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New Strategy involving dendritc cell mediated immune response
to improve TB vaccination

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1. ABSTRACT

Abstract (English)

Tuberculosis (TB) represents one of the major causes of mortality from an infectious disease. The bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, is the only available TB vaccine despite it doesn't protect against some forms of this re-emerging disease. Therefore, the development of a more adequate vaccine is essential for improving tuberculosis control. It has been suggested that the limited protection conferred by BCG is partly due to his missing expression of critical immunogenic proteins. Hence, it is conceivable that Mtb antigens not expressed by BCG strains could increase the efficacy of BCG vaccination. A recently proposed strategy to improve the efficiency of a vaccine formulation is the encapsulation of antigens into nanoparticles (NPs) that can avoid their rapid clearance by host organisms and can promote their capture by specific antigen presenting cells (APCs).

On the basis of these premises and considering the key role played by human dendritic cells (DCs) in the defences against TB and, more generally, in vaccination, this thesis has been aimed at the discovery of new strategies, involving human DC mediated immune response, to improve the BCG effectiveness.

In particular, the study has been focused on two main goals:

- investigation of the effects of Mtb antigens, HspX and ESAT-6, on human DC activity in order to verify their potential use as vaccine components (task A),
- analysis of a vaccine nano-delivery system based on PLGA or pSi NPs to improve the capture of vaccine formulations by human DCs (task B).

Task A. In this part of the study we found that the addition of HspX or ESAT-6 to BCG stimulated DCs does not affect DC maturation and pro inflammatory cytokine secretion. However DC stimulation with BCG and both HspX and ESAT-6 (BCG/HspX/ESAT-6) greatly enhances the expression of DC maturation markers CD83, CD86 and MHCII, and induces the release of IL-12, TNF α, IL-6, IL-23 and IL-1β from these cells.

Interestingly, DC treatment with BCG/HspX/ESAT-6 improves the ability of these cells to elicit IFN-γ release and CD69 expression by $CD4^+$ lymphocytes and NK cells as compared to DC treatment with BCG alone or with BCG plus a single antigen. Moreover,

a TLR2-blocking antibody decreases IL-12 release by DCs stimulated with BCG/HspX/ESAT-6, as well as IFN-γ secretion by $CD4^+$ lymphocytes co-cultured with these cells. Furthermore, HspX and ESAT-6 improve the capacity of BCG treated DCs to induce the expression of memory phenotype marker CD45RO in naïve $CD4^+$ T cells. Our results indicate that BCG, HspX and ESAT-6 cooperate in enabling human DCs to induce a substantial T lymphocyte and NK cell mediated immune responses through TLR2-dependent IL-12 secretion. Therefore our findings suggest that HspX and ESAT-6 represent good candidates for improving the effectiveness of BCG vaccination.

Task B. Considering their biocompatibility and their relevance in the scientific literature, two different types of NPs have been utilized: PLGA (poly (lactic-co-glycolic acid) and pSi (porous silicon). Our results show that at doses up to 0.2 mg, which are widely considered appropriate for *in vitro* DCs stimulation, both PLGA and pSi NPs do not affect human DC viability. However, at doses exceeding 0.4 mg, PLGA, but not pSi NPs induce DC apoptosis, indicating that pSi NPs are less toxic than PLGA NPs. Hence, the amounts of NPs used in our experiments have been chosen accordingly. In this part of the work we found that PLGA and pSi NPs are unable to modify *per se* the secretion of pro-inflammatory cytokines IL-12, TNF α, IL-6 and IL-23 by both resting and LPS-stimulated DCs. Interestingly, a confocal microscopy analysis reveals that both PLGA and pSi NPs are ingested by human DCs. However, PLGA NPs are more efficiently internalized by DCs than pSi NPs, indicating that PLGA NPs could be a suitable tool to be used for targeting molecules into human DCs.

Take together our results suggest a new vaccination strategy against TB based on i) the combination of BCG/HspX/ESAT-6 in order to stimulate the immune system, and ii) the use of PLGA NPs to target antigens into human DCs.

Abstract (Italian)

La Tubercolosi rappresenta una delle principali cause di mortalità da malattia infettiva. Il bacillo di Calmette-Guérin (BCG), un ceppo vivo attenuato del *Mycobacterium bovis*, è l'unico vaccino attualmente disponibile per la tubercolosi, nonostante non protegga da alcune forme di questa malattia considerata riemergente. Pertanto, lo sviluppo di un vaccino più adeguato ed efficace è essenziale per migliorare il controllo della tubercolosi. E' stato proposto che la limitata protezione conferita dal BCG possa

essere parzialmente dovuta alla sua mancata espressione di importanti proteine immunogeniche. Dunque, è concepibile pensare che antigeni del Mtb che non sono espressi dai ceppi BCG, possano incrementare l'efficacia della vaccinazione da BCG. Una recente strategia di vaccinazione proposta per aumentare l'effetto della formulazione di un vaccino è l'inserzione di antigeni in nanoparticelle (NP) che possono evitare la loro rapida distruzione da parte dell'organismo ospite e possono promuovere la loro cattura da parte di specifiche cellule presentanti l'antigene (APC).

Sulla base di queste premesse e considerando il ruolo chiave svolto dalle cellule dendritiche umane (DC) nelle difese contro la TB e più in generale, nella vaccinazione, questa tesi è stata finalizzata alla scoperta di nuove strategie, coinvolgenti la risposta immunitaria mediata dalle DC, per migliorare l'efficacia della vaccinazione da BCG. In particolare, lo studio si è focalizzato su due principali argomenti:

- Indagine degli effetti degli antigeni di Mtb, HspX e ESAT-6, sull'attività delle DC umane al fine di verificare il loro potenziale utilizzo come componenti del vaccino (task A),
- Analisi di un nano-sistema di trasporto basato su NP di PLGA (acido polilatico co-glicolico) o pSi (silicio poroso) per potenziare la cattura delle formulazioni vacciniche da parte delle DC umane (task B).

Task A. In questa parte dello studio abbiamo scoperto che l'aggiunta dei singoli antigeni ESAT-6 o HspX alle DC stimolate con BCG non modifica né la maturazione di tali cellule né la secrezione di citochine proinfiammatorie. Tuttavia, la stimolazione delle DC con BCG e con entrambi HspX e ESAT-6 (BCG/HspX/ESAT-6), aumenta significativamente l'espressione dei marcatori di maturazione CD83, CD86 e MHCII, e induce il rilascio di IL-12, TNFα, IL-6, IL-23 e IL-1β da queste cellule. Inoltre, il trattamento delle DC con BCG/HspX/ESAT-6 aumenta la capacità di queste cellule di indurre il rilascio di IFN-γ e l'espressione di CD69 dai linfociti $CD4^+$ e dalle cellule NK rispetto al trattamento delle DC con solo il BCG o con il BCG e i singoli antigeni. Abbiamo anche visto che il trattamento con un anticorpo bloccante anti-TLR2 riduce il rilascio di IL-12 dalle DC stimolate con BCG/HspX/ESAT-6 ed anche la secrezione di IFN-γ da linfociti $CD4^+$ cocoltivati con queste cellule. Inoltre, HspX e ESAT -6 migliorano la capacità di DC trattate con BCG di indurre l'espressione del marcatore del fenotipo "memory", CD45RO nei linfociti T naive. I nostri risultati indicano che BCG, HspX e ESAT-6 cooperano per consentire alle DC umane di

indurre una sostanziale risposta immunitaria mediata dai linfociti T e delle cellule NK attraverso la secrezione di IL-12 TLR2-dipendente. Pertanto, i nostri risultati suggeriscono che HspX e ESAT -6 risultano essere due buoni candidati per migliorare l'efficacia della vaccinazione BCG .

Task B. Considerando la loro biocompatibilità e la loro rilevanza nella letteratura scientifica, sono stati utilizzati due diversi tipi di NP: PLGA e pSi. I nostri risultati mostrano che a dosi fino a 0,2 mg , che sono ampiamente considerate appropriate per la stimolazione *in vitro* di cellule dendritiche, sia le NP di PLGA che di pSi non influenzano la vitalità delle cellule. Tuttavia, a dosi superiori a 0,4 mg , le NP di PLGA , ma non quelle di pSi, inducono apoptosi delle DC, indicando che le pSi sono meno tossiche rispetto alle NP di PLGA. Quindi, la quantità di NP utilizzate nei nostri esperimenti sono state scelte di conseguenza. In questa parte del lavoro abbiamo trovato che nè le NP di PLGA nè quelle di pSi sono in grado di modificare di per sé la secrezione di citochine pro-infiammatorie quali IL-12, TNFα, IL- 6 e IL-23 sia in DC non stimolate, sia in DC stimolate con LPS. È interessante notare come l'analisi di microscopia confocale abbia rivelato che sia le NP di PLGA che le pSi vengono ingerite dalle DC. Tuttavia, è stato possibile osservare che le NP di PLGA vengono più efficacemente internalizzate dalle DC rispetto alle pSi. Queste evidenze suggeriscono che le NP di PLGA potrebbero essere uno strumento adatto da essere impiegato per il trasporto di molecole all'interno di cellule dendritiche umane .

L'insieme dei nostri risultati, pone l'attenzione su una nuova possibile strategia di vaccinazione contro la TBC basata su i) la combinazione di BCG/HspX/ESAT-6 al fine di stimolare il sistema immunitario, e ii) l'uso di NP di PLGA per indirizzare gli antigeni nelle cellule dendritiche umane.

2. INTRODUCTION

Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells (APC) that captures antigens and are able to stimulate T and B cells thus initiating a primary immune response [1]. DC were first seen as Langerhans cells (LC) in the skin in 1868, and in 1973 Steinman and Cohn recognized them as major cells of the immune system [2].

When DC were first characterised, researchers noticed that high levels of major histocompatibility complex (MHC) class II molecules are expressed on their cell surface [3]. MHC class II molecules are the protein complexes that present antigenic peptides derived from the exogenous peptides internalised from the extracellular fluid by antigen-presenting cells [4]. In addition high levels of the costimulatory molecules CD86 and CD80 (also called B7.2 and B7.1) and CD40 are expressed on DC surface [5]. These membrane proteins are responsible for the full activation (through signal transduction pathways) and proliferation of T cells after they are first stimulated. The large quantities of these molecules on DC was immediately recognised to be responsible for the unique capacity of dendritic cells (among other professional antigen-presenting cells) to prime naïve T cells, as well as to stimulate all T cells extremely efficiently. DC expressing high levels of MHC class II molecules on their surface were isolated from several different tissues, suggesting that all DC constitutively initiate immunity in vivo. With time, however, it has become evident that dendritic cells are not always activated but also reside in tissues as inactive or “immature” cells [2].

DC have evolved to monitor the environment, detect pathogens and trigger T cell activation, providing a link between the innate and adaptive immune system. Therefore, they are present in tissues which are in contact with the environment: in skin [6] and airways, stomach and intestines[7, 8], in the interstitial spaces of many organs tissues [9], lymphoid and they can also be found in blood [10, 11].

To maintain tolerance as well as immunity, “sentinels” in the periphery of the body that patrol the boundaries against foreign enemies are essential. DC have long spiky arms, called dendrites, hence the name “dendritic cells”. Dendrites enable DC to contact a large number of other cells at one time so confering a high surface-to-volume ratio. DC circulate

throughout the body looking to acquire antigens from other cells, proteins or other molecules belonging to invaders (e.g., bacteria, fungi, viruses, toxins, allergens) or normal self-antigens.

DC in peripheral tissues are mainly found as immature dendritic cells with high paghocytic capacity. Immature DC are specialized in capturing and processing antigens to form MHC peptide complexes, yet they have little ability to activate T cells. Immature DC can release cytokines to communicate with other cells of the immune system. Various stimuli, such as tissue damage, inflammatory mediators, microorganisms and cytokines, cause the DC to mature and migrate into the blood and finally to the secondary lymphoid organs, where they can present their captured antigens to T cells (Figure 1)[12].

This maturation process involves an ordered series of signal-dependent events that result in specific alterations of gene expression, intracellular protein targeting, secretion and organelle biogenesis. The morphology of mature DC differs from that of immature DC, mature DC have an irregular shape with many finger-like projections that increase its surface area to present more molecular structures for interaction with lymphocytes. These projections extend and retract from the cell body to provide motility. Mature DC have transformed so that they are no longer able to capture antigens but have increased antigen presentation. This increased antigen presentation results in potent stimulation of T cell immunity. For the mature DC to activate naïve T cells, secondary signals, such as costimulatory and adhesion molecules, are required to initiate a primary response. The adhesion molecules ensure T cell contact and the stability of the immunological synapse, whereas the costimulatory molecules (CD80, CD86) ensure T cell activation and amplification of the signaling process [13, 14]. Thus, the main function of the immature or tissue DC is to absorb and process antigens, while the mature DC presents the antigen [15] (Figure 1).

