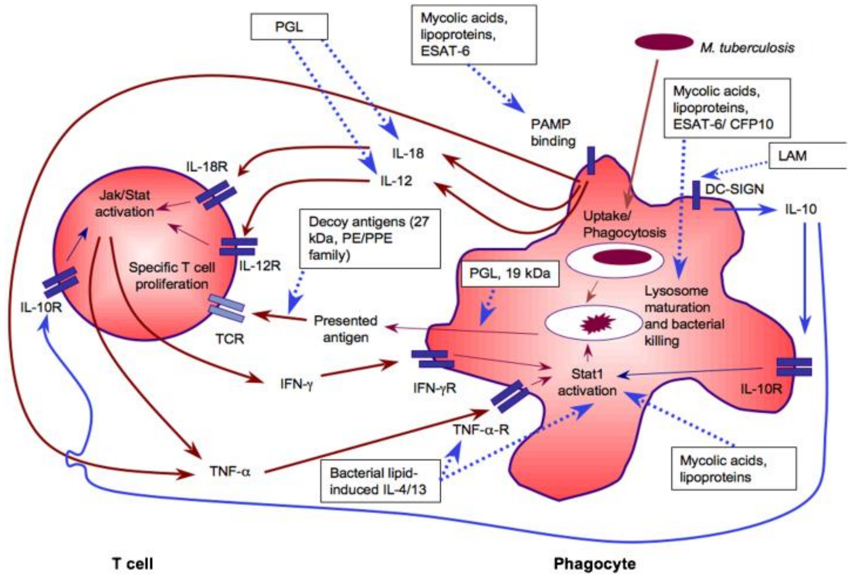


Fig. 3 Innate immune response of TB *(APMIS, 2009).*

1.2.2 Toll like receptors during *M. tuberculosis* infection

Toll-like receptors play a pivotal role in the induction of an innate immune response to various infectious agents, including *Mycobacteria* spp. TLR2 was classically recognized as a principal inducer of the pro-inflammatory signal, tumor necrosis factor (TNF)-α, induced by whole MTB (Underhill *et al*., 1999). Several lines of evidence suggested the protective role of TLR2 in mycobacterial infection. TLR2-deficient mice are impaired in host resistance and neutrophil responses to the infection with virulent *M. avium* strain (Feng et al., 2003). Recent studies, using a human-like infection model, show that certain TLR knockout mice are more susceptible than wild-type mice at an early stage of respiratory tract infection; in particular, TLR2–/– mice are more susceptible than TLR4–/– or wild type mice (Tjarnlund et al., 2006). In addition, defective capability of intracellular killing, preferentially in TLR2–/– macrophages, was correlated with impaired production of TNF-α (Tjarnlund *et al*., 2006), which is vital for containment of mycobacterial infections (Roach et al., 2002).

Recent studies by Liu et al. (2006) revealed a mechanism whereby TLR2/1 function by regulating the responses to infection with MTB. They showed that TLR2/1 signaling mediated by MTB augments the expression of the vitamin D receptor and the vitamin D hydroxylase,

thereby leading to increased expression of antimicrobial peptides (Liu et al., 2006). TLR4 may contribute to resistance to MTB infection, but consensus has not been reached on this issue. Studies using an intranasal infection model with live MTB demonstrate that TLR4 plays a protective role in host defense against murine pulmonary TB *in vivo*, as reflected by an increased mortality and mycobacterial load in the lungs of mice with a non-functional TLR4 (Branger et al., 2004). In addition, an increased susceptibility to MTB was reported in C3H/HeJ mice which have a non-functional TLR4, as reflected by an enhanced mycobacterial outgrowth and an increased mortality (Abel et al., 2002). In contrast, TLR4-deficient animals are indistinguishable from wild-type controls in a model of *M. avium* infection (Feng et al., 2003).

Importantly, Reiling highlighted the effect of inoculum sizes in studies assessing the relative importance of TLR4 and TLR2 in resistance to airborne MTB infection. High-dose aerosol MTB challenge revealed TLR2-, but not TLR4-deficient mice to be more susceptible than control mice, whereas TLR2- and TLR4-deficient mice were as resistant as control mice at a low-dose challenge with MTB (Reiling et al., 2002). Whereas molecules that generate TLR2-dependent signals are entirely dependent upon myeloid differentiation primary response protein 88 (MyD88), TLR4

generates both MyD88-dependent and MyD88-independent signaling. It is possible, then, that host defense could be affected by TLR4 in a MyD88-independent manner. In summary, the role of TLR4 in MTB infection *in vivo* remains unclear, with results depending on the infection model, strains of MTB and inoculum size. TLR9 also plays a role in response to MTB. Mice lacking both TLR9 and TLR2 were significantly more susceptible to MTB infection than mice with single deficiency of either TLR2 or TLR9 (Bafica et al., 2005), indicating a significant role for TLR9 and cooperation between TLR9 and TLR2 in host defense to mycobacterial infection. In addition, induction of interleukin (IL)-12 and CD86 levels expression by DCs infected with *M. bovis* BCG is not exclusively dependent on TLR2, and is abrogated only in TLR2/4/9- deficient DCs, supporting a pivotal role of TLR9 in the recognition of *M. bovis* BCG by murine DCs (von Meyenn et al., 2006). Taken together, these data suggest a role for signaling by multiple TLRs in protection against mycobacterial infection, particularly in the acute phase of respiratory tract infection (Tjarnlund et al., 2006).

1.2.3 Macrophages and antimycobacterial mechanisms: Reactive Oxygen Intermediates (ROI) and Nitrogen Oxides

It is well established that murine macrophages possess antimycobacterial function in tissue culture system (Lurie 1942; Suter 1952; Mackaness 1969). When activated by supernatants of immunologically stimulated lymphocytes, macrophages exhibited various degrees of antimycobacterial activity (Chan et al., 1994). Hydrogen peroxide (H_2O_2), one of the reactive oxygen intermediates (ROI) generated by macrophages via the oxidative burst, was the first identified effector molecule that mediated mycobactericidal effects of mononuclear phagocytes (Walker et al., 1981), but their role is still controversial in host defense against *M. tuberculosis*. Later, IFN-γ was found to be the key endogenous activating agent that triggers the antimycobacterial effects on murine macrophages (Rook e al., 1986; Flesh et al., 1986), but reports of the effect of IFN-γ treated human macrophages on the replication of *M. tuberculosis* range from being inhibitory (Chan et al., 1994) to enhancing (Douvas et al., 1985). This inconsistency aroused several doubts on the antimycobacterial capability of human mononuclear phagocytes, until the demonstration that 1,25-dihydroxy vitamin D_3 [1,25-(OH)2D_3], alone or in combination with IFN-γ and TNF-α, was able to activate macrophages to inhibit and/or kill *M. tuberculosis* in the

immune system (Crowle et al., 1987; Rook et al., 1988; Denis et al., 1991). Moreover, 1,25-(OH)2D_3 was reported to induce the expression of the NOS_2 and *M. tuberculosis* inhibitory activity in human HL-60 macrophage-like cell line (Rockett et al., 1998). This observation identifies NO and related RNI as the putative antimycobacterial effectors produced by human macrophages.

High output nitric oxide (NO) production by immunologically activated macrophages is a major antimicrobial mechanism (Fang et al., 1997; MacMicking et al., 1997; Chan eta l., 1999). These phagocytes, upon activation by appropriate agents such as IFN-γ and TNF-α, generate NO and related RNI via NOS_2 using L-arginine as the substrate. The significance of these toxic nitrogen oxidants in host defense against *M. tuberculosis* has been well documented, both in vitro and in vivo, particularly in murine system (MacMicking et al., 1997; Chan et al., 1999; Shilio et al., 2000). In the mouse, RNI play a protective role in both acute and chronic infection (MacMicking et al., 1997; Flynn et al., 1998). More importantly, accumulating evidence strongly supports a role for RNI in host defense in human tuberculosis (Nicholson et al., 1996; Wang et al., 1998). In addition, the levels of exhaled NO was shown to be increased in tuberculosis patients (Wang et al., 1998). While the role of macrophage NOS_2 in host defense against *M. tuberculosis* is well

established, the significance of toxic oxygen species in the control of tuberculosis remains controversial. Despite the demonstration that H_2O_2 generated by cytokine-activate-macrophages was mycobactericidal (Walker et al., 1981), the ability of ROI to kill *M. tuberculosis* remains to be confirmed (Flesch et al., 1987; Chan et al., 1992). Indeed, mycobacteria are capable of evading the toxic effect of ROI by various means (Chan et al., 1994). For example, mycobacterial components Lipoarabinomannan (LAM) and phenolic-glycolipid I (PGL-I) are potent oxygen radical scavengers (Chan et al., 1989; Chan et al., 1991). In addition, mycobacterial sulfatides interfere with the oxygen radical-dependent antimicrobial mechanism of macrophages. Despite these findings, a role of ROI in defense against the tubercle bacillus cannot be entirely excluded. Mice deficient in the NADPH oxidase complex exhibit modestly enhanced susceptibility to *M. tuberculosis* infection (Adams et al.,1997; Cooper et al., 2000).

1.3 Adaptative immune response

It has been suggested that invasion of phagocytes not yet activated is important for the bacteria's survival because exposure of macrophages to IFN-□ and/orTNF-□ before but not after infection, decreases the ability of pathogenic mycobacteria to inhibit

phagosome maturation and function (Deretic et al., 2006), at least partially, by up-regulating the production of reactive oxygen and nitrogen derivatives (Axelrod et al., 2008; Davis et al., 2007; Nathan et l., 2006; Schon et al., 2004; Green et al., 1994). However, the production of these cytokines is dependent on activating the adaptive arm of the immune response.

Most individuals respond initially to *M. tuberculosis* infection by producing IFN-γ, and it has been hypothesized that the unconventional T-cell subsets γδ, NK-T and CD-1 restricted cells (Ladel et al., 1995; Ladel et al., 1995), whose receptors are far less variable than that of T cells restricted by conventional MHC I and II molecules, act as a bridge between the innate and the adaptive immune responses by 'kick starting' cytokine production (Schaible et al., 2000; Ulrichs et al., 2000). It is known that γδ T cells and CD1-restricted T cells expand considerably during the early phases of *M. tuberculosis* infection, (Schaible et al., 2000; Ulrichs et al., 2000) and by targeting molecules that conventional T cells do not (such as lipids and glycoproteins), they expand the number of cues that the host immune system may respond to (Behar et al., 2007). Data from genetic knockout models of unconventional T cells have shown only minor effects (Ladel et l., 1995; Ladel et al., 1995) and it may be that cytotoxicity against infected APC by TCR1γδ T cells, and

amplification of APC function via non-cognate cytokine production in the early phases of infection by TCR- γδ T cells is their primary function (Caccamo et al., 2006; Dieli et al., 2004). By secreting IFN-γ, they may help activate APCs – boosting the expression of MHC and co stimulatory molecules and amplifying IL-12 and IL-18 production, resulting in a positive feedback loop for IFN-γ production (Caccamo et al., 2006). The importance of IL-12 is highlighted by the observation that gene polymorphisms may affect susceptibility to TB, protection being associated with genotypes leading to high production, and vice versa, while functional mutations in the IL-12 receptor are associated with extreme susceptibility to mycobacterial disease (de Jong et al., 1998; Tso et al., 2004). Control of IL-12 expression is key to the expansion and activation of IFN-γ-secreting CD4+ T cells, which (even more than activation of CD8+ T cells) is most crucial for immunity to TB, as shown by the susceptibility of animals or patients defective in CD4+ T cell function or IFN-γexpression or recognition (Cooper et al., 1993; Flynn et al. 1993; Otthenoff et al., 2005; Saunders et al., 2002; Smayga et al., 2000).

While CD4+ T cells apparently contribute more to the early IFN-γ response, CD8+ T cells are considered to become more important in the later phases of disease possibly via cytotoxic activity

and/or IFN-γ production (Lazarevic et al., 2005; Sud et al., 2006; Woodworth et al., 2006). Activating Th1 responses has thus been a major objective for the vaccines under development. However, *M. tuberculosis* seems to have developed the ability to subvert the host's immune response, in part by directly countering Th1 function and development. Live bacteria or *M. tuberculosis* cell wall extracts may inhibit some of the downstream effects of IFN-γ, although the mechanism is not yet fully defined (Kincaid et al., 2003; Ting et al., 1999; Manca et al., 2005), so that even if IFN-γ is produced, its activity may be reduced. In addition, IFN-γ recall responses are generally reduced in patients with advanced TB (Sodhi et al., 1997), while IL-4 is elevated (Jalapathy et al., 2004; Jimenez-Martinez et al., 2004; Roberts et al., 2007) and the level of IL-4 gene expression appears to correlate with both the disease severity in TB patients (Jalapathy et al., 2004; Jimenez-Martinez et al., 2004) and the risk of subsequent disease in healthy but TB-exposed individuals (Ordway et al., 2004; Wassie et al., 2008). The observation that the IFN-γ/IL-4 ratio increases in most patients during therapy, but decreases in contacts who become ill, suggests that this state is directly related to the disease (Wassie et al., 2008). Consistent with this is the observation that increased production of splice variants that antagonize IL-4 activity (such as IL-4δ2) appears to be characteristic

of individuals who are controlling TB in its latent stage (Demissie et al., 2004). Similar observations have also been made in animal models of TB (Rodhes et al.,2006). Thus, cell wall components such as phosphor-glycolipids or the 19 kDa antigen, which induce IL-4 and IL-13 production, may act as potent virulence factors in clinical strains (Briken et al., 2004; Manca et al., 2004; Reed et al., 2004). Likewise, other factors such as LAM binding to the DC-SIGN receptor on the surface of DC may inhibit IFN-γ production and function by inducing IL-10 (Appelmelk et al., 2004; van Kooyk et al., 2003; Redpath et al., 2001).