The relative scarcity of dendritic cells (they represent only 0.1% of all leukocytes) makes it difficult to isolate them in large quantities and so an intensive effort was made to generate these cells in vitro from bone marrow precursors. Several laboratories have established protocols for the production of primary dendritic cells of mouse and human origin. These methods, which use precursor cells from various sources, have also underlined the importance of specific cytokines for dendritic cell differentiation. For example, granulocyte-macrophage colony stimulating factor (GM-CSF) is indispensable for the generation of dendritic cells from bone marrow precursors. In addition to GM-CSF,

interleukin 4 (IL-4) is required to generate dendritic cells derived from human blood monocytes as was originally shown by Federica Sallusto and Antonio Lanzavecchia [14, 16].

Monocyte-derived dendritic cells (moDC) are at this moment the easiest accessible "source" of human dendritic cells. These cells are generated in 5 days from monocytes cultures in the presence of GM-CSF and IL-4. At day 5 they lose CD14 which is a monocyte lineage marker and gain CD1a, considered as the classical monocyte-derived dendritic cell marker. "Classical" stimuli like lipopolysaccharide (LPS) can stimulate the "immature" moDC to produce factors such as IL-6, IL-8 and IL-12. In some respects, moDC do not show the same behaviour or capability as dendritic cells isolated ex vivo, but they are often used for research, as they are still much more readily available than genuine DC. Moreover, a review published by Serbina et al. showed the importance of circulating blood monocytes in supplying peripheral tissues with macrophage and dendritic cell precursor and, in the setting of infection, in contributing to immune defence against microbial pathogens [17].

Maturation of dendritic cells was first observed in vitro with Langerhans cells. Freshly isolated Langerhans cells needed two or three days in culture to acquire both the morphological features of dendritic cells and their immunostimulatory activity. Maturation therefore became the key event for dendritic cell function, allowing a population of dendritic cells to synchronise antigen capture, antigen presentation and migration to the lymph nodes [18, 19].

In the past few years, several classes of molecules responsible for DC maturation have been characterised: bacterial products like LPS, viral products, inflammatory cytokines (such as IL-1β and TNF-α) and costimulatory receptor stimulation (such as CD40 ligand on T cells) [18]. So, the initiation of maturation results from the integration of signals from various origins. Although we have been talking about DCs maturation as if it is a homogenous process, recent reports suggested that combinations of various maturation stimuli might affect dendritic cell activity differently. The factors used to stimulate DCs in vitro have different impacts on gene expression patterns and on their potential to stimulate T cells. Taken together, DCs comprise a unique system of APC designed to capture antigens at the side of entry and then to identify cognate T cells to prime a specific immune response. Modulators of this critical defense system would be powerful tools to better control pathogenic DCs mediated processes.

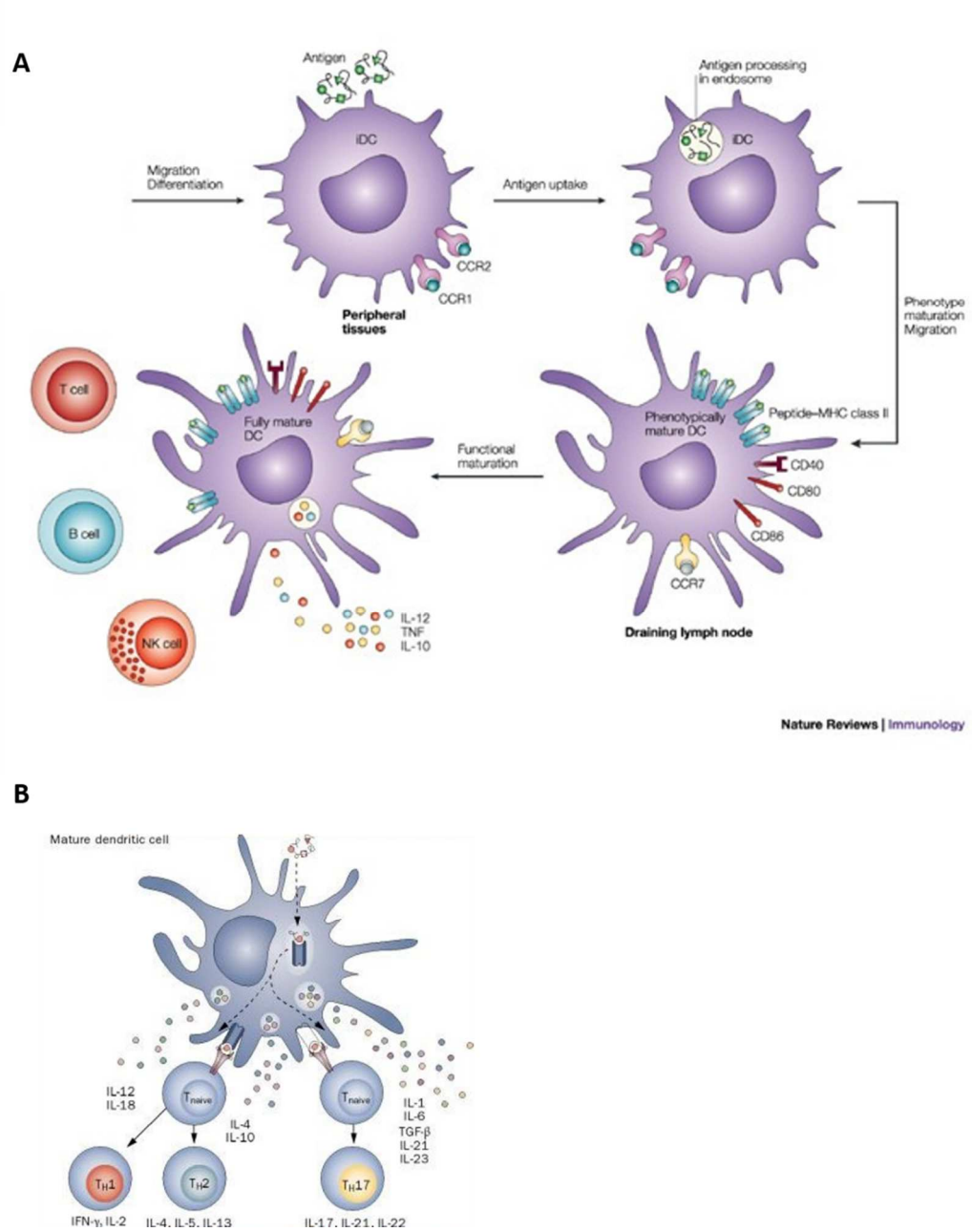

Figure 1. DC maturation and effector cells activation.
A) Resident DCs are activated and migrate to the draining lymph nodes upon pathogen binding in peripheral tissues. At the same time, monocytes are recruited into peripheral tissues. There, they rapidly differentiate to antigen-capturing DCs and upon maturation migrate to the draining lymph node carrying high levels of peptide-MHC complexes and costimulatory molecules. By physical interaction through an immunological synapse, naïve T or B cells achieve stimulation and become committed to proliferate. Moreover DCs are able to activate natural killer cells. B) Sustained TCR stimulation by continuous contact with DC and polarizing cytokines (such as IL-12 or IL-4) promote T cell differentiation and polarization, (e.g. Th1, Th17 or Th2) to nonlymphoid tissue-homing effector cells.

Dendritic cells can internalise large quantities of soluble or particulate antigen in a highly regulated fashion. Many microbes possess their own cell adhesion molecules, which enable them to infect cells, whereas DC can activate an actin cytoskeleton-driven uptake mechanisms called phagocytosis, upon binding of particulate ligands such as microbes to specific cell surface receptors.

Two classes of cell-surface receptors mediate phagocytosis, opsonic and nonopsonic receptors. The first, Fc receptors, recognise serum components which are deposited on microbes and interacts with immunoglobulin and receptors which recognise complement. In contrast, nonopsonic receptors must themselves discriminate between harmless host cells and molecules or surfaces of potentially dangerous microbes. DC possess germline-encoded pattern recognition receptors (PRRs) that recognize and are triggered by evolutionarily conserved molecules essential to pathogen function, which are absent from the host. These so-called pathogen associated molecular patterns (PAMPs) are widespread. Bacteria possess an abundance of PAMPs, from cell wall components (LPS, lipoproteins, peptidoglycans, lipoarabino-mannan) to DNA containing unmethylated CpG motifs. Yeast and fungal cell walls have PAMPs in the form of mannans and beta-glucans, retroviruses have a genome of dsRNA, and protozoa express several unique immunoglycosylated proteins and lipids. Dendritic cells express at their surface widespread antigen receptors such as C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). They activate signaling pathways that induce upregulation of costimulatory molecules, antimicrobial effector responses and inflammation upon recognition of PAMPs. Many of them also are internalized and carry antigens to the MHC class II presentation pathway.

Tuberculosis

From the past to the resumption

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The disease affects 1.7 billion people/year which is equal to one-third of the entire world population and more than 8 million new cases are diagnosed each year.

Mycobacterium tuberculosis is the etiologic agent of tuberculosis in humans, which are the only reservoir for the bacterium. The bacterium Mycobacterium tuberculosis is annually responsible for nearly 2 million deaths worldwide. Pulmonary TB is known since the

time of Hippocrates as phthisis, which is derived from the greek for “wasting away”. During the European Middle-Age, TB was most commonly found in children because of the bacillum spread by unpasteurized milk from infected cows. In 1680 F.F. Sylvius carried out anatomic-pathologic studies in pulmonary nodules from TB patients, which he named “tubercula” observing their evolution to lung ulcers. In 1722, Dr. B. Marten proposed that TB could be transmitted through the “breath” of a sick person. Finally, in the 1882, Dr. Robert Koch isolated and cultured M. tuberculosis from crushed tubercles. In the following years, Dr. P. Ehrlich provided the basis to develop the Ziehl-Nielsen staining, which is an important tool to diagnose TB. Fifteen years after the isolation of the etiologic agent of human TB, the scientist-couple A. Calmette and C. Guerin isolated the bovine variant. After 39th passage in dispersed culture they observed a morphological variant which was avirulent in many animal models, moreover conferring a protection against subsequent challenges with virulent M. tuberculosis. The vaccine currently known as BCG has become widely used to combat TB, it relies on a prophylactic administration of live attenuated bacilli to children.

In the mid 1980 a mixture of 4 antibiotics was introduced as a TB chemotherapy, since then the TB mortality rates were considerably reduced. Although vaccination and chemotherapy have been established to combat TB, epidemiological data indicate that the disease has been eradicated in developed nations, while in developing countries is a long lasting problem. Currently, the WHO (World Health Organization) indicate that there have not been great effects on the global problem since the time of Koch [20] 60. TB resumption can be attributed to several factors: such as the association with HIV, the aging of the world population, aerosol route of transmission amongst environments of human accumulation (such as prisons and hospitals), the increase of immigrants from developing countries to developed ones and the increased number of cases of multiple drug-resistant (MDR) TB [20]. The treatment requires a six to 12 month regimen with at least two antibiotics. Failure to complete the full course of drug therapy can lead to M. tuberculosis organisms that are resistant to one or more anti-tuberculosis drugs, severely limiting effective treatment options.

So, more than a solved problem, TB has reemerged as a serious public health threat worldwide. For this reason, many worldwide organizations decide to fund “The Global Plan to Stop TB” which is a comprehensive assessment of the action and resources needed to implement the “Stop TB strategy” and make an impact on the global TB burden. Its goal is to

dramatically reduce the global burden of tuberculosis by 2015 by ensuring all TB patients, including for example, those co-infected with HIV and those with drug-resistant TB, benefit from universal access to high-quality diagnosis and patient-centered treatment. The strategy also supports the development of new and effective tools to prevent, detect and treat TB.

The microrganism

Within the genus Mycobacterium a number of species are grouped into complexes that include bacterial species with a high degree of genetic similarity as well as cause similar disease syndrome.

Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. The genus includes pathogens known to cause serious diseases in mammals, including tuberculosis and leprosy. The Latin prefix "myco—" means both fungus and wax; its use here relates to the "waxy" compounds in the cell wall. Mycobacterium tuberculosis is a small, rod-like bacillus, obligate aerobe growing more successfully in tissue with high oxygen contenct such as lungs. M. tuberculosis is highly hydrophobic with a high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains so they are classified as acid-fast Gram positive bacterium due to their lack of an outer cell membrane. M. tuberculosis divides every 15 to 20 hours, extremely slowly compared to other bacteria, which tend to have division times measured in minutes (for example, E. coli can divide roughly every 20 minutes). It can withstand weak disinfectants and can survive in a dry state for weeks. Most mycobacteria are found in habitats such as water or soil. However, a few are intracellular pathogens of animals and humans.

Mycobacterium tuberculosis, along with M. bovis, M. africanum, and M. microti all cause the disease known as tuberculosis and are members of the tuberculosis species complex. Each member of the TB complex is pathogenic, but M. tuberculosis is pathogenic for humans while M. bovis is usually pathogenic for animals.

The genome of the H37Rv strain was published in 1998 [21, 22]. Its size is 4 million base pairs, with 3959 genes. 40% of these genes have had their function characterized, with possible function postulated for another 44%. 90% of complete genome sequences is highly conserved (there is no diversity between one set of genes from a range of clinical isolates). The genome contains 250 genes involved in fatty acid metabolism, with 39 of these involved

in the polyketide metabolism generating the waxy coat. Such large numbers of conserved genes shows the evolutionary importance of the waxy coat to pathogen survival.

The cell wall structure of Mycobacterium tuberculosis deserves special attention because it is unique among prokaryotes, and it is a major determinant of virulence for the bacterium (Figure 2).

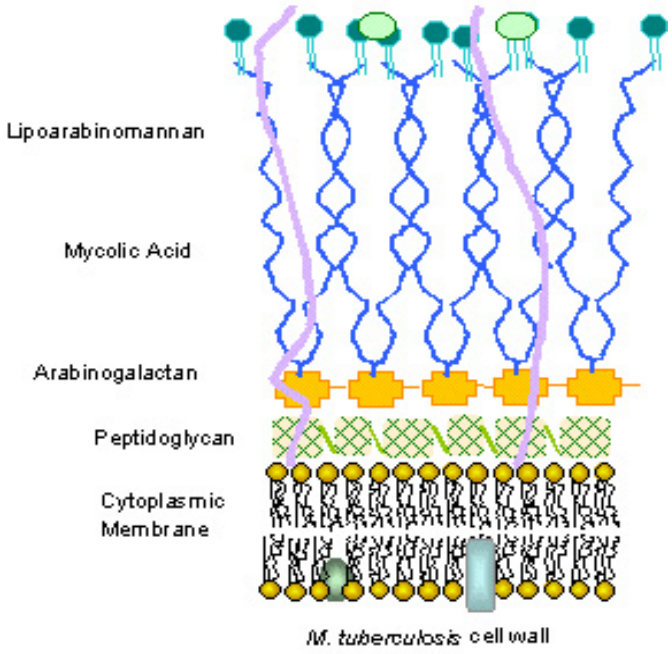

Figure 2. M. tuberculosis cell wall.

The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of M. tubeculosis's cell wall consists of three major components, mycolic acids, cord factor, and wax-D. Mycolic acids are unique alpha-branched lipids found in cell walls of Mycobacterium and Corynebacterium. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in M. tuberculosis. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum. Cord Factor is responsible for the serpentine cording (chains of cells in smears made from in

vitro-grown colonies). Cord factor is toxic to mammalian cells and is also an inhibitor of PMN migration. Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA). The high concentration of lipids in the cell wall of Mycobacterium tuberculosis have been associated with various properties of the bacterium, such as impermeability to stains and dyes, resistance to many antibiotics, to killing by acidic and alkaline compounds, to osmotic lysis via complement deposition and to lethal oxidations and survival inside of macrophages.

M. tuberculosis antigens

To better understand the differences between M. tuberculosis, M. bovis, and the various BCG daughter strains, their genomic compositions were studied by performing comparative hybridization experiments on a DNA microarray [23]. As compared with M. tuberculosis, the vaccine strain lacks an entire genomic region, known as RD1 (region of deletion-1) which is intact in virulent member of the M. Tuberculosis complex. The locus RD1 contains region for secreted proteins such as ESAT-1 [24]. 10% of genome is highly variable and correspond to a family of sequences that encode low molecular weight proteins which are secreted by M. tuberculosis when is cultured in vitro.

One gene family of particular interest encodes Esx proteins, immunodominant T cell antigen that are secreted by a delicate apparatus [25]. The locus Esx1 encodes two family members of originally unknown function, CFP-10 (culture filtrate protein 10 KDa) also called MTSA-10 (M. tuberculosis secreted antigens 10 KDa) and ESAT-6 (early secreted antigen target 6 KDa). Like all other bacteria, mycobacteria have essential general secretion pathways which function to deliver bacterial proteins into the host cell phagosome or cytosol and manipulate the host response to infection. These types of export pathways are usually encoded on so-called “pathogenicity islands”, regions of the genome that are associated with virulence. The likely functional equivalent of these systems, termed ESX-1, has recently been identified [25]. This newly identified protein secretion system is required for growth during M. tuberculosis infection. Since the identification of MTSA-10 and ESAT-6, several studies suggested that these proteins are important for virulence, and recently Hsu et al. has suggested a possible role of ESAT-6 such as a toxin able to directly lyse cellular membranes. ESAT-6 has been extensively studied in mice and human models [26, 27]. One of the first reports has demonstrated that ESAT-6 is a M. tuberculosis antigen recognized by T cells from

a high number of tuberculosis patients, thus suggesting a potential role as an antigen for detection of clinical disease [26]. ESAT-6 has also been proposed for the potential vaccine role in an experimental model. Vaccination by using ESAT-6 delivered in a combination of an adjuvant mix, elicited a strong ESAT-6-specific T-cell response and protective immunity comparable to that achieved with Mycobacterium bovis BCG [28]. MTSA-10 has been demonstrated to bind to the human macrophage surface and induce TNF-α and NO release [29]. Other studies with human monocytes and alveolar macrophages have described promotion of the intracellular replication of bacilli by TNF-α [30, 31], thus raising the possibility that TNF-α secretion induced by secreted mycobacterial antigens, may also serve as an evasion mechanisms for M. tuberculosis. In this paper they speculate that the regulation of macrophage TNF-α and NO production in tuberculosis patient by a soluble secreted antigen, might have an advantage to enable mycobacteria to affect a wider population of macrophages release [29]. Moreover, another article have shown the capacity of MTSA-10 to lead the differentiation of bone marrow cells to dendritic cells, in the absence of any growth or differentiation-inducing factor [32] . These DC expressed various typical DC markers and were able to mature, stimulate T cells and secrete cytokines, upon the use of classical maturation stimuli [32]. This results indicate a putative physiological role for MTSA-10 and possibly mimic the early events that would ensue during an infection, wherein the differentiation of DC by secreted antigens of M. tuberculosis might be followed up by the release of bacteria from macrophages, and eventually leads to a down-regulation of pro-inflammatory responses to mycobacteria.

Antigen 85 complex (three highly related proteins of approximately 30 KDa) is a family of fibronectin-binding proteins involved in the mycobacterial cell wall biosynthesis [33]. It is a major secretory product in Mycobacterium tuberculosis and M. bovis BCG culture fluids and strongly immunogenic (able to cause antibody synthesis and T-cell-mediated reactions). This antigen amongst others have been used as potential DNA vaccine candidates [34, 35]. In the last years many efforts were made to improve TB vaccination: a gene-modified DC-based vaccine expressing antigen 85A was developed, these DC were able to elicit a remarkable CD4 and CD8 immune response conferring protection from M. tuberculosis challenge in mice model [35]. Other investigators have tried to use a fusion protein of ESAT-6 and Ag85B [36] or vaccine-targeted DC [37]. Although many studies seems

to be promising, most of them are really preliminary, so they will tell us in few years the real efficacy for TB vaccination.

One-third of the entire human population may be latently infected with dormant bacilli. Clearly this delay in appearance of the active disease only complicates an already difficult problem, and new strategies which will allow the eradication of latent organisms are required. Such strategies will arise only through an improved understanding of the mechanisms by which the tubercle bacilli remains viable for such extended periods.

As an initial effort to characterize the metabolic changes which allow M. tuberculosis to remain viable for such extended periods of time, Yuan et al. sought to characterize proteins which may be specifically synthesized during late exponential and stationary phase growth in vitro [38]. They identified the 16-kDa antigen of M. tuberculosis (α-crystallin) and demonstrated that it is encoded by a gene that, among the members of the genus Mycobacterium, is present only in the slowly growing M. tuberculosis complex organisms. The antigen α-crystallin is specifically expressed during the transition to stationary phase and is related to the broad family of heat-shock proteins (HSPs). An examination of the α-crystallin domain of the M. tuberculosis homolog reveals that although the sequence homology is not high, the mycobacterial HSP possesses functional similarities with the proteins in this family, the chaperone function. They were able to demonstrate that M. tuberculosis over-expressing this protein displayed an enhanced resistance to autolysis following the end of log-phase growth [38] . Although few studies have hypothesize the role of α-crystallin during infection, they failed to provide information directly related to persistence during latent infections [39, 40]. They do suggest that this protein may be induced in response to such stresses (substrate starvation, oxygen limiting condition, etc…) as are encountered during an infection. The ability to persist following log-phase growth is a common theme in bacterial metabolism.

However, despite the large volume of data available on these antigens the fundamental question regarding their physiological roles at sites of infection has not been answered yet.

Current and novel approaches for Tuberculosis vaccine

Despite immense efforts in TB vaccine research we continue to immunize with Mycobacterium bovis bacillus Calmette-Guerin (BCG) vaccine, which was approved for human use at the beginning of the 20th century. BCG was proposed as a live vaccine against TB by Albert Calmette and Camille Guérin only few years after the discovery of the intracellular pathogen Mtb as the causative agent of TB by Robert Koch in 1890. M. bovis BCG was generated after continual passaging of the parental M. bovis strain for 13 years (a total of 230 passages) in media containing bile, which resulted in an attenuated strain with reduced virulence in animals. BCG vaccine production started in 1927 that then led to generations of daughter BCG strains with different genomic composition [23]. BCG vaccine is administered worldwide as a single intradermal inoculation dose.

The use of heat-inactivated or live attenuated pathogens was the preferred choice for many years in traditional vaccinology since they contain a vast array of antigens. Because of this, a broad and diverse immune response is induced resulting in protection against the pathogen. However, BCG vaccination affords only partial protection in humans. Infact, BCG protects against miliary TB and meningitis TB in infants, but fails to protect against pulmonary and latent TB in adults of both sexes and all ages, including children [41]. The variable efficacy of BCG, ranging from 0 to 80% in randomized control trials is attributed to a number of factors, including geographical location of human population, loss of virulence genes essential in protective immunity, an insufficient induction of CD8+ T cell response, exposure to environmental (non-tuberculous) mycobacteria or helminthic infection prior to BCG vaccination [42].

TB vaccines in clinical trials

Due to protection conferred by BCG in childhood, current vaccines strategies in clinical trials are either focused on engineering BCG to be more immunogenic or boosting prior BCG vaccination with new vaccine regimens in the hopes of increasing protection. There are also a few new live vaccines being tested to entirely replace BCG due to the associated risk of infection with the viable vaccine in TB-HIV population [43].

Live mycobacterium-based vaccines

New-generation mycobacterial vaccine vectors utilize current BCG as backbone to express known T-cell immunogens from virulent Mtb or mutants of BCG that are capable of escaping the phagosome to induce CD8+ T cell response are being tested as vaccine candidates. BCG shares many proteins in common with Mtb, however many of these proteins are not well recognized by vaccinated humans or animals. One such antigen is the molecule known as antigen 85 B (Ag85B), a major secretory protein produced by Mtb previously shown to induce protection to TB in mice and guinea pigs [44]. Although BCG produces this protein, little or undetectable immune response to this molecule is observed after vaccination. However, a recombinant BCG vaccine (rBCG30) overexpressing and secreting ~5.5 fold more Ag85B than conventional BCG induced an order of magnitude greater immune response when compared to the parental BCG vaccine. A Phase I clinical trial with rBCG30 has been successfully completed [45]. Another interesting recombinant BCG-based vaccine is VMP1002 [46]. VMP1002 was engineered using specific properties of listeriolysin O, a secreted thiol-activated cholesterol-binding hemolysin from Listeria monocytogenes. This protein forms multimeric forms that allow engineered VMP1002 cells to escape from phagolysosomes (pH5.5) to the cytosol of the host, where the microbial vaccine antigens can be processed by the MHC Class I machinery and then presented to CD8+ T cells [47].

Booster vaccines to augment BCG immunity

The rationale behind this strategy is to boost anti-TB immunity induced by prior vaccination with BCG. The goal is to continue implementing BCG immunization in neonates but subsequently boost at an older age with another TB vaccine regimen to augment immunity and protection afforded by prior BCG vaccination. Several booster vaccines could be used including recombinant viral poxviruses and adenoviruses vectors, which are highly immunogenic and currently being evaluated as vaccine delivery systems for HIV/AIDS and other diseases [48]. MVA85A is a replication-deficient vaccinia virus Ankara (MVA) used as delivery system for the mycobacterial anti- gen 85A, which augmented BCG-induced immunity in humans [49].

A replication-deficient strain of adenovirus used a delivery vector elicits high magnitude and functional CD4+ and CD8+ T cell responses to a vaccine candidate [50].

Indeed the engineered Ad5Ag85A, a recombinant adenovirus serotype 5 (Ad5) vaccine vector expressing the Mtb antigen 85A is currently being tested in a phase I safety and immunogenicity study in BCG-vaccinated and nonvaccinated healthy adults in Canada. Another variation of this vaccine uses a non-replicating adenovirus serotype 35 expressing anti- gens 85A, 85B, and TB10.4 and is in clinical trial under the name of AERAS-402 [51] (Abel et al., 2010). The advantage of Ad35 over other delivery viral vectors is the low frequency of anti-adenovirus neutralizing antibodies and low-levels of pre-existing immunity found in human

Another strategy to boost BCG-induced immunity is to use protein based subunit vaccines as boosting agents. Subunit vaccines currently in clinical trials include Mtb72F/ASO1/ASO2A, which consists of a 72 kDa recombinant protein containing Mtb32 (Rv1196) and Mtb39 (Rv0125) antigens previously shown to induce strong CD4+ and CD8+ T cells responses in laboratory animals but more importantly, in healthy, PPD-positive individuals [52]. Another vaccine candidate in clinical trial is AERAS-44, which is a fusion protein called Hybrid 1 consisting of antigens 85B [47] and TB10.4 (Rv0288) [53]. The H1 vaccine combined with adjuvants IC31, a synergistic combination of single stranded oligodeoxynucleotide and a peptide (KLKL5KLK) that induces the Toll-like receptor-9 (TLR9) had promising preclinical data in that the vaccine elicited Th1 responses and increased protection against Mtb in mice [54]. H56-IC3 is another candidate vaccine in early human trials. This promising vac- cine formulation contains novel latency-associated TB antigen, Rv2660c, along with Ag85B, ESAT-6, and the IC31 adjuvant [55].