A poor prognosis in TB is associated with a low IFN-□ /IL-10 ratio just as seen for IFN-γ/IL-4 (Wassie et al., 2008; Hussain et al., 2007; Sahiratmadia et al., 2007). Altering the balance between IFN-γ and IL-4 or IL-10 production and function thus seems to be a second major survival strategy for *M. tuberculosis*. An equally important molecule for protection is TNF-α (Jacobs et al., 2007), as shown by the rapid reactivation of latent *M. tuberculosis* infection in people treated with TNF-α receptor antagonists (Anon. 2004; Gomez-Reino et al., 2002). The expression of TNF-α is associated with protection in animal models (Flynn et al., 1995; Ogawa et al., 1991), but in the presence of elevated levels of IL-4, TNF-α appears to promote tissue damage rather than protection (Seath et al., 2001; Sharma et al.,

2001). In addition, infection with *M. tuberculosis*, but not avirulent mycobacteria, promotes the shedding of TNF-α receptors by infected macrophages (Lawn et al., 2000; Tsao et al., 2000), which may then serve as soluble antagonists. This paints a picture similar to that seen for IFN-γ: *M. tuberculosis* target both gene expression of IFN-γ and TNF-α and also affect their downstream signal induction. Perhaps not surprisingly, in light of the earlier discussions, TNF-α blockade also seems to have a negative effect on phagosome maturation (Harris et al., 2008). Thus, *M. tuberculosis* seems to have multiple mechanisms targeted toward inhibiting both IFN-γ and TNF-α function and production, and this inhibition has negative consequences for the development of the bactericidal phagosome and the expansion of an effective adaptive immune response.

1.4 CpG oligodeoxynucleotides (ODNs)

More than a century ago Coley discovered that bladder cancer patients showed a regression of tumor, by continued injections of bacterial extracts containing Streptococcus pyogenes, alone or in combination with Serratia marcescens (Coley, 1893; Wiemann et al., 1994). Since this treatment was responsible of different secondary consequences, it was substituted with the employment of Bacillus

Calmette-Guérin (BCG) per parenteral route (Zuany-Amorim et al., 2002). Successively it was observed that clinical results, found with BCG, could be reproduced by administration of bacterial extract containing DNA (Tokunaga et al., 1999). Actually it is documented that bacterial extracts may contain different forms of DNA, as unmethilated CpG motifs which exert protective effects against different pathologies. In fact, in some murine models, CpG motifs activate innate immune response, protect from lethal infections against a wide range of pathogen and contrast pathologies, as asthma, several allergies and different forms of cancer (Klinman et al., 1999). In particular it was documented, in C57BL/6 murine strain, that only one CpG treatment is able to protect from viral, bacterial and parasitic infections (Sethi et al., 2002). In this contest, the protection is evident in early 48 hours from administration and it maintains for some weeks. Moreover, a periodic treatment with CpG allows to conserve the resistance status also for several months (Klinman et al., 1999). CpG motifs are also very effective as vaccine adjuvants, in mice and non human primates, increasing the adaptive (humoral and cellular) immune response (Lipford et al., 1997; Kim et al., 1999). In mice CpG motifs induce a Th1 immune response, which is stronger than the one of other well known adjuvants, as, for example, the Complete Freund Adjuvant (CFA) (Davis et al., 2000).

CpG, structurally, is a dinucleotide constituted by a cytidine-phosphate-guanosine recognized, in specific manner, by the pattern recognition receptors (PRRs) which mediate the innate immune response with mechanisms non completely elucidated (Janeway et al., 2002). In particular, it was reported that CpG may modulate some biological responses like IL-6, IL-12, IL-18 and TNF-α secretion, in dendritic cells and macrophages. These cytokines determinate NK cells activation increasing antigen presenting cells activity through a feedback, IFN-γ dependent, mechanism. Moreover CpG motif causes the induction of antibodies dependent citotoxicity (ADCC) phenomena, because of the enhancement of the Fc receptors expression in poly-morphonucleate phagocytes. Efficacy of antitumor antibodies used in clinical therapy seems to derive, at least partially, from ADCC mechanisms (Krieg et al., 2002).

About CpG motifs recognition it was reported that some cells of the immune system are able to recognize their sequences, artificially reproduced, into wider flanking sequences (8-30 nucleotides), named oligodeoxynucleotides (ODNs). These ODNs are easily synthesized and are often formed by palindromes where is contained at least one CpG motif (Agrawal et al., 2002).

Comparative analysis between frequencies of bacterial and mammalians ODNs has evidenced that the sequences GACGTC,

GACGTT, AACGTC and AACGTT are the examers with the major capacity of stimulate B lymphocytes proliferation. These examers have a frequency in human genome of $0.72 \pm 0,13 \times 10^{-4}$, while in E. coli DNA it is $2,67 \pm 0,86 \times 10^{-4}$ (Han et al., 1994).

CpG dinucleotides are more frequent in bacterial genomes (Yamamoto S. et al., 1992; Kuramoto et al., 1992; Yamamoto S. et al., 1992), where they are methylated and induce B lymphocytes proliferation and also immunoglobulin secretion (Messina et al., 1991). By the way, the motifs with the widest biological activity are non-methylated CpG dinucleotides, flanked by two Purines at the 5' end and two Pyrimidines at the 3' end (Krieg et al., 1995) which are recognized by TLR9.

1.4.1 Toll Like Receptor 9 (TLR9)

TLR9 detects bacterial DNA, which is able to activate the innate immune cells, in particular the CpG motifs. In human it is expressed only in memory B cells (Bernasconi et al., 2003; Bourke et al., 2003) and DCs (Horng et al., 2002; Kadowaki et al., 2001; Krug et al., 2001). TLR9 expression on human monocyte-derived DCs and monocytes has been reported, but it is still under question (Hoene et al., 2006; Saikh et al., 2004). In contrast, murine TLR9 expression is

not limited to B cells and pDCs, but is also detected in monocytes, macrophages and DCs (Edwards et al., 2003; Hemmi et al., 2000). It was shown that, in those cells, TLR9 are expressed in endosomal/lysosomal compartments (Heil et al., 2003; Latz et al., 2004).

TLRs are transmembrane receptors comprising an extracellular Leucine-rich repeat and a cytoplasmic TIR [Toll/IL-1 (interleukin-1) receptor] domain, connected through a transmembrane domain (Lasker et al., 2006). The TIR domain has a structural homology with the IL-1 receptor. In general, upon encountering a PAMP, the TLRs recruit an appropriate adaptor protein to the TIR domain (O'Neill et al., 2007). This leads to the engagement of IRAK (IL-1 receptor associated kinase), TLR-specific additional adaptor proteins, and TRAF6 (tumor-necrosis-factor-receptor-associated factor 6) in the signalling pathway, resulting in the activation of transcription factors activator protein-1 (AP-1), nuclear factor κB (NFκB) and others, depending on the signalling pathway activated (O'Neill, 2006) (Fig.4).

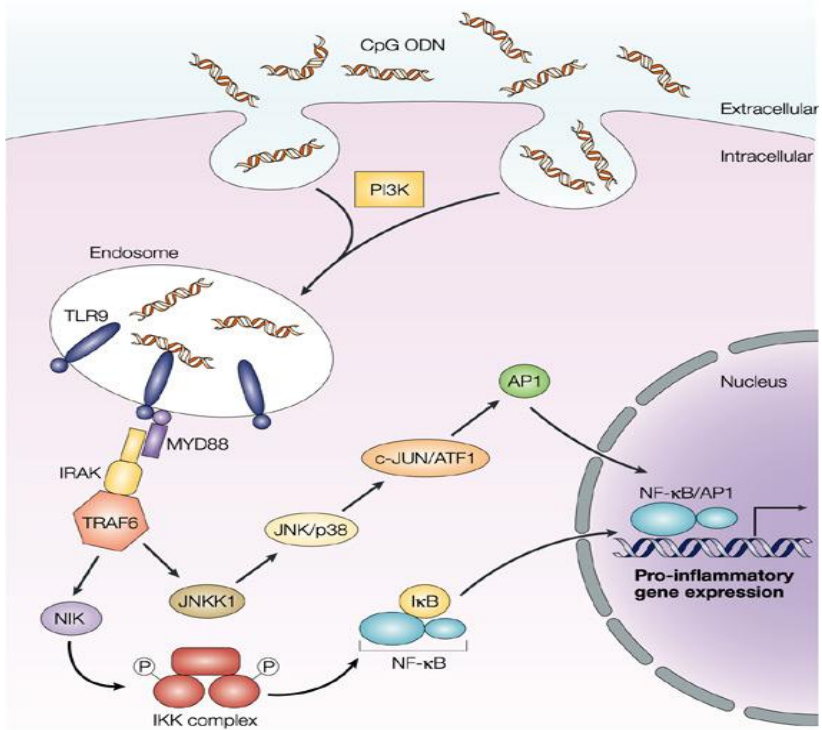


Fig.4 CpG signalling pathway (*Nature Immunology*).

Upon cellular activation, TLR9 traffics to endosomal and lysosomal compartments where the receptor interacts with endocytosed CpG-DNA at acid pH, a condition that is thought to be necessary for DNA recognition (Latz et al., 2004; Leifer et al., 2004; Rutz et al., 2004).

The CpG uptake into the cell needs a transport mechanism. It is known that the uptake of DNA occurs through endocytosis, involving the cytoskeleton rearrangement (Yakubov et al., 1989). In particular it has been shown that vesicles containing clathrin may be directed towards the endosome and may fuse with sub-cellular structures with the following release of their content into the endosome (Beltinger et al., 1995). Upon DNA uptake endosomal maturation occurs, with the passage of the endosomes in the lysosomes compartments (Mellman et al., 1986). The endosomes fusion and the vesicles trafficking are regulated by the intracellular pH: in fact some compounds, able to interfere with the intracellular acidification, may affect the endosomal fusion and the biological action of CpG, as bafilomycin 1 and chlorochine (Macfarlane et al., 1999; Strekowsky et al., 1999).
In monocytic/macrophagic system the post-receptorial events seem to depend upon the activation of the myeloid differentiation activation factor (MyD88), which is followed by cytokines delivery, like IL-6, IL-12, etc. (Yi et al., 1998; Chu et al., 2000). The CpG-mediated transductionl mechanisms are also MyD88-independent, comprising those regulated by the “stress” kinases activation as Jun NH_2- terminal kinase 1/2 (JNK1/2) and p38 MAPK (Hemmi et al., 2000; Hacker et al., 2000; Chuang et al., 2002). JUN1/2 activation

causes the stimulation of AP-1, that promote the synthesis of several cytokines, as IL-12, involved in the control of the Th1 immune response (Riendeau et al., 1994; Nick et al., 1996; Cuenda et al., 1997).

The CpG ODN, through MyD88 activation, promotes the Cicloxygenase-2 (Cox-2) activation, able to catalyze the conversion of Arachinonic Acid (AA) in prostaglandins (PGE_2) (Chen et al., 2001). The CpG effect on Cox-2 is confirmed by recent experimental evidences which show the capacity of CpG to stimulate Phospholipase A_2 (PLA_2) and AA delivery (Yeo et al., 2003).

Furthermore, it was recently demonstrated (Lee et al., 2007) that TLR9 activation stimulates the cytosolic PLA_2 ($cPLA_2$) activity via p38 MAPK, or Akt pathways, and mediates the inducible Nitric Oxide Synthase (iNOS) expression. The importance of $cPLA_2$-mediated Reactive Oxygen Species (ROS) signalling processes in the regulation of monocyte chemoattractant protein-1 (MCP-1) release was shown to be a result of TLR activation (Lee et al., 2008). It was also documented that the biologic effect exerted by CpG may involve Reactive oxygen Species (ROS) (Aramaki et al., 2002).

1.4.2 CpG and *M. tuberculosis*

Immunological effects of CpG during infection with *M. tuberculosis* are still not completely clarified, nevertheless different approaches have been used to investigate this issue. CpG-ODN have been shown to facilitate the control of a variety of intracellular pathogens, including *Listeria monocytogenes, Leishmania major* and *Francisella tularensis* in murine models infection (Krieg et al., 1998; Ito et al., 2005; Ito et al., 2004; Zimmermann et al., 1998; Elkins et al., 1999).