DCs as a target for nanotechnology applications

Given the peculiar characteristics of DCs as professional antigen presenting cells, it became clear that targeting nanoparticles (NPs) to DCs provides a promising strategy for improving immune response in certain conditions. In fact, NPs can modulate the immune response and might be potentially useful as effective vaccine adjuvants for infectious disease and cancer therapy. Although there is no accepted international definition for NPs, it is generally assumed that NPs are particles having one or more structural features on a scale of less than 100 nm that cause their properties to be different from that of the bulk material [56].

A wide variety of NPs can be designed with particular properties (certain size, shape, and composition) to be used as vehicles for specific DC targeting. There are different strategies to use NPs as effective vehicles, such as NPs conjugated to antigens, which are recognized by specific receptors, antigens encapsulated within NPs, which offer the ability to protect the antigen from degradation and labeled NPs, which are also recognized by specific receptors and permit an effective tracking of their migration (Figure 3). These different strategies can be exploited for a variety of applications such as cancer immunotherapy and vaccines that manipulate the immune system for therapeutic benefits and minimize adverse effects. Tracking is also an important application that can be used to evaluate the correct migration of the NP to its target. Figure 3 summarizes the applications of DCs in immunotherapy that can be further exploited by the interactions with different engineered NPs, particularly dendrimers, polymeric NPs, metallic NPs, magnetic NPs, and quantum dots [56]. The electronic and optical properties and the chemical reactivity of small clusters are completely different from the better known property of each component in bulk or on extended surfaces. This is because a NP has a large surface area in relation to its size and is consequently highly reactive. For this reason the appropriate NP must be designed to be used as an effective vehicle that interacts correctly with DCs, so as to finally develop a desired response.

Mechanisms of action mediated by the interaction between DCs and NPs

NPs conjugated with groups that permit specific recognition of DCs allow a more precise localization of these cells. This active targeting is based on the use of a wide variety of ligands such as antibodies, polysaccharides, peptides, and drugs that bind to a protein, for example a cell surface receptor. Another way to target DCs is by cellular uptake, capturing the NPs via endocytocis or pinocytosis [57, 58].

An efficient delivery avoiding cytotoxicity during passage through the DC membrane must be arranged to reach the intracellular compartment. For this reason, various biomaterials such as liposomes, polymeric NPs, and quantum dots, among others, are being used to target specifically DCs and finally accelerate their maturation.

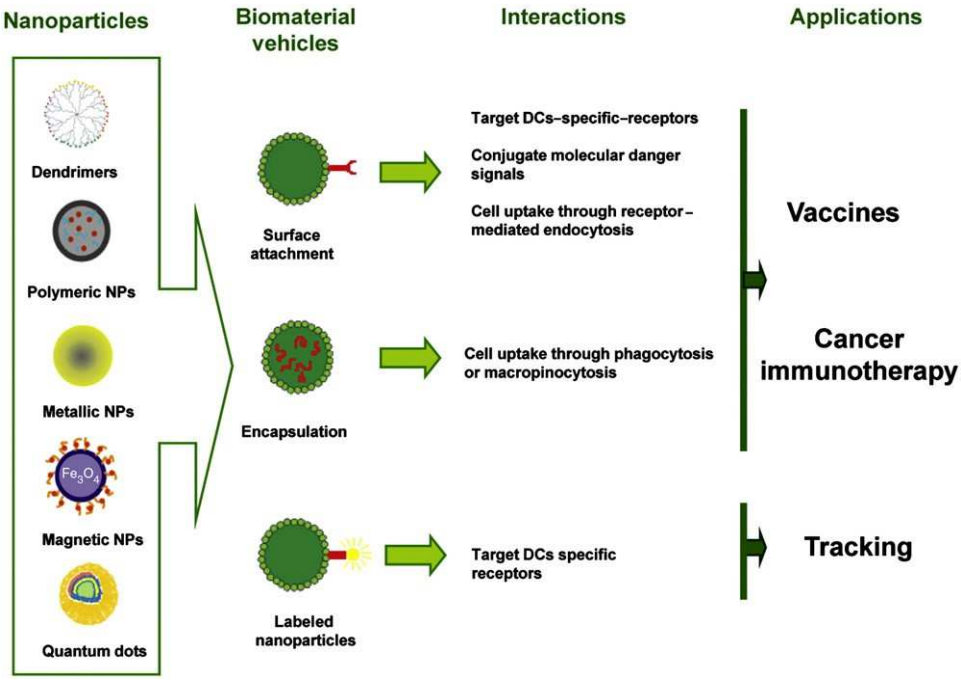

Figure 3. Dendritic cells (DCs) and nanoparticle (NPs) targeting.
The use of multifunctional nanosystems based on NPs (dendrimers, polymeric NPs, metallic NPs, magnetic NPs, and quantum dots) affords convergence of technologies (surface attachment, encapsulation, or labeled NPs) for simultaneous or sequential target-specific delivery of multiple drugs, antigens, or tracking molecules to DCs. The interactions involve the targeting of DC-specific receptors, and uptake of NPs through receptor-mediated endocytosis, phagocytosis, or macropinocytosis, so as to achieve a specific desired response in applications in the fields of vaccine research or cancer immunotherapy, where DCs are known to be key immune-based regulators.

Precisely defined and engineered molecules with specifically designed functions can be attached to NPs and activate DCs Toll- like receptors (TLRs). An example of this strategy can be highlighted in the case of polyethylenimine-based small interfering RNA nanocomplexes (siRNA-PEI NPs), which activate specific TLRs expressed by tumor-associated DCs [59]. Other NPs are designed to avoid trafficking to degradative lysosomes, which are one of the most important cell barriers for effective gene transfer. Therefore, a high transfection efficiency can be achieved with NPs such as polymer nucleic acid complexes [60], used to prevent degradation. Moreover, most NPs are trapped in endosomes by receptor-mediated endocytosis. An example of an antigen carrier trapped in endosomes is the methoxypolyethylene glycol and poly (lactide-coglycolic acid) (PEG-PLGA) NP, which encapsulates a recombinant hepatitis B antigen able to react with an anti-HBs antibody [61]. Magnetic NPs have also been demonstrated by transmission electron microscopy images

and confocal microscopy to internalize DCs and localize mainly in the lysosomal compartment [62]. Other properties of the NPs such as the size that can have a significant effect on the endocytic process, have to be kept in mind. In particular the latter point has been investigated with magnetic NPs, and it has been concluded that endocytosis is not size selective within a size range of 10-200 nm [62]. Other data about size selective targeting of DCs with polystyrene NPs have been reported to show that large particles (500-2000 nm) traffic to the lymph nodes in a DC-dependent manner, whereas small NPs (20-200 nm) and virus-like particles drain freely to the lymph nodes, where they are taken up by lymph node resident DCs and macrophages [63].

An alternative strategy to specifically activate DCs can be carried out indirectly, by interacting with other cell types. This is the case of natural killer T (NKT) cells, which display potent antiviral and antimetastasis function after activation of DCs [64, 65]. For this reason, α-galactosylceramide (a glycolipid antigen presented by CD1d receptors on NKT cells) functionalized NPs are an attractive DC-mediated immunomodulatory strategy. The NKT cells, thus stimulated by TCR signaling, may finally activate DCs by upregulating co-stimulatory molecules and intracellular trafficking via the MHC class I pathway.

Take together these evidences suggest that NPs, for their ability to bring both antigens and adjuvants into cells, have a great potential as targeting systems for cancer vaccines.

3. AIM OF THE STUDY

The main purpose of this thesis has been to design and characterize on the immunological point of view a new vaccine strategy that could be useful to fight TB, a reemerging disease. The relevance of this study is underlined by the awareness that despite TB is the leading cause of death in the world from a bacterial infectious disease, the vaccine currently used, known as BCG, does not confer an effective protection against various forms of the disease, especially adults pulmonary TB. Hence, it is clear that the development of new and effective tools to prevent and/or treat TB is urgently needed.

Considering the key role played by DCs in the defense against TB and in vaccination in general, the present study has been aimed at the discovery of new strategies to improve the human DC-mediated immune response against Mtb. In this regard, the focus of the research presented herein has been not only the investigation of the effects of new Mtb antigens useful as vaccine components on DC activity (Task A), but also the analysis of a vaccine nano-delivery system that could improve the capture of vaccine formulations by DCs, increasing the immune response mediated by these cells (Task B).

<u>Task A: analysis of ESAT-6 and HspX as new BCG adjuvants.</u>

BCG induces a good protection against TB in childhood, but is poorly efficient in some forms of this disease in adults. For these reasons the research on TB vaccination field is mainly aimed to ameliorate the protection conferred by BCG, either by engineering more immunogenic BCG formulations, or by boosting prior BCG vaccination with new adjuvants. However, there are also a few new-line vaccines being tested to entirely replace BCG. The limited efficacy of BCG vaccination against some forms of TB is partly due to the missing expression of critical immunogenic proteins. Among these, ESAT-6 and HspX have been found to be good immune-modulatory agents in various animal models, but their effects on human immune cells and, most importantly, their potential role as vaccine components has not been completely elucidated.

On the basis of these premises, the first task of the investigations reported here has been to analyze whether the single or combined addition of ESAT-6 and HspX to BCG

could improve the human DC-mediated immune response and could induce a better immunological memory than the one elicited by BCG alone.

Task B: Designing and testing an efficient nano-delivery system for human DC targeting.

The development of delivery systems able to target various molecules toward immune cells could play a critical role in the future of vaccinology, and this is the reason of the emerging attention on nanoparticles as potential carriers of various molecules. Regarding the design of vaccine formulations, the encapsulation of antigens into nanoparticles can avoid their rapid clearance by host organisms and can promote their capture by a specific APCs. Hence, the selective targeting of vaccine formulations into DCs would be a valuable strategy to improve the immune response mediated by these cells. Various kinds of materials can be used for nanoparticle production. However, on the basis of their biocompatibility and their relevance in the scientific literature, two different types of nanoparticles have been utilized in this study: PLGA (poly (lactic-co-glycolic acid) and pSi (porous silicon).

The purpose of task B has been to design and to test a pSi or PLGA nanoparticle-based delivery system able to selectively bring vaccine components into DCs improving the antigen capture by these cells and consequently, the immune response.

2. MATERIALS AND METHODS

Reagents and antibodies

The following reagents were used for DC preparation and stimulation: RPMI 1640 and low-endotoxin fetal bovine serum (FBS) were obtained from Lonza (Walkersville, MD); recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and human IL-4 were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany); gamma-irradiated whole cells of *M. tuberculosis* (strain H37Rv) were obtained through BEI Resources, NIAID, (NIH NR-14819); α-crystallin (Gene Rv2031c), a purified native protein from *M. tuberculosis* (strain H37Rv, NR-14860) was provided by NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH; M. bovis BCG kindly provided by Dr. G. Batoni (Dept. Of Experimental Pathology, Medical Biotechnology, Infectivology and Epidemiology, University of Pisa, Italy) was prepared as described in [66] and killed at 55°C for 30 minutes; rdESAT-6 was provided by Statens Serum Institut (Copenhagen, Denmark); ultrapure lipolysaccharide (LPS) from E. coli (0111: B4 strain) and palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) were purchased from InvivoGen (San Diego, CA). All of the above reagents contained less than 0.125 endotoxin units/ml, as measured by the Limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD).

Flow cytometric analysis of surface marker expression and cytokine intracellular staining was performed using the following mouse anti-human antibodies: CD83 (HB15e), CD4 (SK3) and CD1a (HI149) (Becton Dickinson, San Jose, CA); CD56 (HCD56), CD69 (FN50), CD86 (T2.2), HLA-DR (L243) and IFN-γ (4S.B3) (Biolegend, San Diego, CA); CD4 (VIT4), CD45RO (UCHLI), CD45RA (T6D11) (Miltenyi Biotec); IL-17AF (20LJS09) (eBioscience, San Diego, CA). In the viability assays the percentage of apoptotic cells was determined using the Annexin V staining kit (Miltenyi Biotec).

The following blocking antibodies were used in our experiments: anti- Toll-like Receptor (TLR) 2 (T2.5) (eBioscience, San Diego, CA); anti-IL12p70 (20C2) [67, 68] kindly provided by Dr. G. Trinchieri (Center for Cancer Research, NCI, Frederick, MD). Monoclonal mouse IgG1 (eBioscence) was used as the isotype control antibody.

PLGA nanoparticles (PLGA NPs) have been synthetized and characterized by the group of Professor U. Monaco (Biocrystallography Lab, Dep. of Biotechnology, University of Verona, Verona, Italy). Silicon nanoparticles (pSi NPs) have been synthetized and characterized by the group of Dr. N. Daldosso (Fluorescence Lab., Dep. of Computer Science, University of Verona, Verona, Italy).

Preparation and culture of DCs, lymphocytes and NK cells

After written informed consent and upon approval of the ethical committee, buffy coats from the venous blood of normal healthy volunteers were obtained from the Blood Transfusion Centre of the University of Verona. Monocytes were isolated from buffy coats by Ficoll-Hypaque and Percoll (GE Healthcare Life Science) density gradients and purified using the human monocyte isolation kit II (Miltenyi Biotec), as previously described [69]. The final monocyte population was 99% pure, as measured by FACS analysis. To generate immature DCs (iDCs), monocytes were incubated at 37°C in 5% CO_2 for 5-6 days at 1 x 106/ml in 6-well tissue culture plates (Greiner, Nürtingen, Germany) in RPMI 1640, supplemented with heat-inactivated 10% low endotoxin FBS, 2 mM L-glutamine, 50 ng/ml GM-CSF, and 20 ng/ml IL-4. The final DC population was 98% $CD1a^+$, as measured by FACS analysis.

To perform the experiments regarding the task A, DCs were treated for 24 hrs with: Mtb (50 μg/ml), Pam3CSK4 (10 μg/ml), LPS (100 ng/ml), BCG (50 μg/ml) alone or combined with HspX (10 μg/ml) and/or ESAT-6 (10 μg/ml). In the experiments performed with blocking antibodies immature DCs were pre-incubated for 15 min at room temperature with anti-IL-12p70, anti-TLR2 antibodies or an isotype antibody IgG1.

To perform the experiments regarding the task B, iDCs were treated for 24 hrs with various doses of particles or, when necessary, with 100 ng/ml LPS.

NK cells and autologous total and naïve $CD4^+$ T cells were isolated from the lymphocyte fraction of the Percoll gradient with EasySep™ Negative Selection Human Cell Enrichment kits (StemCell Technologies, Vancouver, Canada). The final populations were 98% pure, as measured by FACS analysis. To preserve T cells during differentiation of monocytes into DCs, the cells were spinned down, resuspended in freezing medium (low endotoxin FBS + 10% DMSO), and kept in a liquid nitrogen freezer. In order to study their

effects on T lymphocytes, mature DCs were co-cultured for 7 days with total $CD4^+$ T cells, or for 9 days with naïve $CD4^+$T cells. The DCs:T-cell ratio was 1:10.

NK cells were incubated for 24 hrs with conditioned media (added to 1:1 ratio) from DCs treated with Mtb (50 μg/ml) or BCG (50 μg/ml) alone or combined with HspX (10 μg/ml) and/or ESAT-6 (10 μg/ml). For the experiments with blocking antibodies, the supernatants were pre-incubated for 15 minutes at room temperature with anti-IL-12p70 and with an isotype antibody IgG1.

ELISA assay

The quantification of cytokine production in the cell culture supernatant was determined by specific Enzyme-Linked ImmunoSorbent Assay (ELISA). The Ready-Set-Go ELISA kits purchased from Bioscience (San Diego, CA) were used to assay the protein levels of: IL-12 (range 4-500 pg/ml), IL-23 (range 15-2000 pg/ml), TNF-α (range 4-500 pg/ml), IL-6 (range 2-200 pg/ml), IL-1β (range 4-500 pg/ml), IL-10 (range 2-300 pg/ml), IFN-γ (range 4-500 pg/ml), IL-17AF (range 30-4000 pg/ml) according to the manufacturer's protocol.

Flow cytometric analysis

To perform cell surface protein staining experiments, the cells were washed twice with PBS salt solution and incubated for 30 min with 10% human serum to prevent non-specific binding. For direct immunofluorescence staining, mouse anti-human CD1a, CD83, CD86, HLA-DR, CD69, CD56, CD4, CD45RO, and CD45RA were used (see reagents).

To perform a cytokine intracellular staining, the T cells were treated with 20 ng/ml PMA, 1 μM ionomycin and 10 μg/ml brefeldin A (Biolegend) during the final 6 hrs of culture [70]. After staining with a fluorescent-conjugated antibody anti-CD4, the cells were incubated with fixation/permeabilization buffer (420801 and 421002, Biolegend). Subsequently, they were stained with anti-IL17AF and IFN-γ fluorescent-conjugated antibodies (see reagents). The percentage of apoptotic cells was determined using the Annexin V (see reagents). Samples were acquired on a seven-color MACSQuant Analyzer (Miltenyi Biotec) and FlowJo software (TreeStar, Ashland, OR, USA) was used for data analysis.

Immunofluorescence and confocal microscopy analysis

DCs were seeded on 13 mm poly-L-lysine-coated coverslips; after treatment with NPs, the cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature and quenched with 50 mM NH4Cl. The cells were then permeabilized with PBS-0.1% Triton X-100 and blocked with 1% BSA for 30 min. After washing, the coverslips were incubated 30 min with Phalloidin-Rhodamine (Cytoskeleton, Denver, CO, USA) to visualize F-actin. In some experiments cells were also incubated for 10 min with DAPI (Sigma-Aldrich) to stain nuclei. The cells were washed and mounted in glycerol based anti-fading medium. The images were acquired by confocal microscopy (Leica-Microsystems, Wetzlar, Germany) at 400× magnification by using the 63× oil immersion objective (1.25 NA). Z-stacks were acquired and the maximum intensity projections (MIPs) were obtained by using the LAS-AF software (Leica-Microsystems). The images were processed for brightness and contrast with Adobe Photoshop.

Statistical analysis

All the data presented are expressed as the mean + SD. The Statistical analysis, including Student's t test and one-way ANOVA with Bonferroni test, were performed using SigmaStat 3.0 for Windows (Systat Software, San Jose, CA).

3. RESULTS AND DISCUSSION

5.1 TASK A: analysis of ESAT-6 and HspX as new BCG adjuvants.

RESULTS

ESAT-6 and HspX improve the ability of BCG to stimulate human DC pro-inflammatory cytokine release and maturation

It is well known that the interaction of DCs with pathogenic microorganisms or their derivatives elicits the production of various cytokines that orchestrate the immune response 34 [71]. In this regard, we first examined the capacity of BCG, ESAT-6 and HspX to induce cytokine secretion by DCs. For this purpose, monocytes purified from healthy donors as described in methods, were cultured for 5 days with GM-CSF and IL-4 to obtain immature DCs (iDCs). The latter cells were challenged with BCG, ESAT-6 or HspX, as well as with Mtb as a positive control [69]. After a 24 hr treatment, culture supernatants were collected and cytokine secretion was analyzed by ELISA. Preventive to their use, the amounts of bacteria and antigens have been chosen on the basis of previous dose-response experiments.

The apoptosis has been checked by using Annexin V staining. The concentration of bacteria and antigens used for the experiments described herein did not lead to any apoptosis events (data not shown).

The ELISA assays revealed that BCG induced a weak release of various pro- and anti- inflammatory cytokines, as indicated in figure 1. Since the reintroduction of the ESAT-6 gene restores the ability of BCG to activate mouse immune cells [72], we wanted to determine whether the addition of the ESAT-6 protein, alone or simultaneously with HspX, which also augments the immune stimulatory effects of BCG in mice [73, 74] , could increase human DC response to BCG stimulation. As shown in figure 1 the addition of HspX alone to BCG-treated DCs did not significantly influence cytokine release, whereas ESAT-6 increased the secretion of IL-23 but not the other cytokines. Interestingly, the simultaneous addition of HspX and ESAT-6 to BCG-stimulated DCs significantly increased

the secretion of IL-12, IL-1β, IL-23, IL-6, and TNFα but not IL-10, as compared to DCs challenged with BCG alone or when combined with the single antigens (Fig. 4).

It is important to note that DC challenge with a purified HspX and/or a recombinant ESAT-6 protein in the absence of BCG, did not induce any cytokine production from DCs (results not shown).

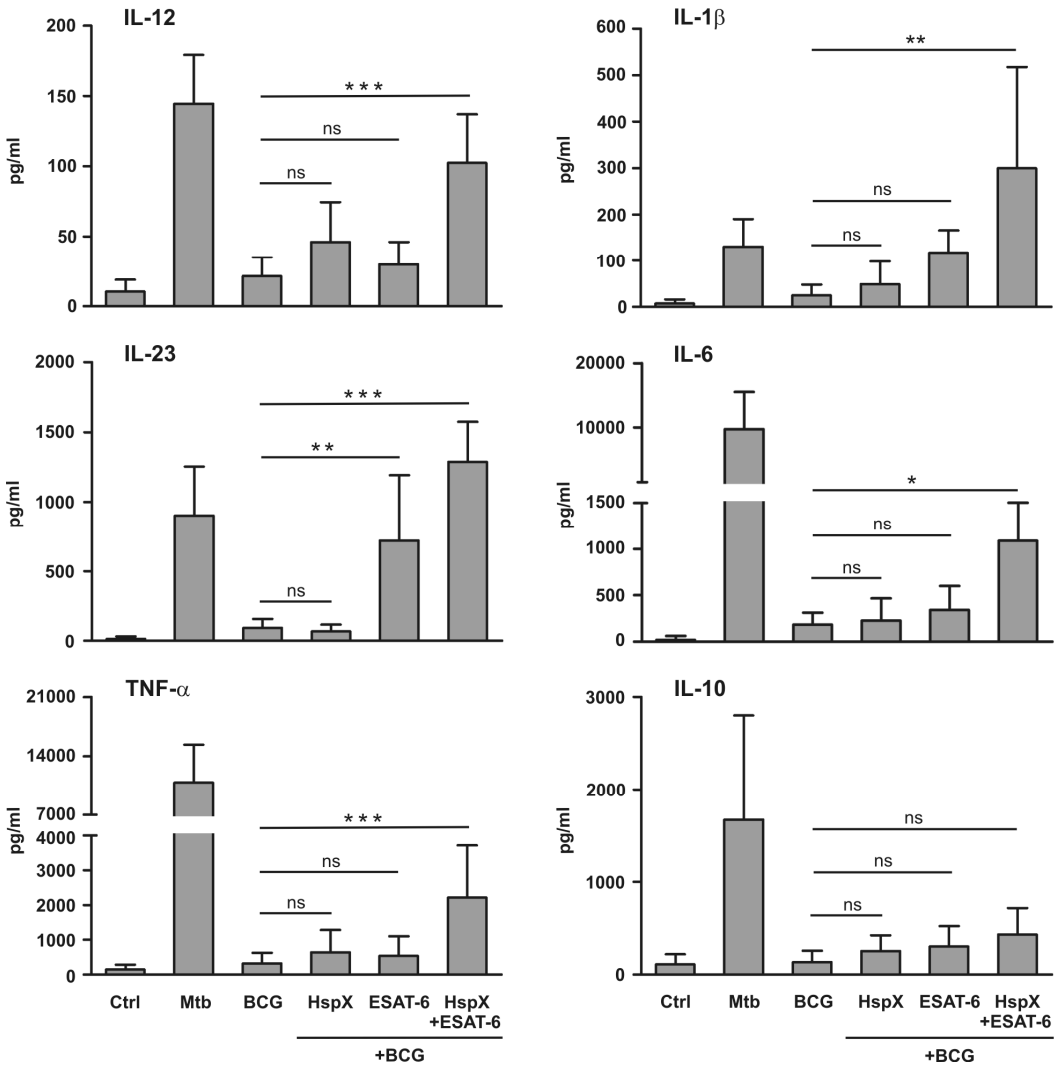

Figure 4. Effect of HspX and ESAT-6 on BCG-induced cytokine secretion by DCs.
Monocytes were treated (5 days) with 50 ng/ml GM-CSF and 20 ng/ml IL-4 to obtain immature DCs, that were subsequently cultured (24 hrs) in the absence (CTRL) or presence of 50 µg/ml BCG, alone or combined with 10 µg/ml HspX and/or 10 µg/ml ESAT-6. DCs were also cultured with 50 µg/ml Mtb as a positive control. Release of the indicated cytokines in culture supernatants was evaluated by ELISA. Results are expressed as the mean value + SD of seven independent experiments. Statistical analysis: DCs treated with BCG alone vs BCG plus HspX and ESAT-6 added alone or in combination; ns P>0.05, *P<0.05, **P<0.01, ***P<0.001.

DC maturation is another process that occurs when immature DCs interact with pathogens or their antigens. This process is accompanied by the above mentioned cytokine secretion and enables DCs to activate immune effectors cells [71]. This event leads to the formation of DCs with increased expression of CD83, CD86 and HLA-DR on the membrane surface [75, 76]. Therefore, in parallel to the cytokine detection, we checked the maturation state of the DCs already used for ELISA (Fig. 1), analyzing the membrane expression of CD83, CD86 and HLA-DR. We found that BCG did not induce significant CD83 and CD86 up-regulation and that it inhibited HLA-DR basal expression, as compared to untreated DCs (Fig. 5A and B). However, the simultaneous addition of ESAT-6 and HspX to BCG-treated DCs significantly increased CD83, CD86, and HLA-DR expression, as compared to DCs incubated with BCG alone (Fig. 5A and B). Finally, we found that DCs did not mature upon cell stimulation with ESAT-6 or HspX in the absence of BCG, nor with BCG in the presence of single ESAT-6 or HspX (results not shown).