In particular, mice vaccinated with BCG plus CpG-ODN showed a greater reduction in bacterial loads after infection with *M. tuberculosis* strain Erdman than those vaccinated with BCG alone (Friedag et al., 2000). More recently Juffermann et al., (2002) found that mice infected with virulent *M. tuberculosis* are protected by CpG-ODN and that this effect is abrogated in IFN-γ gene-deficient mice. Previous results indicated that IFN-γ knock-out mice were more susceptible to infection with *M. tuberculosis*. In contrast to murine studies, IFN-γ treatment of human macrophages *in vitro* consistently produces a paradoxical stimulation in the intracellular growth of virulent *M. tuberculosis* strains (Cooper et al., 1993; Flynn et al., 1993). This effect is surprising since IFN-γ has been found to

directly activate human macrophages co-cultured with autologous lymphocytes, primed with IFN-γ plus *M. tuberculosis* antigen, and that macrophages treated with IFN-γ are mycobactericidal (Bonecini-Almeida et al., 1998). These results suggest that IFN-□ may function as a co-stimulatory factor in the human immune response against *M. tuberculosis*, rather than a primary activator of macrophages anti-mycobacterial activity. The currently accepted idea that increased levels of IFN-γ may be used as a single marker of protection in TB should be re-evaluated because, recently, it was shown that increased levels of IFN-γ, induced by culture filtrate proteins (CFP) antigen and CpG-ODN immunization, do not confer significant protection against MTB infection because the immunized mice presented severe pulmonary injury accompanied by necrosis, despite a strong Th1 response. Only mice immunized with CFP and incomplete Freund's adjuvant (IFA) were protected after *M. tuberculosis* infection, preserving better lung parenchyma (Morais de Fonseca et al., 2007).

Instead, there are not much studies performed on human cells system. But some works highlighted an antimycobacterial activity showing a direct relationship between host phospholipase-D (PLD) activity and human macrophage ability to control intracellular mycobacterial replication (Auricchio et al., 2003) and that CpG ODN induces a dependent Ca^{2+} PLD activity, leading to phagosome

maturation and intracellular growth inhibition in human monocytes (Greco et al., 2006).

1.4.3 CpG, oxidants (Nitrogen Oxide and Reactive Oxygen Species) and intracellular pH (pHi)

Upon cellular activation, TLR-9 traffics to endosomal and lysosomal compartments where the receptor interacts with endocytosed CpG-DNA or synthetic CpG-ODN at acidic pH (6.5-5.0), a condition that is thought to be necessary for DNA recognition (Latz et al., 2004; Leifer et al., 2004; Rutz et al., 2004). Compounds that interfere with endosomal acidification such as the weak base Chloroquine and Bafilomycin A1, an inhibitor of the ATP-dependent acidification of endosomes, consequently prevent CpG-DNA driven TLR-9 activation (Hacker et al., 1998; Yi et al., 1999). Moreover, acidification of endosomal CpG-ODN is coupled to the rapid generation of ROS (Yi et al., 1998). ROS are generally considered to be cytotoxic when produced in excess and have been implicated in pathogenesis of a wide variety of diseases (Sen et al., 1996). However, moderate concentrations of intracellular ROS influence genes expression, and two well-defined transcriptional factors, NF-κB and AP-1 have been shown to be regulated by the intracellular

redox state (Sen et al., 1996). Yi et al., (1996) reported that responses of both B cell and monocyte-like cell lines to DNA containing CpG-ODN were sensitive to endosomal acidification for the production of cytokines like IL-6, IL-12 and TNF-α. Furthermore, CpG-ODN-induced ROS has been linked to the activation of NF-κB, which, in turn, induces leukocyte gene transcription and cytokine secretion (Yi et al., 1998).

Nevertheless, NFκB is not the main responsible of IL-12 secretion in macrophages stimulated with CpG-ODN, because the cytokine secretion is suppressed by NADPH-oxidase inhibitors, suggesting that ROS are involved in cells activation, but these inhibitors don't influence activation of NFκB that is the factor implicated in IL-12 gene trascription (Aramaki et al., 2002). Moreover, different studies highlighted the importance of CpG in ROS generation through the signaling pathways mediated by TLR9, with the involvement of the cytosolic Phospholipase A_2 ($cPLA_2$) and the release of Arachidonic acid (AA). In particular, the stimulation of macrophages with CpG resulted in the up-regulation of ROS via JNK- cPLA2 pathway and this increase of ROS was positively associated to Monocyte Chemo attractant Protein 1 (MCP-1), which influences Monocytes migration to the sites of inflammation (Lee et al., 2007). In this context another study showed the importance of CpG in iNOS expression through

$cPLA_2$ in murine macrophages (Lee et al., 2007). However, the molecular mechanisms responsible for the immunostimulatory effects of CpG-ODNs have not been yet fully elucidated.

1.4.4 CpG ODNs influence cytokines profile through TLR9 activation

Microbial infection is sensed by Toll-like receptors (TLRs) present on innate immune cells. Among the ten so far defined TLRs, TLR9 and its ligand are peculiar. TLR9 recognizes bacterial DNA characterized by the abundance of unmethilated CpG dinucleotides, which distinguish bacterial DNA (CpG DNA) from mammalian one.
Interestingly, synthetic oligonucleotides containing CpG motifs have been shown to induce cell proliferation, differentiation, and cytokine production in B cells, DCs and macrophages through a TLR9-dependent mechanism. *In vitro* and *in vivo* studies have shown that CpG-ODN may strongly stimulate macrophages and DCs to induce the production of various cytokines like IL-10, IL-12 and IFN-γ, and the up-regulation of MHC class II and co stimulatory molecules. Further maturation of DCs and B cells proliferation facilitates the activation of T helper cell type 1 (Th1) immune response (Wagner et al., 1999; Yamamoto et al., 2000; Krieg et al., 2002). Adjuvant

activity of CpG-ODN is based on the induction of IL-12 in macrophages, DC and B cells (Ballas et al., 1996; Cowdery et al., 1996; Brazolot Millan et al., 1998; Krieg et al., 1998; Jakob et al., 1999; Wagner et al., 2004). In contrast, IL-10 has been shown to be a major counter-regulatory cytokine that may affect the immunomodulatory effects of IL-12 directly or indirectly (Snijders et al., 1996; Aste-Amezaga et al., 1998; Akdis and Blaser, 2001; Yi et al., 2002). During infection, the recognition of intracellular pathogens CpG DNA skews and fine-tunes the ongoing immune response and induces long-lasting Th1 milieus. Thus, CpG DNA might play an important role in driving the immune system to a Th1 profile, preventing undesired Th2 milieus that might favor the induction of allergic responses. Since CpG DNA may be synthesized with high purity and sequence fidelity, synthetic CpG DNA will become an important agent for Th1 instruction and an effective adjuvant during vaccination. The Th1-polarizing ability of CpG ODN suggested the therapeutic use of these motifs in allergy treatment and as adjuvant in vaccination.

1.5 CpG as vaccine adjuvants.

CpG ODN stimulates cells that express TLR9, initiating an immunomodulatory cascade that culminates in the production of Th1 and pro-inflammatory cytokines and chemokines. CpG ODNs also improve the antigen presenting function of DCs, monocytes and macrophages, induce the proliferation of B cells, stimulate the immunoprotective activity of NK cells, and recruit T cells to the site of ODN administration (Sun et al., 1998; Lipford et al., 1997; Jakob et al., 1998). These diverse effects of CpG ODN on the host's immune milieu underline their value as vaccine adjuvants. Findings from multiple laboratories indicate that CpG ODN may act as immune adjuvants, triggering a response that promotes humoral and/or cell-mediated responses against co-administered antigens. These findings are consistent with the hypothesis of Fearon and Locksley, who suggest that stimulating the innate immune system creates an immune milieu conducive to the development of antigen-specific immunity (Fearon et al., 1996). There is consistent evidence that CpG ODN function as adjuvants when co-administered with conventional protein-based vaccines, boosting antigen-specific Abs and cell-mediated immune responses. CpG ODN may both accelerate and magnify vaccine-specific immunity. These effects could be of considerable benefit when the rapid induction of a

protective immune response is required (Klinmann et al., 1999). Yet the adjuvant effect of CpG ODN appears to be strongest when the amount of immunogen being administered is sub-optimal. This is usually referred to as an “antigen-sparing effect”. When high doses of vaccine are administered, the magnitude of the immunologic boosting attributable to CpG ODN is modest. The utility of CpG ODN is underscored by their ability to enhance mucosal as well as systemic immunity. This is of considerable importance for pathogens that gain access to the host through the respiratory, gastrointestinal and reproductive tracts. Several studies show that the co-administration of CpG ODN with vaccines significantly increases antigen-specific IgA levels at mucosal sites and IgG levels systemically (Moldoveanu et al., 1999; McCluskie et al., 1998; Horner et al., 1998). An additional benefit of CpG ODN is their ability to boost immunity in groups with reduced immune function, such as newborns, the elderly, and the immunosuppressed. Preclinical studies involving non-human primates confirm the expectation that CpG ODN selected for their ability to stimulate human immune cells are active *in vivo*.

Clinical studies designed to evaluate the safety and activity of CpG ODN in humans are ongoing. Available results suggest that these agents are reasonably safe, and in some cases boost the

immunogenicity of co-administered vaccines. Efforts continue to i) identify ODNs of different classes that are optimally active in humans when co-administered with specific vaccines, ii) determine how these different ODN regulate discrete elements of the immune response, iii) monitor the long-term safety of CpG ODN and iv) establish the optimal dose, duration and site(s) of vaccine/ODN delivery. It is important that these efforts to further improve the utility of CpG-based vaccines for the induction of protective immunity against infectious pathogens.

2. Goal of the study

CpG motifs are sequences, located mostly in bacterial genomes, with immunostimulatory capacities, able to promote antibacterial effects against numerous intracellular pathogens, as *M. tuberculosis* (Greco et al., 2009). These motifs improve their immune effects when flanked by specific sequences constituted by TC dinucleotide at the 5' end and pyrimidine at the 3' side. Besides, during last decade the confirmation of their safety has been an attractive feature for their use in therapeutic strategies, but, despite their importance in innate immune response, there are not many studies fully elucidating the CpG ODNs activities. Their adjuvant properties have been mainly described in mice but little is known about the effects of CpGs in humans.

In this scenario, the aim of our study consisted in investigating the effects of different CpG ODNs on human macrophages to better comprehend the molecular and cellular mechanisms involved in their immunostimulatory features, and the specific role played by the flanking sequences.

We divided our study in two parts:

- Analysis of the eight different CpG ODNs treatment outcome in human macrophages, with particular attention to:

  a. pHi variations, given that acidification is necessary for

TLR-9 recognition of DNA; b. ROS production, given that they influence the macrophages gene expression and are involved in the respiratory burst. Moreover, we tried to describe the human macrophages milieu prior to infection, examining, at the transcriptional level, a cytokines pattern involved in the innate immune response.

- Investigation of the antimycobacterial activity of CpG the ODNs which more significantly affected the MTB growth and the variations in cytokines expression after MTB infection, both at the transcriptional and at the protein levels.

We ended by delineating a new pattern of cytokines, already involved in different biological processes, in the attempt to enlarge the number of host factors involved in a protective innate immune response against MTB infection.

3. Materials and methods

3.1 Cell culture

Monocytes were isolated from Peripheral Blood Mononuclear Cells (PBMC) of healthy donors' buffy coat by density gradient centrifugation using Lympholyte-H (Cederlane, Mayada). Cells were grown in complete medium consisting of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and gentamicin 5 μg/ml at 37°C in a 5% CO_2 atmosphere. The day after monocytes, differentiated in macrophages by adherence, were detached and resuspended 10^6/ml. At the seventh day 10^6 cells were stimulated for 1h and 24h with 1 μM of different phosphorothioate CpG ODNs (Table 1) and GpC ODN (ATGCACTCTGCAGGCTTCTC). In some experiments the cells, after 1 hour of treatment, were infected with MTB at a MOI 10:1 for 3 hours (see next paragraph).

CpG ODN	Sequences
CpG1 CpG1 rev	ATCGACTCTCG**GACGTC**CTC GAG**GACGTC**CGAGAGTCGAT
CpG2 CpG2 rev	ATCGACTCTCG**GACGTT**CTC GAG**GACGTT**CGAGAGTCGAT
CpG3 CpG3 rev	ATCGACTCTCG**AACGTC**CTC GAG**AACGTC**CGAGAGTCGAT
CpG4 CpG4 rev	ATCGACTCTCG**AACGTT**CTC GAG**AACGTT**CGAGAGTCGAT

Table1. CpG ODNs sequences. The active examers are indicated in bold, while the flanking sequences described to be important to enhance their immunomodulatory properties are underlined.

3.2 Infection of macrophages with MTB H37Rv

The infection was performed after seven days of macrophage culture, at a multiplicity of infection (MOI) of 10:1 bacteria: cells, following 1 hour of pre-treatment with 1 μg/ml CpG ODNs. Macrophages infected with MTB, but without CpG ODNs stimulation were used as control cells. After 3 hours of infection the cells were washed twice with warm Phosphate Buffer Saline (PBS) to remove extra-cellular bacteria. The infected macrophages were left in culture for another day, when the culture supernatant was harvested and the cells lysed to extract total RNA. Before infection, MTB bacilli were sonicated to disrupt small aggregates of bacteria.

3.3 Determination of Colony Forming Units (CFU)

Bacilli in the culture and supernatants were plated immediately after 3 h of incubation with cells, to determine the percentage of phagocytosis at the first day of infection. At the same day the intracellular bacteria were obtained lysing the cells with sterile PBS containing 0,1% saponin (Sigma, St Louis, MO) and released bacilli were serially diluted in PBS. Finally bacteria were plated on 7H10 Middlebrook (Becton Dickinson, Franklin Lakes, NJ, USA) medium

with OADC in duplicate. CFU were counted after 21 days of incubation at 37 °C in a 5% CO_2 atmosphere and plates were maintained for 30 days to ensure that no additional CFU appeared.