ESAT-6 and HspX enable BCG-treated DCs to activate $CD4^+$ lymphocytes

Mature DCs are known to regulate the activity of T lymphocytes which play a prominent role in defensive mechanisms against tuberculosis. In particular DCs are involved in the polarization of various T helper (Th) populations, characterized by specific pro- or anti- inflammatory activity. The main pro-inflammatory Th subsets involved in the immune response against TB are Th1 and Th17 populations [77]. As we found that ESAT-6 and HspX improved the BCG-dependent DC maturation and cytokine release (Fig. 4 and 5), we hypothesized that these antigens could influence the capacity of DCs to regulate T lymphocyte activity. Therefore, we investigated whether treatment with BCG, HspX and/or ESAT-6 would enable DCs to induce Th1 and/or Th17 response. For this purpose, DCs induced to maturation with Mtb or BCG, alone or combined with ESAT-6 and/or HspX, were co-cultured with autologous $CD4^+$ lymphocytes. After 7 days the culture supernatants were assayed by ELISA for the presence of IFN-γ and IL-17AF, secreted by Th1 and Th17 respectively. This time point was chosen on the basis of previous time course viability assays (results not shown). The figure 3A shows that DCs incubated with Mtb induced remarkable IFN-γ and IL-17AF production by T cells, whereas DCs stimulated with BCG, alone or in combination with either HspX or ESAT-6, showed a weak ability to activate these responses. However, DCs incubated with BCG and both HspX and ESAT-6

induced a significantly higher IFN-γ secretion by $CD4^+$ lymphocytes than that elicited by DCs challenged with BCG alone or when combined with the single antigens (Fig. 6A). In contrast, IL-17AF secretion induced by DCs incubated with BCG/HspX/ESAT-6 was comparable to that observed when DCs were treated with BCG alone or in combination with either antigen separately.

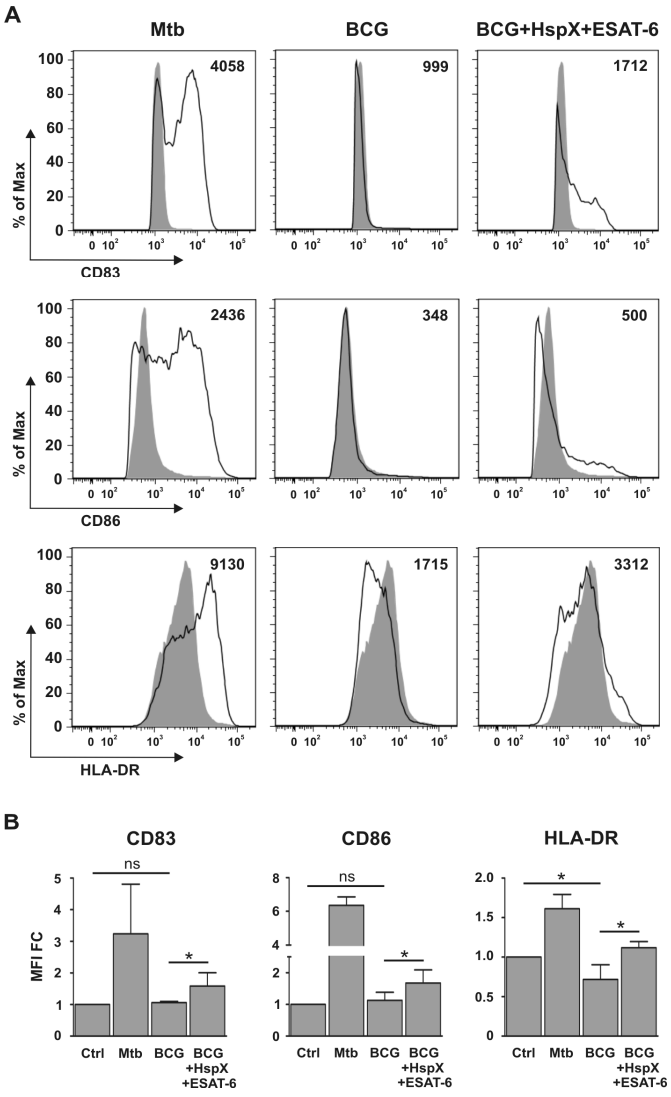

Figure 5. Effect of HspX and ESAT-6 on BCG-elicited DC maturation. DCs were treated with Mtb as a positive control and with BCG alone or combined with HspX and ESAT6. Cells were collected after 24 hrs culture and the expression of maturation markers was analyzed by flow cytometry. (A) Histograms illustrate CD83, CD86 and HLA-DR surface expression in CD1a+ cells and the MFI. Filled histograms represent the control, open histograms indicate treated cells. One of four different experiments is presented. (B) Bar graphs show the CD83, CD86 and HLA-DR MFI value of the four experiments expressed as fold change (MFI FC) over control (CTRL). Statistical analysis: CTRL vs BCG alone; BCG alone vs BCG plus HspX and ESAT6; ns $P>0.05$, *$P<0.05$.

Among the classical Th1 and Th17 cells responsible for IFN-γ and IL-17 production, respectively, a Th17/Th1 subset able to produce both IL-17 and IFN-γ has been discovered [78]. In order to identify the T cell subsets responsible for cytokine production (Fig. 6A), we performed IFN-γ and IL-17AF intracellular staining. Cell viability was evaluated by Annexin V staining.

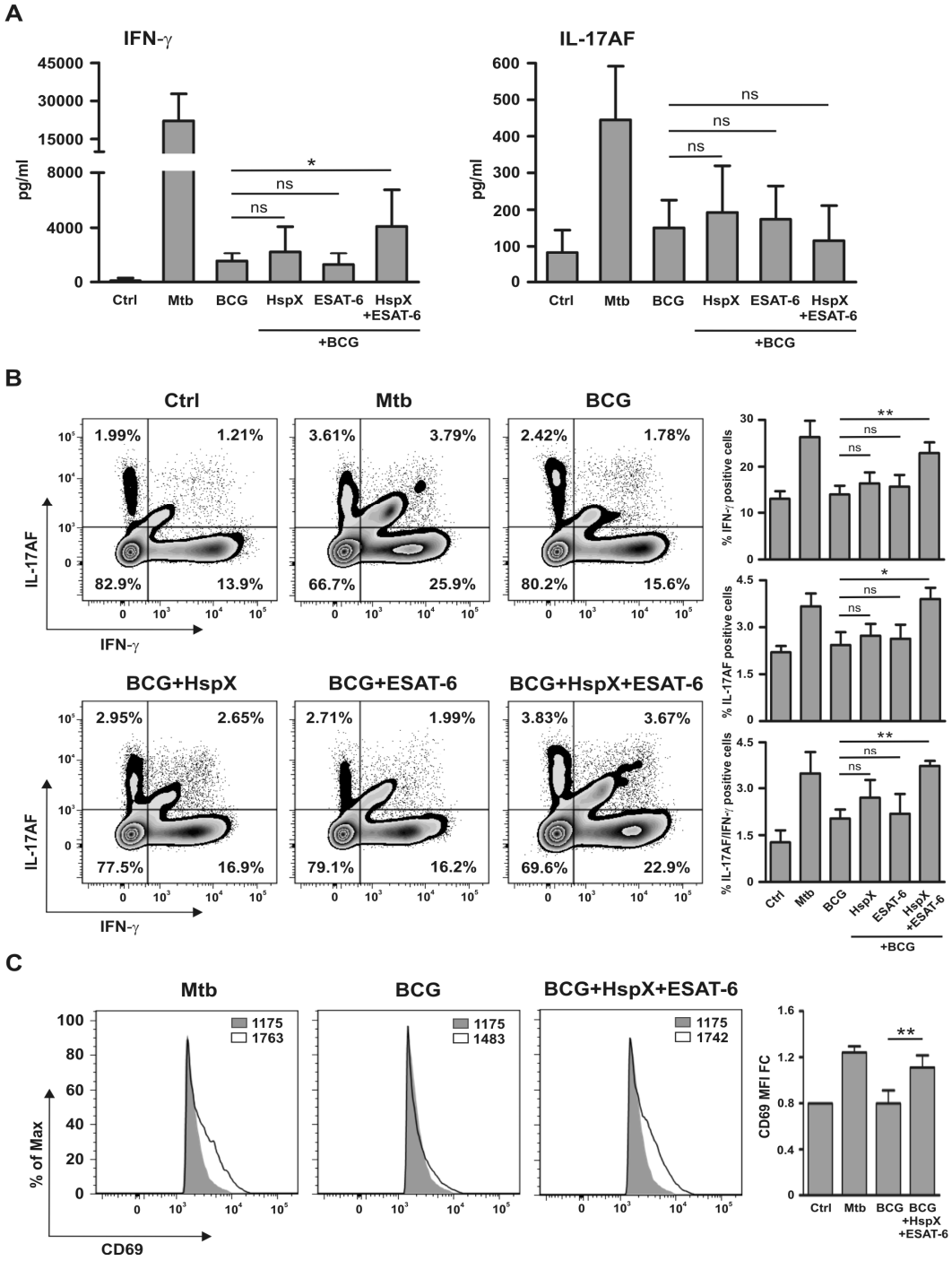

Figure 6. ESAT-6 and HspX enable BCG-stimulated DCs to elicit IFN-γ secretion and to enhance CD69 expression in CD4+ T lymphocytes

DCs stimulated for 24 hrs with Mtb or BCG alone or with ESAT-6 and/or HspX, were co-cultured with autologous CD4+ cells for 7 days. (A) Evaluation of IFN-γ and IL-17AF secretion in culture supernatants by ELISA. Results are the mean + SD of seven experiments. Statistical analysis: DCs treated with BCG alone vs BCG plus HspX and/or ESAT-6; ns P>0.05, *P<0.05. (B) Evaluation of IFN-γ and IL-17AF production detected by intracellular staining and analyzed by FACS in Annexin V-/CD4+/CD1a- cells. Zebra plots illustrate one representative experiment and the percentage of positive cells (left); bar graphs show mean + SD of three experiments (right). Statistical analysis: DCs treated with BCG alone vs BCG plus HspX and/or ESAT-6; ns P>0.05, *P<0.05, **P<0.01. (C) FACS analysis of CD69 expression in Annexin V-/CD4+/CD1a- cells. Panels illustrating one representative experiment with the MFI are shown on the left. Filled histograms represent the control, open histograms represent treated cells. Bar graphs representing the MFI mean + SD of three experiments expressed as fold change over control (MFI FC) are shown on the right. Statistical analysis: DCs treated with BCG vs BCG/HspX/ESAT-6; **P<0.01.

The FACS analysis demonstrated that DC stimulated with BCG, alone or in combination with the single HspX or ESAT-6, showed a weak ability to induce Th1, Th17, and Th17/Th1 differentiation, whereas DC incubated with BCG/HspX/ESAT-6 induced a remarkable development of all these Th cell subsets. (Fig. 6B).

These results demonstrate that IFN-γ and IL-17AF, as detected by ELISA (Fig. 6A), are produced by Th1 and Th17, respectively, but also by Th17/Th1 cells. Although DCs incubated with BCG/HspX/ESAT-6 induced a significant increase in IL-17AF-producing cells as compared to BCG-treated DCs (Fig. 6B), the amount of IL-17AF detected in the culture media of these cells was not significantly different (Fig. 6A). This discrepancy could be due to the fact that a very low percentage of IL-17AF-producing cells is insufficient to generate significant IL-17AF protein secretion in culture media.

We also performed control experiments with $CD4^+$ lymphocytes stimulated with various combinations of BCG, ESAT-6 and HspX in the absence of DCs and we didn't observe any IFN-γ or IL-17AF secretion (results not shown). This result suggests that BCG, ESAT-6 and HspX do not directly modulate T cell activation. Moreover, DCs alone treated with various combinations of BCG, HspX, and ESAT-6 did not secrete IFN-γ or IL-17AF (results not shown), thus ruling out their contribution to cytokine production after co-culture of DCs with T lymphocytes.

Subsequently, we analyzed the effect of BCG, HspX and ESAT-6 on the ability of DCs to induce the expression of CD69, a well-known T lymphocyte activation marker, in $CD4^+$ cells [79]. We found that BCG-treated DCs did not elicit CD69 expression, whereas the simultaneous addition of BCG, HspX and ESAT-6 up-regulated this activation marker in $CD4^+$ cells co-cultured with DCs. Notably, this effect was comparable to that obtained upon DCs challenge with Mtb (Fig. 6C).