3.4 RNA extraction

Cells were drained of media and the adherent cells were resuspended in ice-cold 4M Guanidium iso-thiocyanate (GTC) lysis solution. Total RNA was extracted as described by Chomczynski et al. (2006) and was analysed in a 1,5% denaturing agarose gel for absence of degradation, and quantified by UV spectroscopy at 260/280 nm. The obtained RNA is represented mainly by host RNA (data not shown) and was used to perform real time on the cytokines experiments.

3.5 Reverse transcription (RT) and quantitative real time PCR

One microgram of total RNA was reverse-transcribed using random examers and SuperScript III Reverse Transcriptase (Invitrogen, Paisley, UK), according to the manufacturer's instruction. Quantification of PCR products was performed with ABI PRISM 7000 SDS (Applied Biosystem, Foster City, USA). The

RealMasterMix SYBR ROX (Eppendorf AG, Germany) was used to produce fluorescently labelled PCR products, and we monitored increasing fluorescence during repetitive cycling of the amplification reaction. Primers sets for all amplicons were designed using the Primer-Express 1.0 software system (Applied Biosystem). The sequences and annealing temperatures (Ta) for each primer are as follows:

L34 forward 5'-GGCCCTGCTGCATGTTTCTT-3', reverse 3'-GTCCCGAACCCCTGGTAATAGA-5' (Ta 64°C);

IFN-γ forward 5'- GGCTGTTACTGCCAGGACCCATATGT-3', reverse 3' GATGCTCTTGCACCTCGAAACAGCCAT-5' (Ta 64);

IL-10 forward 5'- AGGCGCATGTGAACTCCCT-3', reverse 3'-CACGGGCCTTGCTCTTGTTTT-5' (Ta 62°C);

IL-12 forward 5'-GCTGCTGAGGAGAGTTCTGCCC-3', reverse 5'- CCAGCTGACCTCGACCTGCC-3' (Ta 62°C);

IL-18 forward 5'-ATCGCTTCCTCTCGCAACA-3', reverse 3' CATTGCCACAAAGTTGATGCA-5';

IL-32 forward 5'-GACATGAAGAAGCTGAAGGCC-3', reverse 3'-ATCTGTTGCCTCGGCACCG-5' (Ta 62°C);

Ezrin forward 5'-AAAGAGCAGATGATGCGCG-3', reverse 3'-ATACGGTCAGCCTCTAGGC-5' (Ta 58°C).

For all primers, the following temperature cycling profile was used: 2 min a 50 °C and 10 min at 95 °C followed by 10 seconds, 30 seconds at Ta and 1 minute at 68 °C for 40 cycles. L34 was used as an internal control because it was shown to be stable with different induction. The relative level for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

3.6 Reactive Oxygen Species detection

It was used fluorophor Dichlorofluorescein Diacetate (DCF-DA) to detect ROS production. This probe was dissolved in DMSO at the concentration of 1 mM, stored at -20°C in the dark and diluted at the final concentration before each experiment. Cells (10^6) were incubated, in PBS, with DCF-DA (1 µM) for 1 hour at 37 °C in the dark. This fluorophore spreads rapidly through cellular membrane and is hydrolysed by intracellular estherases to form DCFH that in presence of ROS is oxidated in DCF and gives out fluorescence. DCF fluorescence intensity is proportional to the ROS amount produced intracellularly (Shen et al., 1996). After incubation, cells were washed twice with PBS, centrifuged for 10 minutes at 2000 RPM, resuspended in PBS buffer and stimulated with CpG ODNs. Fluorescence was measured by FACS (Becton Dickinson).

3.7 Intracellular pH detection

Intracellular pH (pHi) was measured using the intracellular probe 2'-7'-bis (carboxyethil)-5(6)-carboxyfluorescein (BCECF/AM) according to protocol (Grinstein et al., 1989). BCECF/AM were dissolved in dimethylsulfoxyde (DMSO) at the final concentration (1 µg/ml) at 37 °C in dark for 30 minutes. $2x10^6$ cells were incubated with the fluorophore. Na^+-H^+ exchanger's role was detected in Hepes buffer without bicarbonate with the following composition: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM MgCl2, 20 mM Hepes, 10 mM glucose at pH=7.4. Bicarbonate absence allowed to eliminate Na-dependent and Na-indipendent Na exchangers contribution in pH regulation. Fluorescence was measured under continuous magnetic stirring at the controlled temperature (37°C) in a Perkin-Elmer L-S-5 luminescence spectrometer equipped with a chart recorder model R 100A, with excitation and emission wavelengths of 500 and 530 nm using 5 and 10 nm slits. pHi variations are indicated with Fluorescence Arbitrary Units (F.A.U.).

3.8 Human cytokines protein array

The culture media were collected after 24h from infection, upon CpG ODNs treatment. All focused protein array analyses were performed according to the manufacturer's instructions. Briefly, we blocked membranes with Array Buffer 4 for 1 hr; in the meanwhile we prepared samples by adding up to 1 ml of each sample to 0,5 ml of Array Buffer 4, adjusting to a final volume of 1,5 ml with Array Buffer 5. Than we added 15 µl of Cytokines Detection Antibody and incubated for 1 hr at room temperature. After we incubated overnight at 4 °C samples on membranes and the day after we performed washes with distilled water. Then we diluted Streptavidin-HRP in Array Buffer 5 and it was dripped on membranes incubated for 30 minutes at room temperature. After washes each membrane was incubated with chemiluminescent detection reagent.

The culture media were each measured using the human cytokine array panel A (proteome profilerTM) (R&D Systems, Minneapolis, MN, USA). Streptavidin-HPR was used to detect protein expression and the data were captured by exposure to Kodak BioMax Light film by chemioluminescent ECL (Amersham Biosciences, GE Healthcare, UK). The arrays were scanned into a computer and optical density measurements were obtained with Array Vision 7.0 software (Imaging Research Inc., Canada). Positive controls were

located in the upper left-hand corner (two spots), lower left-hand corner (two spots) and lower right-hand corner (two spots) of each array kit.

3.9 Statistical analyses

GraphPad Prism 4 (Graphpad software, San Diego, CA) was used for graphical and statistical analysis. Statistical significance was assessed by analysis of variance (ANOVA), followed by Bonferroni’s post test; differences were considered significant at $p<0.05$.

4. Results

4.1 CpG1-4 examers genome composition and incidence in MTB modulated genes

CpG are non methylated motifs, expressed mostly in bacterial genomes, with immunostimulatory effects, and enhancing the innate immune response. Our initial approach consisted in finding the number of these examers into different bacterial genomes using TubercuList World-Wide web server (http://genolist.pasteur.fr/TubercuList/). In figure 4 we show that CpG1 (see Materials and Methods section) is the most represented in the mycobacterial genomes. In particular in *M. tuberculosis* chromosome there are 5498 sequences of CpG1. In contrast, CpG4 is the less represented examer in mycobacterial genomes: in fact in *M. tuberculosis* we counted 890 CpG4 examers. CpG2 and CpG3 show the same frequency in all the genomes because they are the reverse complement of each other and they are enumerated on both the DNA strands. The other bacteria analysed show a different CpG incidence given that, in comparison to mycobacteria, CpG1 is the least and CpG4 the most represented among the others CpG. For example, in *B. subtilis* we have 1010 CpG1 and 2120 CpG4 examers.

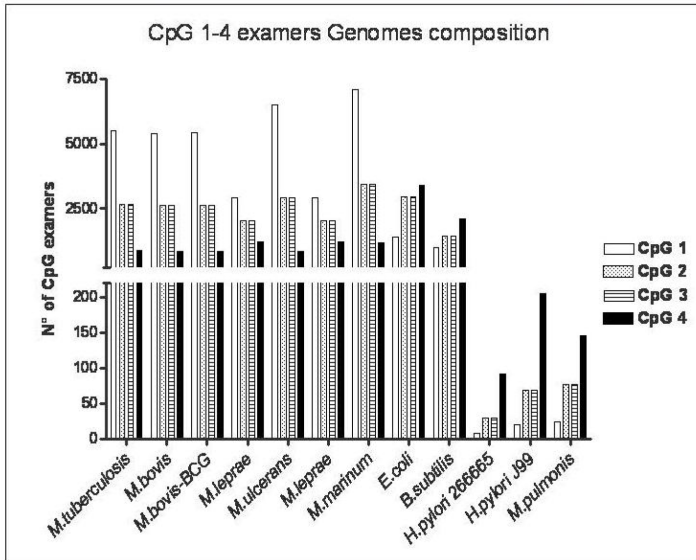


Figure 4. Analysis of the distribution of the selected four CpG examers in different bacterial genomes using TubercuList Web Server, for *M. tuberculosis* genome search (http://genolist.pasteur.fr/TubercuList/), BoviList (http://genolist.pasteur.fr/BoviList/), for *M. bovis* genome, BGCList (http://genolist.pasteur.fr/BCGList/), for BCG genome, Leproma (http://genolist.pasteur.fr/Leproma/), for *M. leprae* genome, BuruList (http://genolist.pasteur.fr/BuruList/) for *M. ulcerans* genome, MarinoList (http://genolist.pasteur.fr/MarinoList/) for *M. marinum* genome, Colibri (http://genolist.pasteur.fr/Colibri/) for *E.coli* K12 genome, SubtiList (http://genolist.pasteur.fr/SubtiList/) for *B. subtilis* 168, PyloriGene (http://genolist.pasteur.fr/PyloriGene/) for *H. pylori* 266665 and J99 genomes, and MypuList (http://genolist.pasteur.fr/MypuList/) for *M. pulmonis* genome.

In a previous work (Cappelli et al., 2006), we characterized the gene expression profile of MTB in Sauton's synthetic medium and in infected human macrophages, identifying a set of genes differently expressed in the two culture conditions: the induced, repressed and conserved genes in macrophages as compared to synthetic medium culture. Therefore, we asked whether the incidence of the employed four examers significantly differed in these three groups of genes (Fig.5).

Interestingly we found that only one CpG examer, namely CpG1, was significantly modulated in human macrophages, and consistently more than its incidence in MTB genome, while the other three were less represented than their percentage incidence in MTB genome, except for CpG4 which resulted over-represented in the group of genes repressed in human macrophages as compared to medium culture.

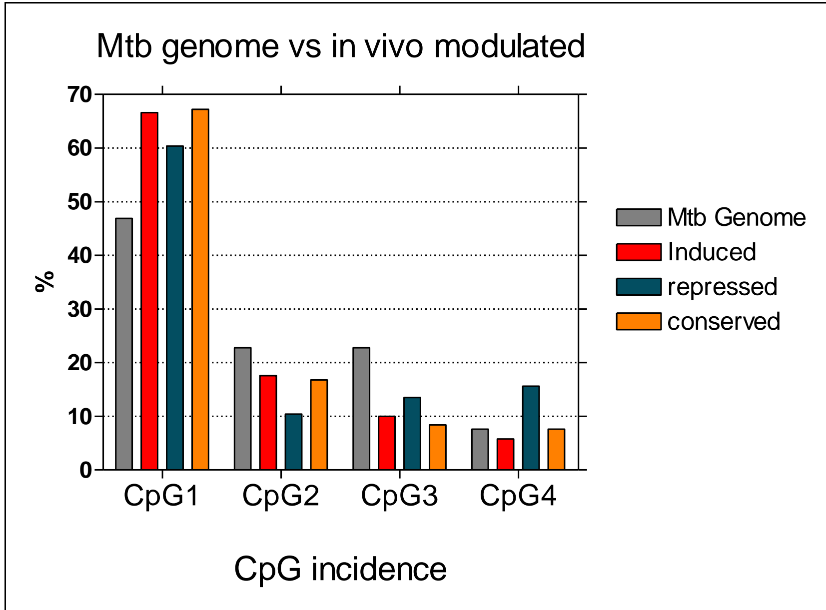


Figure 5. Percentage values of the 1-4 CpG mycobacterial examers occurrence in MTB genome and in the group of genes which resulted, in a previous study, induced, repressed and conserved in human macrophages as compared to synthetic medium culture (see reference Cappelli et al., 2006).

4.2. H37Rv MTB infection of macrophages pre-treated with different CpG-ODNs

Our four CpG examers were synthetically flanked by specific DNA sequences, composed by 2 Purines at the 5’ end and 2 Pyrimidines at the 3’ end which were already shown to increase the immune response (Yi et al., 1998; Hartmann et al., 1999; Hartmann et al., 2000) (Fig. 6).

5’ TC 2PuCpG2Pi 3’ CTC

CpG1	ATCGACTCTCGGACGTCCTC
CpG2	ATCGACTCTCGGACGTTCTC
CpG3	ATCGACTCTCGAACGTCCTC
CpG4	ATCGACTCTCGAACGTTCTC

Figure 6. CpG examers, flanked by 2 Purines at the 5’ end and 2 Pyrimidines at the 3’ end.

To examine whether the pre-treatment with CpG-ODNs would stimulate the human macrophages to inhibit the intracellular MTB growth, the cells were treated with 1 µg of CpG ODN, or GpC-ODN used as control, for 1 hour, and then infected with MTB H37Rv at a MOI of 10. To exclude a different phagocytosis of MTB by CpG-treated macrophages, the supernatants, after 3 hours of infection, were harvested and plated. In figure 7 we show that in supernatants

there are not significant differences of CFU between macrophages stimulated with the 1-4 CpG-ODNs, neither among them nor in respect with control cells.

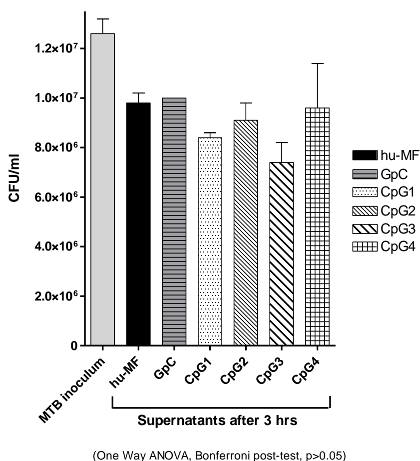


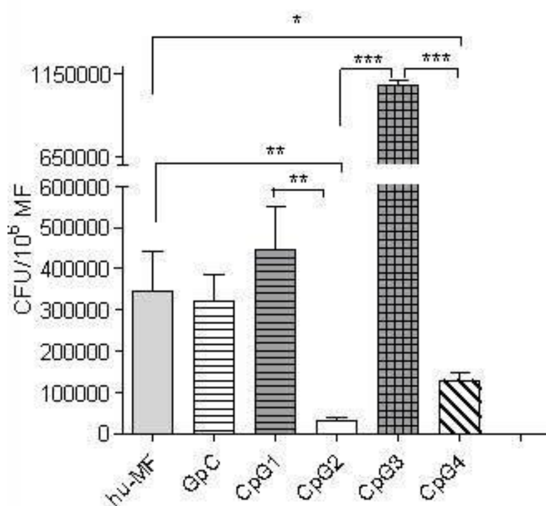
Figure 7. Colony Forming Units determination in Supernatants of differently treated human macrophages. After three hours of mycobacteria exposition the cells were washed and the supernatants were plated on 7H10 medium to determinate the number of bacteria not phagocytosed by human cells. Statistical significance was assessed by using One-Way ANOVA (Bonferroni post-test); differences were considered significant at $p<0.05$. As it is shown no significant difference in the number of extracellular mycobacteria, left in supernatants, was found among the different CpG treatments.

In figure 8a we show the intracellular CFU number after 24 hours of infection; the macrophages pre-treated with CpG2 and CpG4 ODNs, display a significant reduction of CFU: specifically, the macrophages treated with CpG2 ODN reduce the mycobacterial growth 6-fold as compared to untreated macrophages ($p<0.001$), while CpG4 treatment determines a 1,5-fold growth reduction versus control macrophages ($p<0.05$). On the contrary, CpG3 ODN pre-treatment significantly increases the intracellular MTB growth 5 fold ($p<0.001$) in comparison to control cells, while CpG1 treatment does not significantly affect MTB growth after 1 hour treatment ($p>0.05$).
The immunostimulatory effects of the ODNs are enhanced if the ODN have a TpC dinucleotide at their 5' end and a lot of pyrimidine at their 3' side (Yi et al., 1998; Hartmann et al., 1999; Hartmann et al., 2000). The immunostimulatory properties of a particular ODN or fragment of DNA are also affected by the number and spacing of the CpG motifs, the presence of poly G sequences or other flanking sequences in the ODN, and the ODN backbone (Pisetsky et al., 1998; Yi et al., 1998).
Having this in mind, we built four new ODNs, keeping the same immunostimulatory examers described above, and changing only their flanking sequences, simply using the reverse complement of the first ones: so we have CpG1 rev, CpG2 rev, CpG3 rev and CpG4 rev

ODNs (see Materials and Methods, Table 1). To investigate whether these new ODNs could influence the biological effects of CpG, in particular their antimycobacterial activity, we infected human macrophages with MTB H37Rv, pre-treated for 1 hour with the new CpG ODNs.

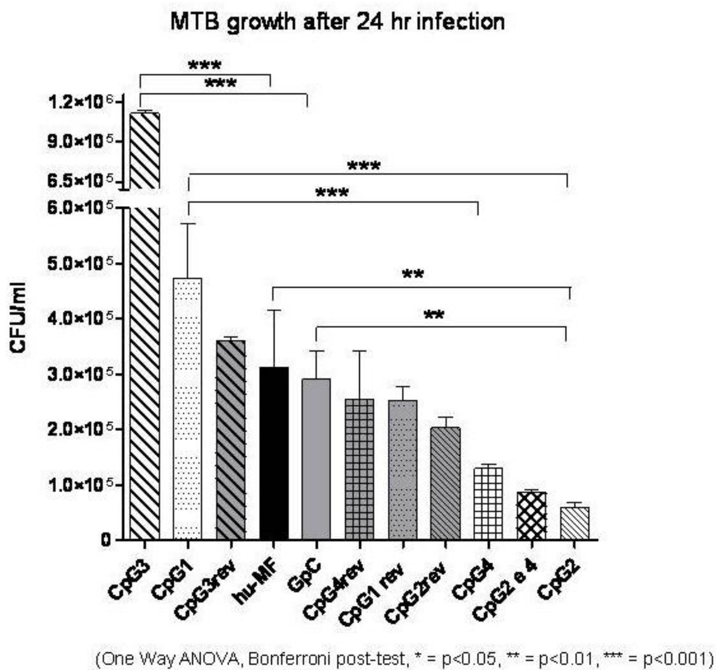
In figure 8b we describe the effects of the reverse complement CpG ODNs in comparison to the 1-4 CpG ODNs, ordered by a decreasing number of intracellular CFU after 24 hours of infection. The pair CpG3-CpG3 rev ODNs is the only one in which there is a statistically significant difference in CFU number, in that the CpG3 rev ODN does not stimulate the MTB intracellular growth to the same extent than CpG3 ODN. The pairs CpG2-CpG2 rev and CpG4-CpG4 rev ODNs show the tendency to loose their mycobactericidal properties in the wrong sequence scaffold, without the proper flanking sequences, even if the data did not result statistically significant. Finally, the pair CpG1-CpG1 rev ODNs is not particularly affected by the flanking sequences, given that neither CpG1 showed a considerable mycobactericidal effect. Finally, we analysed also the possible synergic effect of CpG2 and CpG4 ODNs, which are responsible of the highest decrease of MTB intracellular growth, and we observed an intermediate result, because these ODNs are able, together, to reduce MTB replication of 4 fold than control.

Essentially, CpG2 and CpG4 ODNs cause a strong antimycobactericidal effect and show a slight synergic effect between them in contrasting MTB growth.



a)

Figure 8. see next page.



b)

Figure 8. Colony Forming Units in cell lysate of differently treated human macrophages. 1 hour after the CpG ODN (1 μM) treatment, the cells were infected with MTB at a MOI of 10 for 24 hours. The resulting CFU are shown as the mean value ± SD of 3 independent experiments performed in duplicates. Statistical significance was assessed by using One-Way ANOVA (Bonferroni post-test); differences were considered significant at p<0.05 (*), and p<0.01 (**). a). Only the 1-4 CpG were tested, in comparison with non treated and GpC-treated macrophages; b). Each of the 1-4 CpG was tested in parallel with its corresponding reverse complement CpG sequence.

4.3 CpG ODNs effects on intracellular pH (pHi) in human macrophages

It is shown that TLR9 binds directly and sequence-specifically to single-stranded unmethylated CpG-DNA. TLR9-CpG-DNA interaction occurs at the acidic pH (6.5-5.0) found in endosomes and lysosomes (Rutz et al., 2004). To describe the macrophage milieu after CpG treatment, and prior to MTB infection, we analyzed the pHi trend during a period of 30 minutes, given that we observed that this time is sufficient to reveal the CpG effects (data not shown). In figure 9 are described the effects of CpG ODNs and reverse complement CpG-ODNs on pHi variation.

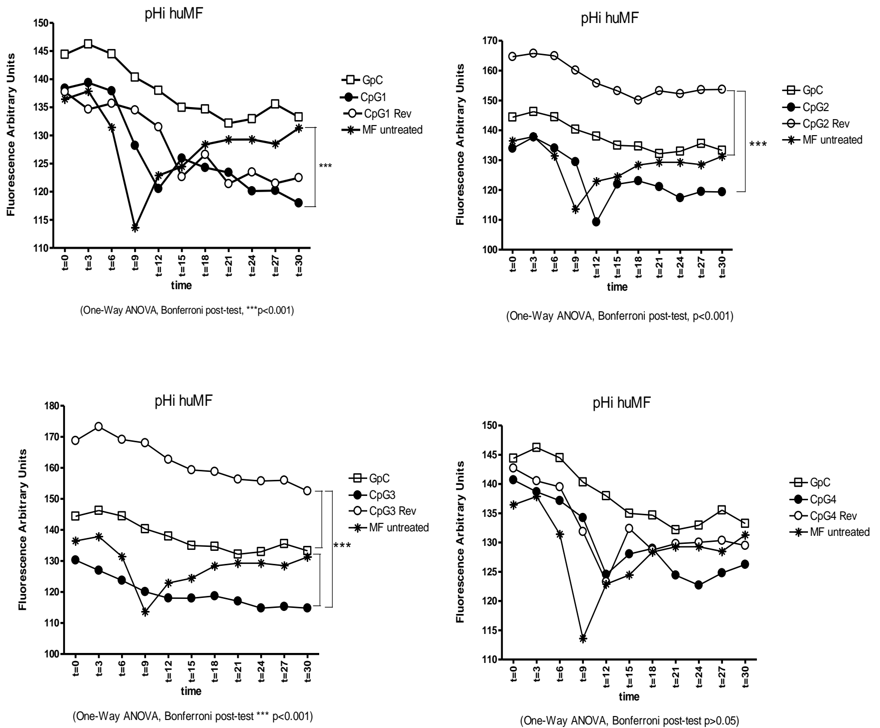


Figure 9. Time course of intracellular pH (pHi) variations in human macrophages treated with 1 µM of the different CpG ODN for 1 hr: in a, b, c and d are described the pHi trend in cells of each CpG and its specific reverse complement sequence. pHi detection was performed in Hepes buffer as described in Materials and Methods section. Statistical analysis was performed for each time interval, and the differences resulted significant in the last time range (24 to 30 minutes) by using One-Way ANOVA (Bonferroni post-test); differences were considered significant at p<0.05(*), p<0.01 (**), and p<0.001 (***).

We found that in control cells the pHi, at 9 minutes from the beginning of measures, shows a considerable decrease, as described in a previous study in human granulocytes, where it was observed an acceleration in the initial rate of acidification after pre-incubation with BCECF (Sullivan et al., 1989). Therefore we considered the significance of the last three times measures (from t=24 to t=30 minutes). Both CpG1 and CpG3 ODNs reduce significantly pHi when compared with control macrophages ($p<0.001$). Their reverse complement ODNs have a different behavior because CpG1 rev ODN slightly decreases pHi and CpG3 rev ODN enhances pHi values in a significant manner as compared to control macrophages and CpG3 ODN ($p<0.001$). Likewise CpG2 ODN shows a higher decrease in pHi values than macrophages treated with CpG2 rev ODN ($p<0.001$), which determines a significant increase in Fluorescence Arbitrary Units in comparison with control macrophages ($p<0.001$). Finally, macrophages treated with CpG4 ODN show a slight pHi decrease as compared to control macrophages, but the difference did not result significant.

Overall, CpG1 and CpG3 ODNs are the sequences that induce the highest and significant decrease in pHi values and it seems that there is a correlation between specific CpG sequences and pHi variations, given that only CpG ODNs, and not CpG ODNs reverse

complement, induce a decrease when compared to control macrophages.

4.4. Effects of different CpG-ODNs on macrophages' ROS production

We next investigated whether ROS generation was detectable in macrophages after CpG treatment and whether the flanking sequences could influence this production. The intracellular production of ROS in cells was measured using the cell-permeable oxidation-sensitive dye, DCF-DA. CpG stimulation of cells results in an increase of the DCF fluorescence intensity, indicative of oxidation by hydroxyl radicals. As shown in figure 10, the fluorescence indicates that CpG1 and CpG3 ODNs treatment increase the ROS generation in a significant manner as compared to control macrophages ($p<0.01$).