TLR2-dependent IL-12 secretion is involved in $CD4^+$ lymphocyte activation by DCs stimulated with BCG, ESAT-6 and HspX

It is well accepted that IL-12 plays a key role in the induction of IFN-γ secretion by T lymphocytes [80]. Hence, we hypothesized that among the cytokines secreted upon DC treatment with BCG/ESAT-6/HspX (see Fig 4), IL-12 could be the one mainly involved in the stimulation of IFN-γ secretion by T lymphocytes (see Fig 6). In this regard, we wanted

to examine whether IL-12 blockage could affect IFN-γ secretion. To test this hypothesis, the cells were incubated with an antibody able to bind IL-12p70 and, specifically, to block IL-12 without affecting IL-23 [67, 68]. The figure 4 shows that IL-12 blockage decreased the ability of DCs challenged with BCG, alone or combined with HspX and ESAT-6, to induce IFN-γ secretion by $CD4^+$ lymphocytes. Moreover, the antibody treatment increased IL-17AF production by $CD4^+$ lymphocytes cultured with DCs stimulated with BCG/HspX/ESAT-6 (Fig. 7). Similar results were obtained from control experiments with DCs incubated with the antibody and stimulated with Mtb.

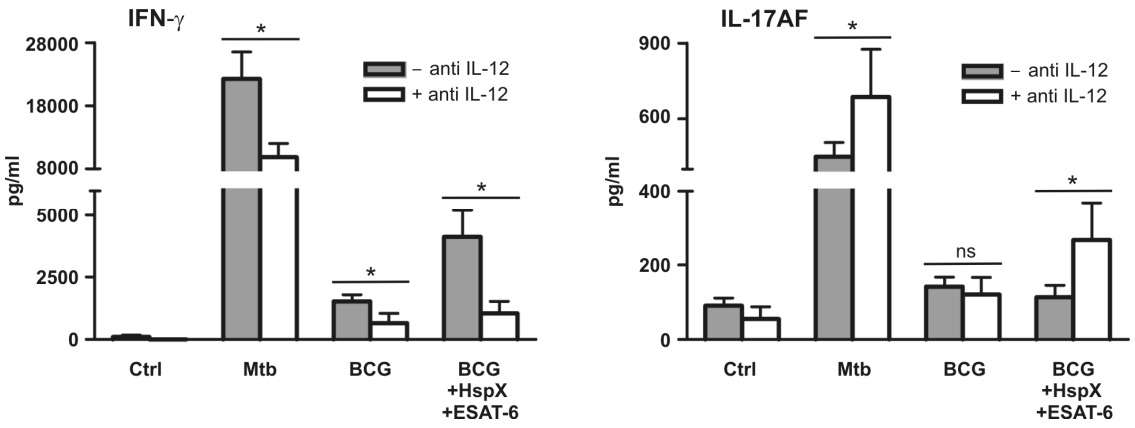

Figure 7. The ability of BCG, ESAT-6 and HspX-treated DCs to elicit IFN-γ secretion by CD4+ cell is mediated by IL-12

DCs pre-incubated (open bars) or not (filled bars) with 20 μg/ml IL-12p70 blocking antibody were stimulated for 24 hrs with Mtb or with BCG alone or with ESAT-6 and HspX and then co-cultured for 7 days with autologous CD4+ T lymphocytes. IFN-γ and IL-17AF secretion was analyzed by ELISA. Results are expressed as the mean + SD of five experiments. Statistical analysis: antibody-treated vs antibody-untreated cells; ns $P>0.05$, *$P<0.05$.

It is important to note that an isotype matched antibody did not affect the capacity of DCs to modulate the cytokine production by T cells (results not shown). These results indicate that ESAT-6 and HspX increase IFN-γ release by T lymphocytes mainly by enhancing IL-12 secretion by co-cultured DCs treated with BCG. Moreover, the ESAT-6- and HspX-dependent increase in IL-12 release inhibited IL-17AF secretion, shifting the lymphocytes toward a Th1 response characterized by a prevalent IFN-γ release.

It has been reported that ESAT-6 [81, 82] and some Mtb heat shock proteins [83] bind TLR2, which plays a critical role in the interaction between DCs and mycobacteria [84]. Therefore, we explored whether a TLR2-blocking antibody could affect BCG, ESAT-6

and HspX cooperation. We found that the antibody reduced IL-12 release by BCG-treated DCs stimulated with ESAT-6 and HspX (Fig. 8A).

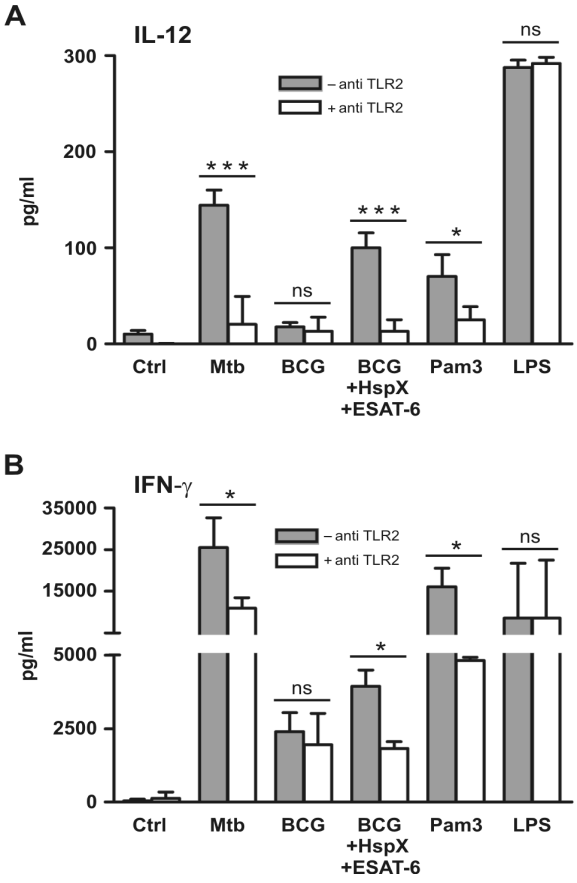

Figure 8. TLR2 is involved in IL-12-dependent IFN-γ secretion by CD4+ cells co-cultured with ESAT-6, HspX and BCG-treated DCs. DCs cultured in the absence (filled bars) or presence (open bars) of 5 μg/ml TLR2-blocking antibody were treated for 24 hrs with Mtb, BCG alone or combined with HspX and ESAT6, 10 μg/ml Pam3CSK4 (Pam3) or 100 ng/ml LPS. (A) Supernatants were collected and IL-12 release was analyzed by ELISA. Results are the mean value + SD of four experiments. Statistical analysis: antibody-treated vs antibody-untreated cells, ns $P>0.05$, *$P<0.05$, ***$P<0.001$. (B) DCs were co-cultured with autologous CD4+ T lymphocytes. After 7 days, culture supernatants were collected and analyzed by ELISA for IFN-γ release. Results are the mean + SD of three experiments. Statistical analysis: antibody-treated vs antibody-untreated cells, ns $P>0.05$, *$P<0.05$.

The antibody also suppressed IL-12 release by both Mtb-treated DCs and control DCs stimulated with Pam3CSK4, a specific TLR2 agonist. In contrast, the antibody did not affect IL-12 production by DCs stimulated with LPS, a TLR4 agonist (Fig. 8A), indicating that it specifically blocks TLR2-dependent IL-12 release. Moreover, an isotype matched antibody did not affect IL-12 release by DCs stimulated with BCG/ESAT-6/HspX, Mtb, Pam3CSK4 or LPS (results not shown). Interestingly, the TLR2-blocking antibody also decreased the ability of DCs incubated with Mtb, as well as with BCG/HspX/ESAT-6, to induce IFN-γ secretion by co-cultured $CD4^+$ lymphocytes (Fig. 8B). Additionally, the capacity to induce IFN-γ secretion by $CD4^+$ lymphocytes was inhibited by the antibody only in Pam3CSK4-stimulated DC and not in LPS-treated DCs (Fig. 8B).

DCs challenged with BCG, HspX and ESAT-6 induce a memory phenotype in naïve T lymphocytes

One of the most important characteristics of a good vaccine component is the ability to induce an immunological memory that permits a rapid and efficient immune cell response in case of infection by its specific pathogen. Therefore, we wondered whether the treatment of DCs with BCG/HspX/ESAT-6 was able to induce a memory phenotype in naïve T cells. It is important to remind that human $CD4^+$ lymphocytes extracted from the blood, contain both naïve and memory T cells. However, these two $CD4^+$ T cell subsets are characterized by a different expression of CD45RO and CD45RA proteins on the membrane surface, that are considered specific memory and naïve T cells markers respectively. Therefore, the naïve $CD4^+$ T cell subset is $CD45RA^+/CD45RO^-$ while the memory subset is $CD45RA^-/CD45RO^+$ [85]. Using specific isolation kits based on these peculiar protein expression patterns, we have been able to separate from the blood only the naïve $CD4^+$ T cell fraction. Subsequently, we co-cultured these naïve $CD4^+$ T cells with DCs stimulated with BCG, alone or with HspX and ESAT-6, as well as with Mtb as a positive control. After 9 days, we analyzed by flow cytometry the expression of CD45RO and CD45RA.

As shown in Figure 9A and B, BCG treatment did not lead to a significant expansion of $CD45RO^+$ cells (22.9%), as compared to untreated cells (21.5%). Very interestingly, we observed that the simultaneous addition of HspX and ESAT-6 enabled BCG-treated DCs to induce the expansion of memory $CD4^+$ T cell population (51.4%), as demonstrated by the up-regulation of CD45RO marker (Fig. 9A and B).

ESAT-6 and HspX improve the ability of BCG-treated DCs to activate NK cells through induction of IL-12 release

It is well known that the soluble mediators released by mature DCs after the interaction with pathogens activate various immune effector cells, including NK cells. Given the important role of NK cells in the host defence against micobacteria [86], we explored whether the cytokines released in culture supernatants by DCs in the experimental conditions depicted in Figure 4, induced NK cell activation. For this purpose, NK cells isolated from blood of healthy donors, were incubated with the conditioned media collected from the cultures of DCs already used for the ELISA assay described in figure 1. In these experiments DCs were treated with BCG, alone or combined with ESAT-

6 and/or HspX, as well as with Mtb as a positive control for 24 hrs. Subsequently, the conditioned media were collected and used to stimulate cultured NK cells.

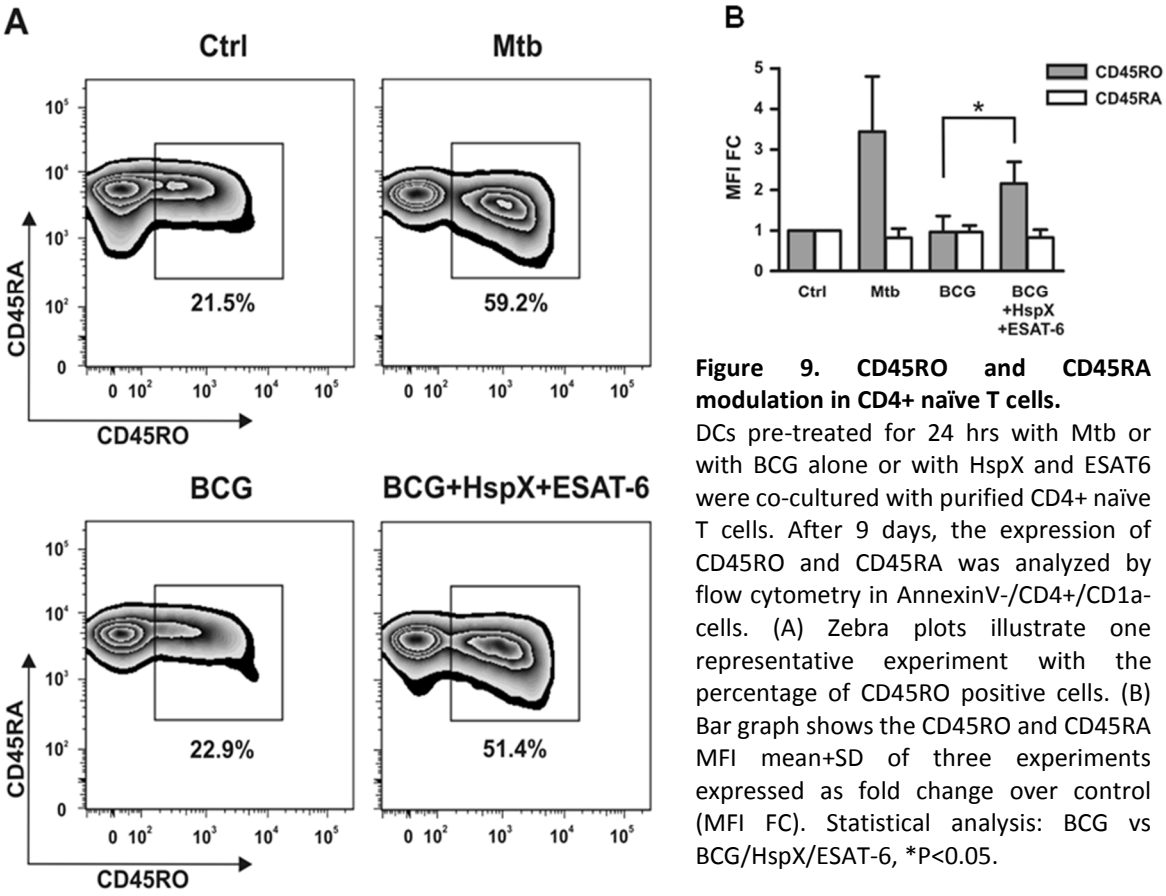

Figure 9. CD45RO and CD45RA modulation in CD4+ naïve T cells.
DCs pre-treated for 24 hrs with Mtb or with BCG alone or with HspX and ESAT6 were co-cultured with purified CD4+ naïve T cells. After 9 days, the expression of CD45RO and CD45RA was analyzed by flow cytometry in AnnexinV-/CD4+/CD1a- cells. (A) Zebra plots illustrate one representative experiment with the percentage of CD45RO positive cells. (B) Bar graph shows the CD45RO and CD45RA MFI mean+SD of three experiments expressed as fold change over control (MFI FC). Statistical analysis: BCG vs BCG/HspX/ESAT-6, *P<0.05.