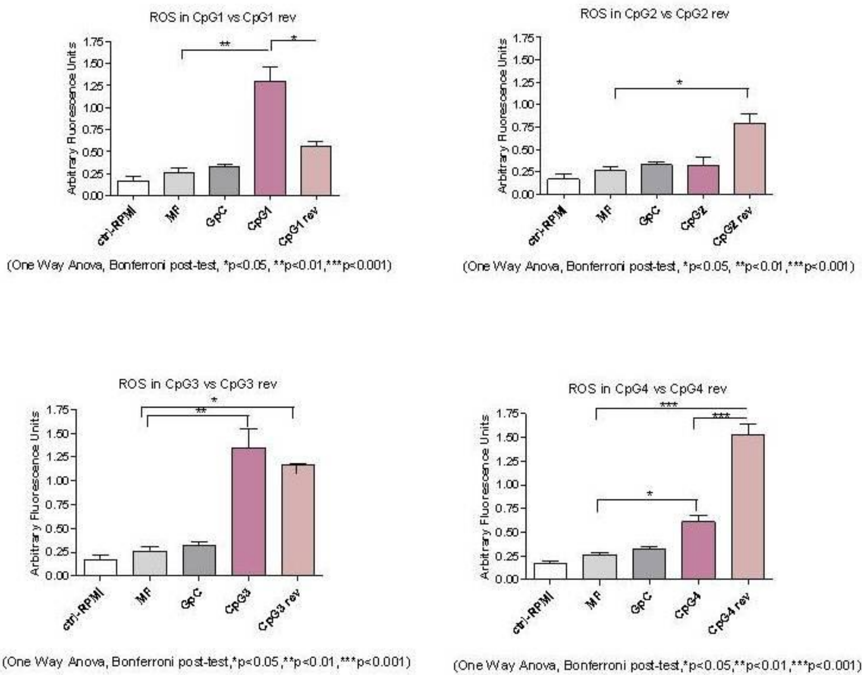


Figure 10. Reactive Oxygen Species (ROS) production by human macrophages treated with 1 μM of each of the 1-4 CpG ODN, and their specific reverse complement sequences, are reported. ROS detection was performed as described in Materials and Methods section. Statistical significance was assessed by using one-way ANOVA (Bonferroni post-test); differences were considered significant at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

Likewise, CpG3 rev ODN determinates a significantly higher ROS release than controls ($p<0.05$). CpG2 ODN treatment of macrophages is the only one which does not induce ROS generation, while CpG2 rev increases it as compared to controls ($p<0.05$). At last, in macrophages treated with CpG4 and CpG4 rev ODN a significant increase of ROS is detected in comparison with control cells (respectively: $p<0.05$ and $p<0.001$). We may then conclude that CpG1 and CpG3 ODNs are responsible for a higher ROS release in comparison to CpG2 and CpG4 ODNs and that there is no correlation between the increase of ROS production and the CpG sequence specificity.

4.5. Cytokine profile in macrophages treated with CpG-ODNs

To describe the macrophage milieu existing before MTB infection, immediately after CpG ODNs treatment, we analyzed a group of cytokines including IFN-γ, IL-12, IL-10, IL-18, IL-32, and also Ezrin, a protein involved in cytoskeleton rearrangement and phagosome maturation (Berryman et al., 1995; Erwig et al., 2006). We focused our attention on mRNA transcription, after 1 hour of treatment with the two CpG ODNs that determinate the most

significant effects on MTB growth, that are CpG2 and CpG3 ODNs. In figure 11 we observe that macrophages treated with CpG2 ODN show a lesser mRNA transcription of IFN-γ and IL-12 in comparison to cells treated with CpG3 ODN. After 24 hours there is an increase of IFN-γ transcription in both CpG ODNs treated macrophages, as compared to 1 hour of treatment, even if CpG2 ODN still induces a lower amount of mRNA than the other two CpG-ODNs.

Among the cytokines involved in Th1 response there is also IL-18, which enhances its expression after 1 hour of stimulation with CpG3 ODN but CpG2 ODN determines its down-regulation. At 24 hours of stimulation the expression of IL-18 mRNA is augmented for both CpG-ODNs treatments.

Interestingly, IL-10 results up-regulated predominantly in cells treated with CpG2 ODN, but such an increase drops down after 24 hr of stimulation.

IL-32, associated to IFN-γ-IL-18 pathway (Netea et al., 2008), results down-regulated in macrophages stimulated with CpG2 ODN and conserved for macrophages stimulatd with CpG3 ODN. After 24 hours there is an up-regulation in comparison to 1 hour for the two CpGs ODNs (fig.11).

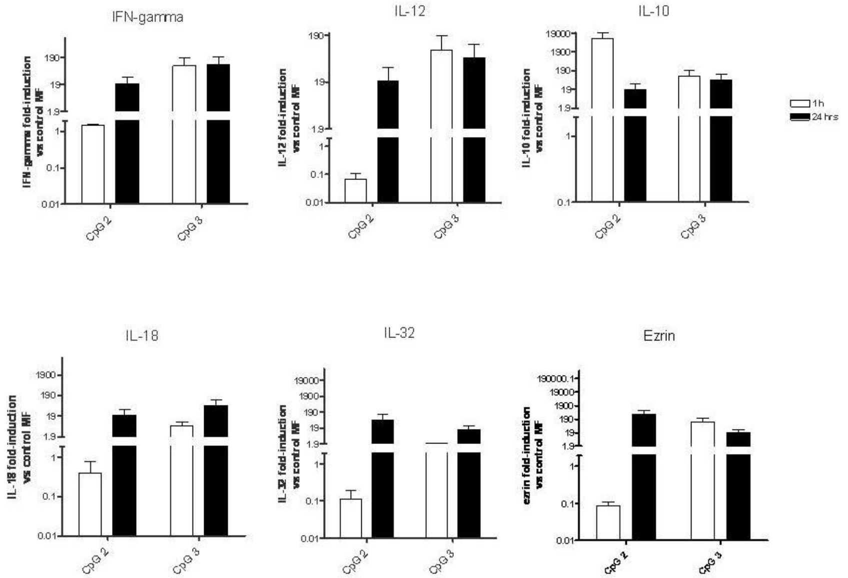


Figure 11. Gene expression profile, by q-rt RT PCR, of human macrophages after 1 hr (white histogram) and 24 hrs (black histogram) of CpG ODN (1 μM) treatments. Total RNA extraction, reverse-transcription and q-rt RT PCR were performed as described in Materials and Method section. Gene expression was normalized to L34 gene expression and the fold-change was calculated with respect to untreated macrophages.

Finally, we analyzed in q-rt Real Time PCR the expression of Ezrin, a membrane cytoskeleton linker. At 1 hour CpG2 ODN determines a consistent down-regulation in comparison to control macrophages, while for CpG3 and ODN we observe an up-regulation. After 24

hours mRNA transcription is enhanced for macrophages treated with CpG2 ODN and decreased for cells stimulated with CpG3 ODN.
To better characterize the previous results we tried to associate the cytokines mRNA fold-induction levels with the relative CFU reduction resulting after 1 hour of CpG treatment and 24 hours of infection. Only the IFN-γ mRNA significantly correlates with the CFU reduction (Pearson correlation $p<0.05$), whereas IL-10, IL-12, IL-32, IL-18 and Ezrin fail to show a similar correlation (fig. 11).
In summary we observed that macrophages treated with CpG3 ODN for 1 hour show a strong Th1 response and a weak production of IL-10, while CpG2 ODN determines the activation of a weak Th1 response and a higher IL-10 transcription. After 24 hour the Th1 response seems to be maintained for CpG3 ODN with a lower quantity of IL-10 mRNA. Instead CpG2 ODN strengthens IFN-γ, IL-12 transcription and reduces IL-10 mRNA levels.

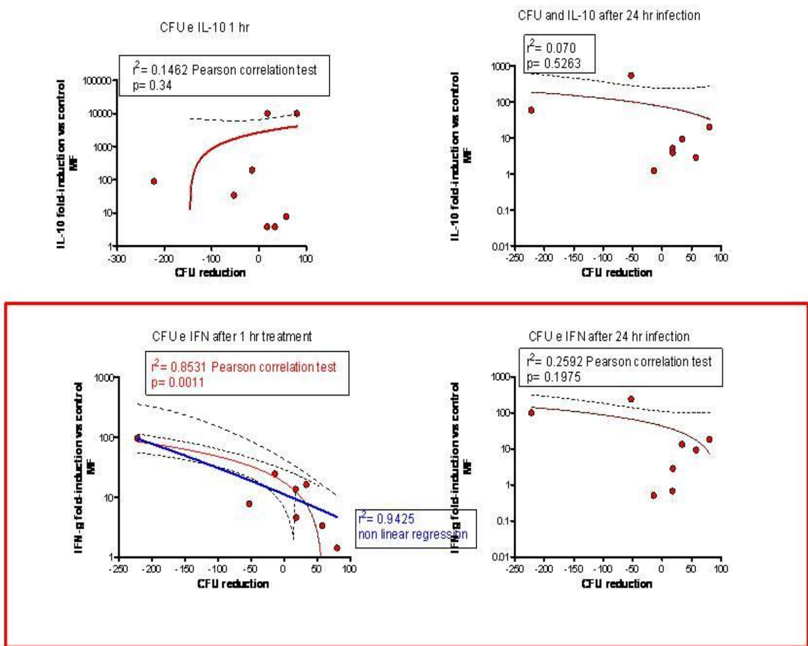


Figure 12. see next page.

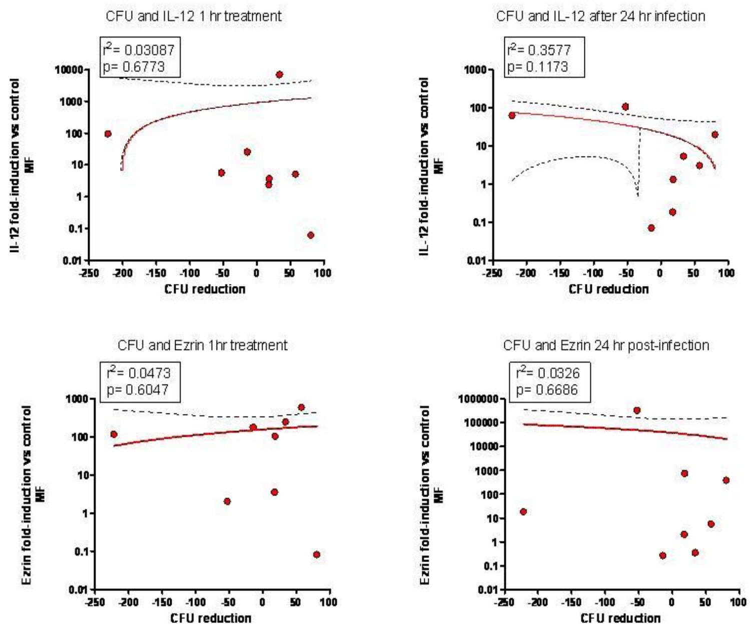


Figure 12. see next page.

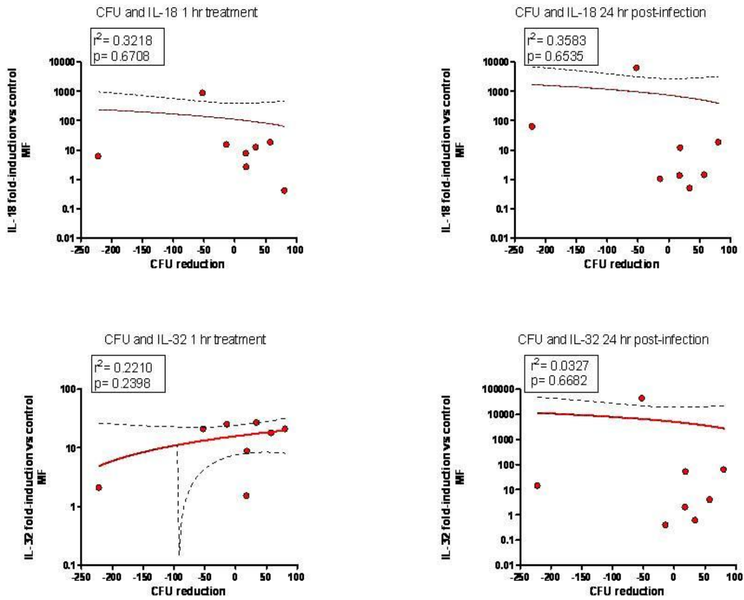


Figure 12. Correlation of pathway genes transcripts levels (after 1 hour of CpG ODN stimulation before and after infection) and CFU. mRNA levels of all molecules were determined by q-rt RT PCR, normalizing against the L34 gene. Correlations between cytokines transcripts and number of CFU are calculated using Pearson correlation test. Values of $*p<0.05$ were considered to be significant. Only IFN-γ and IL-10 correlation are represented.

4.6. Cytokine profile of macrophages infected with MTB H37Rv, after CpG ODNs pre-treatment

To investigate the macrophage intracellular environment taking place after MTB infection, the mRNA levels of six cytokines, and of one protein involved in cytoskeleton reorganization, were analyzed after 1 hour of each CpG-ODNs treatment and subsequent 24 hours of MTB infection, and the data are expressed as fold-induction in respect to untreated macrophages (Fig. 13).

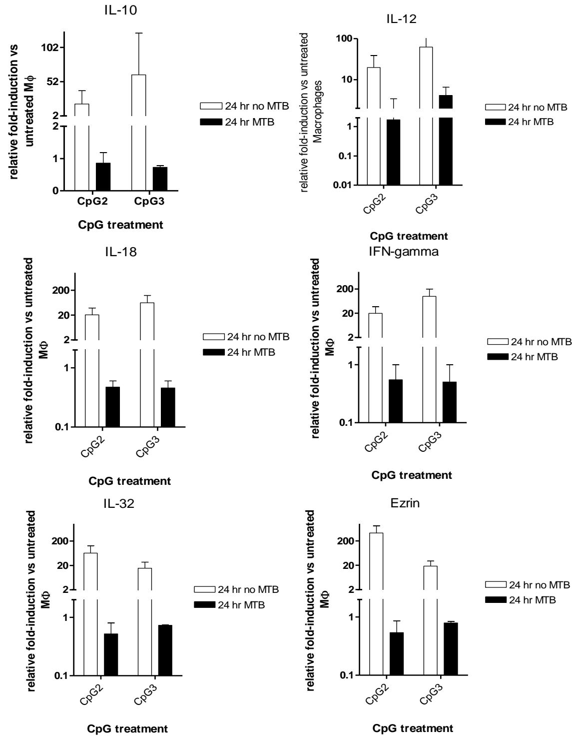


Figure 13: Comparison of expression profile, by rt-q RT PCR after 1h (white istograms) and 24 hrs (black istograms) from CpG2 and CpG3 ODNs treatments. Gene expression was normalized to L34 gene and fold-change was calculated with respect to untreated macrophages.

As we show, each cytokine seems to be affected by *M. tuberculosis* entry and survival in human macrophages. In particular IL-18, IL-32 and Ezrin are completely down-regulated after 24 hours of infection as compared to control cells. There is also a reduction of IL-10

expression for both CpG ODN treatment. IL-12, without infection, is expressed in macrophages treated with both CpG ODNs but MTB infection decrease the cytokine expression for two treatments. The slight up-regulation following CpG3 ODN treatment, after infection, may be due to donors variability. IFN-γ, like IL-12 involved in a Th1 immune response against MTB infecion, shows a particular trend because, before infection, it displays an up-regulation by both CpG ODN treatments, while after infection with MTB H37Rv its mRNA is always down-regulated as compared to controls.

In figure 14 are summarised cytokines expression of macrophages treated with 1 hour and 24 hours of CpG2 and CpG3 ODNs treatments and after 1 hour of stimulation and 24 of infection.

Overall, after 24 hours of infection with MTB, CpG ODNs treatments seem to reduce a Th1 response activated previously, according to Hickam (2002) where infected macrophages are described not able to produce IL-12.

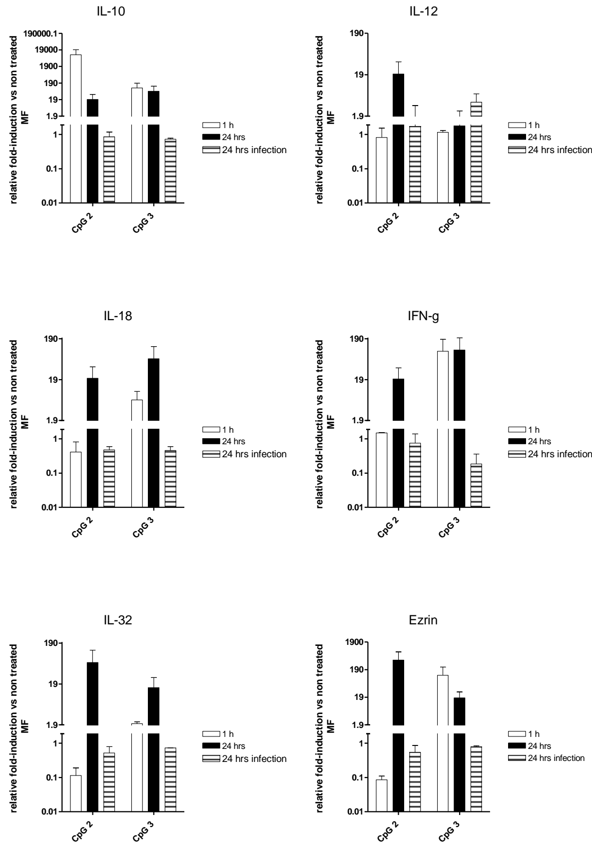


Figure 14. Comparison of expression profile, by rt-q RT PCR upon after 1h (white istograms) and 24 hrs (black istograms) from CpG2 and CpG3 ODNs and after 1 hour of CpG ODNs stimulation and 24 hours of infection (striped istogram). Gene expression was normalized to L34 gene and fold-change was calculated with respect to untreated macrophages.

4.7 Protein array analysis of the effect of *M. tuberculosis* infection on CpG ODNs-treated macrophages

To further elucidate the CpG biological effect, we decided to perform a protein array assay, only for infected macrophages pre-treated with the three CpG ODNs treatments able to reduce the intracellular mycobacterial growth. Therefore, we collected the supernatants from untreated macrophages and macrophages treated with CpG2, CpG4 and both CpG2 and CpG4 ODNs and subsequentltly infected with MTB. The protein array is composed by thirty-six proteins belonging to different families, such as chemokines, cytokines, adhesion molecules and cell surface proteins. In figure 15 are shown the four membranes used for the analysis, which were scanned with a Phosphorimager, and analyzed by the Array Vision software, able to provide us with the normalized density values of each spot.

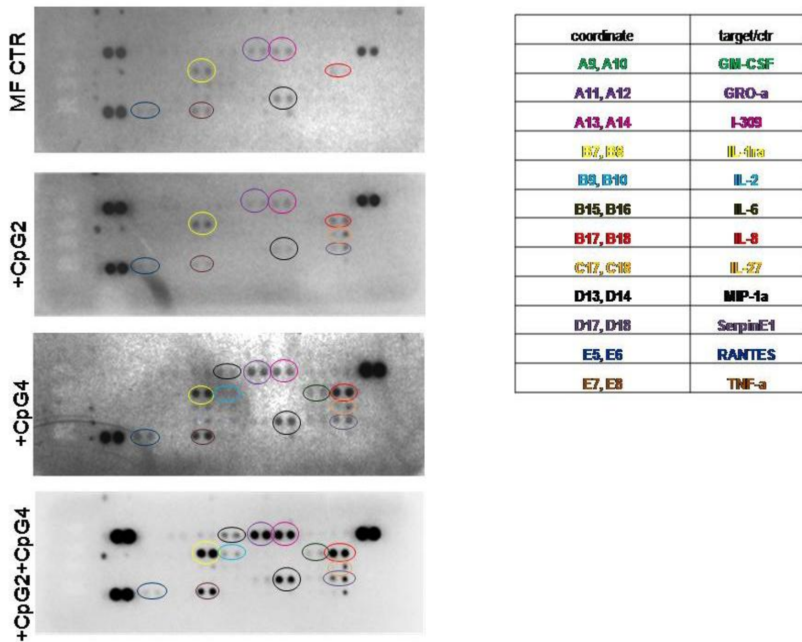


Figure 15. Comparison of expression profile, by q-rt Real Time PCR upon CpG2 and CpG3 ODNs without infection and 24 hrs (black histogram) and after infection (following 1 hour CpG treatment of cells). Gene expression was normalized to L34 gene expression and the fold-change was calculated with respect to untreated macrophages.

To clarify the CpG-induced changes, we divided the proteins in induced, conserved and down-regulated in comparison to those released by control macrophages and the results are described in figure 16.

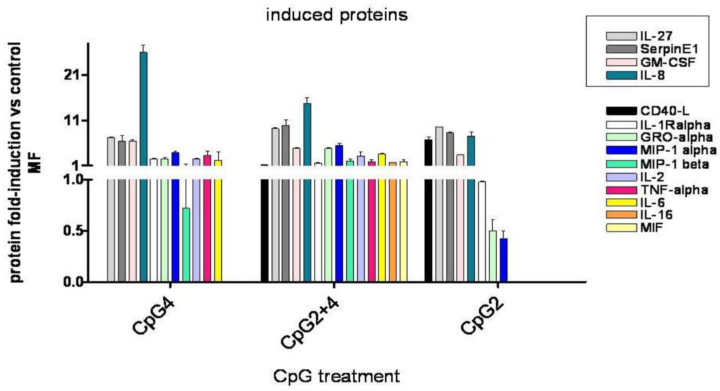
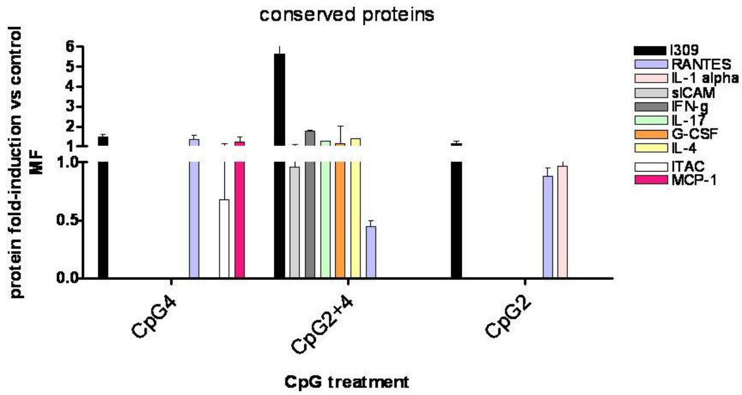


Fig. 16. Next page.



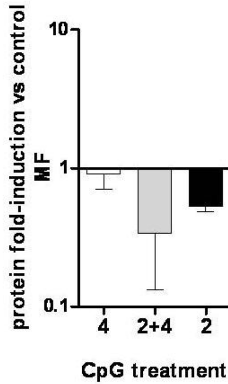


Figure 16. Analysis of proteins obtained by human cytokine protein array, performed collecting supernatatants of human macrophages treated with different CpG ODN (1 μM) and infected for 24 hr with MTB at the MOI of 10. Fold-induction of optical density measurements of CpG treated and infected macrophages vs cells used as control are showed. IL-27, SerpinE1, GM-CSF and IL-8 are induced, I-309, RANTES, IL-1a and IL-Ra are conserved and IL-13 is repressed in macrophages stimulated with, CpG2, CpG4 and CpG2+CpG4. It is representative of one experiments.

Among the CpG-induced proteins there are: IL-27, that promotes a Th1 response with IFN-γ production but inhibits inflammation (Robinson et al., 2008), which is over-expressed upon each CpG ODNs treatment; SerpinE1 (PAI-1), involved in fibrinolysis, which

shows a fold-induction of 8,1, 6 and 9,7 fold respectively for CpG2, CpG4 and CpG2+CpG4 ODNs treatments; IL-8 (CXCL8), which has been previously reported to be secreted from the pulmonary epithelial cell line A549 (Lin et al., 1998) and human bronchial epithelial cells following exposure to *M. tuberculosis* (Wickremasinghe et al., 1999) and is the most induced cytokine, especially upon CpG4 ODN treatment. Finally, there is granulocyte macrophage-colony stimulating factor (GM-CSF), crucially important for macrophages maturation and differentiation (Shitaba et al., 2001; Trapnell et al., 2002) which is up-regulated in all the three CpG ODN treatments. Additionally, also the up-regulation of CD40 Ligand (CD40L or CD154) is important, which could play a role in the contact-mediated inhibition of intracellular *M. tuberculosis* through stimulation of the production of IL-12 and the promotion of Th1 responses (Shu et al., 1995), which is up-regulated only in macrophages treated with CpG2 ODN.

Interestingly, a number of proteins detected upon pre-treatment were not released when the CpG ODNs stimulation were given separately, possibly indicative of different signaling pathways activated. For example we observed that IFNγ, sICAM̤G-CSF, IL-17 and IL-4 are constantly translated in macrophages treated with CpG2+4 ODNs as

compared to control cells, while they are not detectable in CpG2 and CpG4 ODNs separate treatments.
On the contrary, RANTES production was conserved only in CpG2 and CpG4 ODNs treatment, but when both were used the effect was reduced. Moreover we included I-309 in this group because it is conserved both for CpG4 and CpG2 ODNs treatments and is induced by MTB and TLR ligands in several lines of monocytes and macrophages (Jang et al., 2008). Differently, TNF-α, involved in granuloma formation, is over-expressed in macrophages treated with CpG4 ODN and the over-expression in macrophages stimulated with CpG2+CpG4 ODNs seems to be due only to CpG4 ODN contribution, given that it is not detectable in CpG2 ODN-treated cells.
Finally, we observed the IL-13 down-regulation in all the three treatments, a cytokine associated to macrophage deactivation and Th2 immune response.
According to this protein analysis we may conclude that, following infection with MTB, during the early innate immune response, the macrophages treated with CpG2 and CpG4 ODNs may exploit different mechanisms of activation which could depend on several components not yet described before.

5. Discussion

Bacillus Calmette-Guérin (BCG) is still the vaccine currently used in preventing the most severe disseminated form of disease in children and newborns, but its efficacy against active TB in adults has been challenged by several clinical studies (Andersen et al., 2005). There is a new urgency for a TB vaccine as the World Health Organization recently reported alarming rates of "multidrug resistant" and "extensively drug-resistant" TB (WHO, 2008), mostly because of improper observance of a lengthy and costly drug regimen treatment. The variable efficacy afforded by BCG vaccination and the absence of a TB vaccine protective in adults have been the primary rationale for several approaches to identify immunodominant MTB Ags that could be used in a subunit vaccine to boost immune responses leading to improved protection.

Protective immunity against *Mycobacterium tuberculosis* is dependent on the interplay between activated T cells, macrophages and other leukocytes. Proinflammatory cytokines such as IFN-γ, TNF-α and IL-12 are essential for protective immunity against this pathogen (Orme 1993; Jo et al., 2003). IL-10 produced by macrophages is important in regulating the Th1 cytokine balance and down-regulates the proinflammatory response (Murray et al., 1999). So, to make an efficient vaccine is critical the selection of a right

adjuvant, and CpG-ODN is a possible adjuvant candidate because its ability to potentiate Th1 CD4 and CD8 T cell responses was recently reported (Klinman et al., 2006).

During the last years, there has been increasing recognition of the immunological capacities of CpG-DNA, and its safety is an attractive feature for its use in therapeutic strategies. In this respect, Krieg (Krieg et al., 1995) demonstrated that unmethylated CpG dinucleotides, within a particular sequence context, are responsible for the immunostimulatory properties of bacterial DNA. The effects of bacterial DNA may be mimicked through synthetic ODN containing CpG motifs. CpG-ODNs have been shown to facilitate the control of a variety of intracellular pathogens, including *Listeria monocytogenes, Leishmania major and Francisella tularensis* in murine models of infections (Krieg et al., 1998; Ito et al., 2005; Ito et al., 2004; Zimmermann et al., 1998; Elkins et al., 1999). Considering this, the main question we asked in this study was whether different examers, placed in different sequence contexts (CpG ODNs sense and CpG ODNs reverse complement), could influence the *M. tuberculosis* growth inside human macrophages and how, upon CpG-ODNs treatment and infection, macrophages could respond in term of cytokines transcription and proteins release.

Initially we performed the search of the four different CpG examers selected, known to be immunostimulatory (Han et al., 1994), in Mycobacteria and in other pathogens, and we found that CpG1 was the more represented and CpG4 the less represented in all Mycobacteria including *M. tuberculosis*. This descriptive analysis allowed us to perform a finer investigation, only in *M. tuberculosis* genome, on the base of data obtained in our previous work, where we characterized the gene expression profile of MTB in Sauton's medium and in infected human macrophages, identifying a set of genes differently expressed in the two culture conditions (Cappelli et al., 2006). This study allowed to identify three groups of mycobacterium induced, repressed and conserved genes in the course of its survival and replication into human macrophages, and we observed that CpG1 is the most represented in all the three groups. Actually, the study regarded the presence of the four CpG sequences in mRNAs, given that our study consisted in a transcriptional analysis. Nevertheless, we made this search because recently also some CpG-RNA sequences were identified, both double and single stranded, that do exert immunomodulatory effects on human antigen presenting cells (Sugiyama et al., 2005; Sen et al., 2004).

CpG motifs exert their effects through TLR-9 that, upon cellular activation, traffics to endosomal and lysosomal compartments, where

the receptor interacts with endocytosed CpG-DNA at acidic pH, a condition that is thought to be necessary for DNA recognition (Latz et al., 2004; Leifer et al.,2004; Rutz et al., 2004). Actually, compounds which interfere with endosomal acidification, such as the weak base chloroquine and bafilomycin A1, an inhibitor for ATP-dependent acidification of endosomes, accordingly prevents CpG-DNA driven TLR9 activation (Hacker et al., 1998; Yi et al., 1998). Therefore we analyzed the effect of different CpG ODNs on intracellular pH (pHi) in a range of time of 30 minutes. With this study we could assess that there is a relation between the specificity of the DNA CpG sequence and the pHi decrease, because the reverse complement ODNs enhance values of pHi as compared to control macrophages and to cells treated with their corresponding sense ODNs. Interestingly CpG1 and CpG3 ODNs significantly reduce pHi as compared to control macrophages, while CpG2 and CpG4 ODNs are less effective in lowering it.

The pHi is important in MTB infection of macrophages: following phagocytosis, the cell proceeds with the formation of a phagosome and in turn of a mature phago-lysosome, in which the low pH activate the lysosomal proteases. Consequently MTB, to survive therein, have to elude this host defence mechanism, by possibly inhibiting H^{+}-ATPasi V able to decrease pHi (Hackam et al., 1997).

Surprisingly, we observed that the lowering of pHi, induced by some CpG treatments, does not decrease the intracellular CFU of MTB. We hypothesised that MTB may possibly take advantage of the intracellular milieu generated by the excess of H^+ ions. A potential scenario is that the massive balance of the external (intracytoplasmic) acidification, leads a saturated H^+-ATPasi V to block its activity, and therefore it does not allows other H^+ ions to enter in the phagosome. Such a process would consequently inhibit the acidification of phagosome and the formation of a mature phago-lysosome.

Indeed, CpG1 and CpG3 ODNs induce the highest acidification but also an enhanced growth of MTB, while in cells treated with CpG2 and CpG4 ODNs, the pHi reduction is lower and the MTB growth is limited respectively of 6 and 1,5 fold than macrophages used as control.

Acidification of endosomal CpG ODN is coupled with a rapid generation of ROS within 30 minutes from stimulation (Yi et al., 1998). According to our analysis, there could be a correlation between the intracellular acidification and the ROS release after CpG ODN administration: in particular CpG1 and CpG3 ODNs stimulate the highest release of ROS and the highest pHi decrease. In contrast, CpG2 and CpG4 ODNs, that induce a slight acidification,

correlate with a lower ROS release. Antioxidant molecules are, generally, considered to be cytotoxic when produced in excess and have been implicated in the pathogenesis of a wide variety of disease (Sen et al., 1996). ROS production, after CpG stimulation, determines NFκB and MAPKs activation (Hacker et al., 1998; Yi et al., 1998) that leads to the expression of different cytokines genes. In particular, CpG ODN is known to induce MAPKs activation upon endosomal acidification and ROS generation (Hacker et al., 1998; Yi et al., 1998), and the activation of different MAPKs may play differential roles in the production of Th1-type cytokines. Actually, in murine macrophage-like cell line, RAW264.7, ERK and p38 MAPKs activated by CpG DNA are found to play differential role in the regulation of cytokine production. Several lines of evidence support the conclusion that CpG ODN-mediated IL-12 production is, at least in part, regulated by an IL-10-dependent pathway that is triggered by ERK activation (Yi et al., 2002).

Therefore, to better comprehend what are the mechanisms involved in control of MTB growth and associated to intracellular acidification and ROS release, we analysed the environment of macrophages for cytokine expression before (after 1 and 24 hours of CpG ODNs treatment) and after infection (after 1 hour of CpG ODNs stimulation and 24 hours upon host-pathogen contact).

We focused on the two farthest situations, deriving from CpG2 and CpG3 ODNs treatment of human cells. In our analysis, by q-Real Time RT-PCR, at 1 hour of treatment, macrophages treated with CpG3 ODN display a Th1 cytokine response, characterised by the expression of IL-12, IFN-γ mRNA, IL-18 and IL-32 and also of IL-10. IL-10 is usually associated to a balance of the Th1 response and to a down-regulation of the pro-inflammatory response, promoting MTB survival and the suppression of antigen presentation of macrophages to T cells (Bermudez et al., 1993). Thus, macrophages treated with CpG3 ODN are stimulated to express, initially, a group of cytokines involved in a Th1 response, but they also express IL-10, that contrasts with ROS production and antymicobacterial activity (Bodgan et al., 1991).

On the contrary, for CpG2 ODN treatment, there is not a strong Th1 cytokine response activation, with an over-expression of IL-10 and the down-regulation of IL-12 expression.

We could then hypothesize that there is a different regulation in transcriptional pathways activated by different effectors, such as MAPKs, but this biochemical step need to be further investigated.

IFN-γ is a Th1 cytokine usually associated to MTB killing, but several observations showed an abrogative effect of IFN-γ in protection against the pathogen exerted by CpG ODN treatment

(Juffermans et al., 2002). In particular, in infected human macrophages, the CpG ODN inhibitory effect in MTB growth is reversed by IFN-γ treatment, representing an escape mechanism, specific for this bacterium (Wang et al., 2005). Our results show that, accordingly, there is a positive correlation between MTB growth enhancement and the over-expression of IFN-γ (after 1 h of CpG3 ODN stimulation), confirming the contradictory role of this cytokine at the earlier steps.

In our transcriptional studies, we considered also Ezrin, a member of the ezrin/radixin/moesin (ERM) family of proteins, that regulate cytoskeletal-related functions such as cell adhesion, cell survival, and cell motility (Takeuchi et al., 1994; Gautreau et al., 1999; Lamb et al., 1997), and also phagosomal formation (Defacque et al., 2000; Erwig et al., 2006). It is known that this molecule in the human colon cancer cell line HT29 is inhibited by IL-2, IL-8 and IL-10 (Jiang et al., 1996). We noticed that, after 1 h of CpG2 ODN stimulation, a down-regulation in Ezrin transcription and higher expression of IL-10 take place, while macrophages treated with CpG3 ODN expressed comparable quantities of Ezrin and IL-10 mRNAs.

At 24 hours of CpG3 ODN treatment there is a strengthening of the Th1 response induction, and a slight reduction in Ezrin transcription. At this time, also the CpG2 ODN stimulation seem to have the same

effect for pro-inflammatory cytokines, but shows a reduction of IL-10 mRNA levels and an enhancement in Ezrin mRNA production.

According to these results, after 1 hour of treatment, a low activation of macrophages seems to be relevant for the containment of infection and we like to favour the hypotheses that intracellular MTB growth reduction depends on initial low levels of IFN-γ avoiding to revert protective effects of CpG ODN, as shown by previous studies (Wang et al., 2005) and on a later up-regulation in IFN-γ (Th1 response) and Ezrin transcription, efficient in mature phagolysosomal formation.

After 24 hours of infection with MTB there is a general down-regulation of all the cytokines, as described above, probably because the CpG ODNs effects are very early. This is also confirmed in protein array after infection where IL-10, IL-12 and IFN-γ seem to be not released at all.

Through this array we wanted to analyze 36 proteins, released in supernatants of infected macrophages, previously treated for 1 hour with CpG2, CpG4 and CpG2+CpG4 ODNs, that determine the lower MTB intracellular growth, and including chemokines, receptors and cytokines. In this study emerged an important role of IL-8, GM-CSF, IL-27 and SerpinE1 (PAI-1), that resulted induced proteins upon MTB infection.

While IL-8, GM-CSF and IL-27 are already known to perform an important role during MTB infection, SerpinE1 was never associated before to the human cells defence mechanisms against MTB.

IL-8 is expressed by many monocytes/macrophages (Yoshimura et al., 1987), and most cell types produce little, if any, IL-8 constitutively (Matsushima et al, 1989). This cytokine is a chemoattractant for neutrophils and lymphocytes (Baggiolini et al., 1989; Larsen et al., 1989) and it may be released in the early response of macrophages to the phagocytosis of MTB. This result confirms also the CpG ODN importance in the IL-8 release as described in cell line RW264.7 after infection with *M. bovis* and after activation of NFκB (Lee et al., 2006), and in THP-1 cells (Friedland et al., 1992). This cytokine is involved in granuloma formation (Zhang et al., 1995) possibly by acting as a T cell chemoattractant and our results may confirm this finding, given that there is also a relevant release of TNF-α.

Another important cytokine, detected in our treated cells, is GM-CSF, which, in the lungs, is crucially important for macrophage maturation and differentiation (Shitaba et al., 2001; Trapnell et al., 2002), surfactant homeostasis, and host defence (Trapnell et al., 2002; Reed et al., 1999; Yoshida et al., 2001). When this cytokine is lacking, as in gene-disrupted mice, the architecture of the lungs is

altered, and alveolar macrophages become foamy in appearance (Dranoff et al., 1994). GM-CSF also acts locally as a pro-inflammatory cytokine, both in recruitment of leukocytes and the enhancement of APC function (Shi et al., 2006). Moreover it can induce the up-regulation of MHC class II and of costimulatory molecules such as CD80 and CD86 on APC, as well as increasing their phagocytic activity and stimulatory capacity (Larsen et al., 94; Burger et al., 2000; Daro et al., 2000; Daro et al., 2002).

IL-27 may have an important immune regulatory capacity, since it has both pro-inflammatory and anti-inflammatory properties. While IL-27 has been shown to promote inflammation, Th1 responses, and IFN-γ production, it can also inhibit inflammatory responses (Hunter et al., 2005; Stumhofer et al., 2006). Surprisingly, animals deficient in the IL-27 receptor were able to limit *M. tuberculosis* infections more efficiently than controls, and neutralization of IL-27 *in vitro* led to enhanced antituberculosis activity in human monocytes (Pearl et al., 2004; Robinson et al., 2008).

The last protein, up-regulated in each of three CpG ODNs treatments, is SerpinE1, that is a protein never associated before to MTB infection. In fact, this molecule, like Ezrin, is mainly associated to cell migration in cancer (Maier et al., 2004) and in thrombolysis (Brogren et al., 2004). Since Ezrin is involved in

phagosomal formation and is related to cytoskeleton actin, and given that SerpinE1 mediates the contacts between the membrane and the cytoskeleton, there could be a particular mechanism that get to reassembling of component implicated in containment of infection, that induce or not the phagosomal formation, but this issue deserves further examination.

The immune response against Tuberculosis is complex and still debated because, as we can see from our results, a lot of mechanisms are involved in protection against MTB, like intracellular pH, ROS production, cytokines transcription, etc, and it is necessary a precise balance of each component of the delicate network.

Our data confirm that the treatment with specific mycobacterial CpG ODNs may help to improve the efficiency of BCG vaccine, but, also, may highlight that the mechanisms playing a role in protection could be more complex than those studied until now.

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