After a 24 hr incubation, the IFN-γ secretion from NK was analyzed by ELISA, and the NK membrane expression of CD69 activation marker was evaluated by FACS analysis. We found that the incubation with media from Mtb-treated DCs induced IFN-γ release by NK cells (Fig. 10A), whereas culture supernatants from DCs stimulated with BCG, added alone or in combination with either HspX or ESAT-6, showed a slight ability to activate such a response (Fig. 10A). In contrast, media collected from cultures of DCs treated with BCG/HspX/ESAT-6 elicited a significantly higher IFN-γ release than that observed in the media from DCs incubated with BCG added alone or with the single antigens (Fig. 10A). The FACS analysis revealed that supernatants from DCs treated with Mtb or BCG/HspX/ESAT-6, but not with BCG alone, induced CD69 expression by NK cells (Fig. 10B). Importantly, the direct NK cell stimulation with Mtb, BCG and antigens did not induce IFN-γ release or CD69 expression (results not shown), suggesting that in our

experimental conditions NK cell activation is mediated by soluble agonists released by mycobacteria- and antigen-activated DCs.

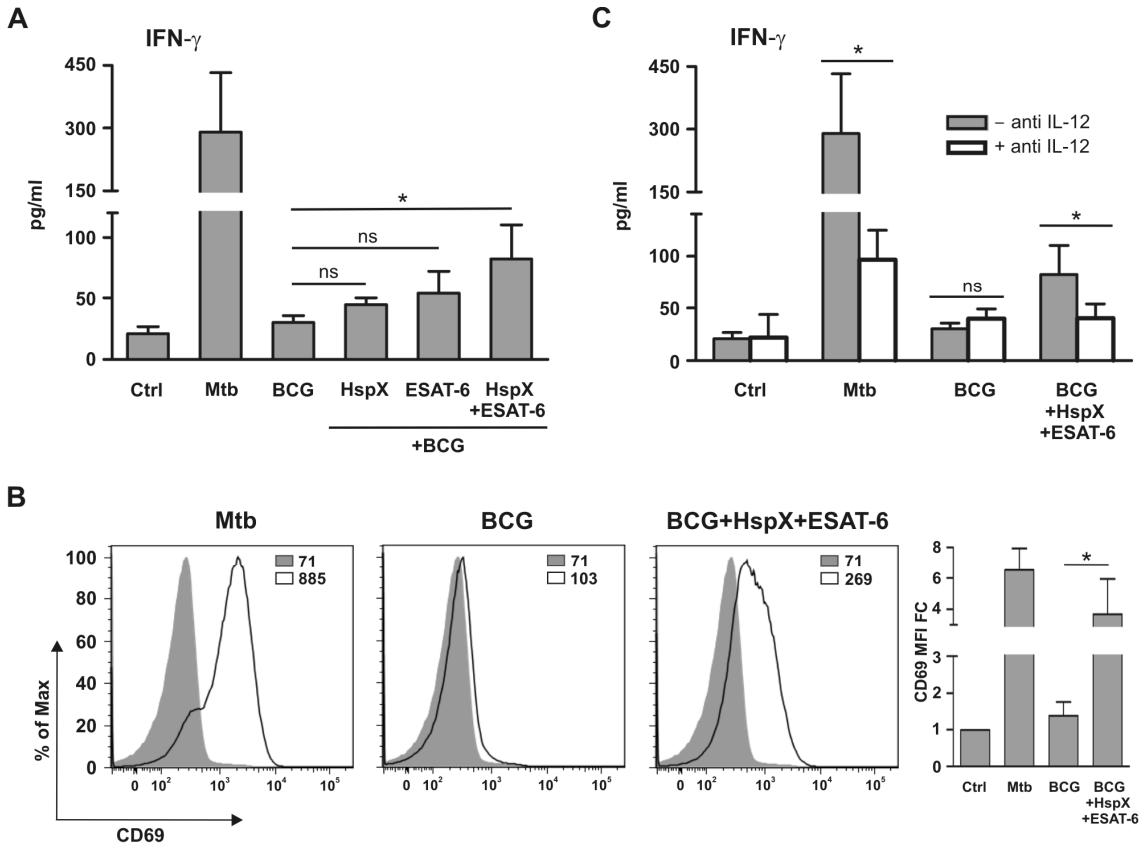

Figure 10. Effect of BCG plus ESAT-6 and HspX on DC-mediated IFN-γ release and CD69 expression by NK cells.

Culture supernatants of DCs treated as described in Figure 1 were incubated without (filled bars) or with (open bars) 20 μg/ml IL-12-blocking antibody and then added to purified NK cells. After 24 hrs, IFN-γ release was measured by ELISA (A and C) and CD69 expression was analyzed by flow cytometry in CD56+ cells (B). (A) Results are the mean + SD of three experiments. Statistical analysis: NK cells stimulated with supernatants from BCG-treated DCs vs supernatants of BCG plus HspX and/or ESAT-6-treated DCs; ns $P>0.05$, *$P<0.05$. (B) Panels illustrate one representative experiment and the bar graph shows the MFI mean value + SD of three experiments expressed as fold change over control (MFI FC). Filled histograms represent the control; open histograms represent the treated cells. Statistical analysis: NK cells stimulated with supernatants from BCG-treated DCs vs supernatants of BCG/HspX/ESAT-6-treated DCs, *$P<0.05$; (C) Results are the mean + SD of three experiments. Statistical analysis: IL-12-blocking antibody-treated supernatants vs untreated supernatants; ns $P>0,05$, *$P<0.05$.

As IL-12 plays an essential role in the NK cell activation [87], we analyzed the effect of the IL-12-blocking antibody, already used for the experiments shown in Fig. 7, on NK cell responses induced by culture media of DCs stimulated with BCG and the two antigens. Antibody addition to the media from DCs treated with Mtb or BCG/HspX/ESAT-6 decreased the ability of these supernatants to induce IFN-γ release by NK cells (Fig. 10C).

An isotype matched antibody did not affect the capacity of culture media from DCs treated with Mtb or with BCG/HspX/ESAT-6 to stimulate IFN-γ release by NK cells (results not shown). These results indicate that ESAT-6 and HspX enhance NK cell activation by increasing IL-12 release from BCG-treated DCs.

DISCUSSION

In the first part of this work it has been found that BCG shows a scarce ability to induce human DC maturation and cytokine release, which results in a subsequent weak capacity of DCs to induce $CD4^+$ lymphocytes and NK cell activation. These findings confirm previous data showing a weak immune cell response to BCG [88] which might, in part, explain why BCG vaccination does not produce strong and persistent protection against adult pulmonary tuberculosis. In this part of the research it has been also found that ESAT-6 and HspX, *per se* or when separately added to BCG-treated DC, do not significantly affect DC activity. However, ESAT-6 and HspX cooperate in increasing BCG-dependent DCs maturation and pro-inflammatory cytokine secretion, suggesting that the addition of HspX and ESAT-6 could confer to BCG important immune stimulatory characteristics. Conversely, anti-inflammatory cytokine IL-10 secretion did not significantly increase, indicating that the cooperation between ESAT-6 and HspX results in a preferential release of mediators that enhance pro-inflammatory immune response. This indication is supported by the evidence that upon stimulation with both these antigens and BCG, DCs become able to activate $CD4^+$ lymphocytes and NK cells. Interestingly, this treatment rendered DCs able to induce a memory phenotype in naïve T lymphocytes, further corroborating the suggestion that HspX and ESAT-6 enhance the ability of BCG to activate immune responses.

Taken together these findings are very important considering that the ability to elicit immunological memory is an essential requisite of vaccine components. Moreover these data are consistent with previous reports that demonstrate as reintroduction of the ESAT-6 gene into BCG improves its capacity to protect mice against Mtb challenge [72].

It has been reported that the addition of HspX [73] or ESAT-6 [26] alone activates IFN-γ production by human PBMC. However these effects were obtained with cells from patients with tuberculosis, whereas healthy or BCG-vaccinated subjects were less or not responsive to HspX [73] or ESAT-6 [26]. It is conceivable, therefore, that in the absence of Mtb infection, stimulation with either HspX or ESAT-6 alone does not efficiently activate immune cells and/or boost BCG-induced cell responses. The results reported so

far in the present work suggest that a more effective stimulation might be obtained by treating human immune cells from healthy subjects with BCG and both the antigens.

It has been demonstrated that a recombinant DNA vaccine encoding ESAT-6 elicits a strong Th1 response in mouse models [89] 17, and that HspX-based vaccines enhance the ability of BCG to stimulate immune response [73] [90-92]. In the present study, however, neither ESAT-6 nor HspX alone activated immune cells on their own or when either was combined with BCG. This discrepancy indicates that, differently from murine cells, stimulation with both ESAT-6 and HspX is needed to induce human immune cell response.

Conversely, the findings obtained during the performance of this task are in line with previous results showing that vaccination with fusion protein composed of two Mtb antigens efficiently increases DCs and T cell response [36, 37, 93]. Importantly, we observed that DCs are necessary for the activation of T lymphocytes and NK cells by Mtb, BCG and antigens. These data indicate that the effect of these agonists is mediated by DCs. A number of studies have suggested that DCs reinforce cellular immune response against Mtb. In fact, DCs are very well represented at the sites of Mtb infection, where they capture antigens, mature and migrate towards lymphoid organs in which they prime T cells through antigen presentation, cytokine secretion, and co-stimulatory molecule expression [94]. In this regard the results presented herein, corroborate these findings, highlighting the essential role of DCs in the mechanisms driving protective immunity against Mtb. In the present work we also report that conditioned media from DCs cultured with BCG and antigens activate NK cells, suggesting that soluble factors released by DCs are sufficient to activate NK cells. These results are crucial, given that NK cells are involved in the control of Mtb infection and activated by Mtb-treated DCs [86, 95]. Importantly, among the many soluble factors secreted in culture media, IL-12 produced upon stimulation of BCG-treated DCs with HspX and ESAT-6 plays a central role in both CD4+ and NK cell stimulation. These findings agree with previous studies showing that IL-12 is the most important cytokine for T cell and NK cell activation [87].

Here we also report that inhibition of IL-12 release leads to increased IL-17AF secretion by T cells cultured with DCs stimulated with BCG, HspX and ESAT-6. This results confirm previous findings that T cells shift from Th1 to Th17 production, depending on the type of cytokines present in the cell environment [78, 96]. In particular, IL-12 elicits

IFN-γ secretion [97], whereas other cytokines, such as IL-1β, IL-23, and IL-6, promote and/or maintain both IL-17A and IL-17F release [98-100]. Despite DC treatment with BCG, HspX and ESAT-6 induces a remarkable increase in IL-1β, IL-23 and IL-6 secretion, the enhanced IL-12 release, obtained in the same experimental conditions, pushes T cells toward a Th1 response and it inhibits Th17 response. This finding is remarkable because Th1 cells are known to play an important role in host defense against Mtb [101]. Although the role of Th17 in host protection against tuberculosis has not been completely clarified, it has been shown that the IL-23/Th17 pathway is not crucial for the control of Mtb infection [102]; therefore, the shift from Th17 toward Th1 response, induced upon HspX and ESAT-6 treatment, might increase the effectiveness of immune response against Mtb.

Moreover, we identified a receptor involved in the effects of ESAT-6 and HspX on human DCs. Little is known about the receptors engaged by Mtb antigens. It has been demonstrated that ESAT-6 and some Mtb heat shock proteins bind TLR2 [81-83], which is involved in the interaction between DCs and mycobacteria [84]. Our results show that TLR2 plays an important role in the mechanisms by which Mtb, ESAT-6 and HspX induce IL-12 release and subsequent Th1 response. The formality of TLR2 recruitment during the coordinated action of BCG and mycobacterial antigens remains to be characterized. However, our results highlight that TLR2 participates in the biological events leading to the activation of immune defense against tuberculosis.

In conclusion, in the first part of the study we found that HspX and ESAT-6 cooperate to enhance the capacity of human BCG-primed DCs to produce IL-12 which, in turn, induces an effective Th1 and NK cell response. Moreover, the cooperation of HspX, ESAT-6 and BCG in IL-12 production occurs through TLR2 receptor engagement. Importantly, this is the first evidence that HspX and ESAT-6 improve the ability of BCG to stimulate human DC-dependent activation of T lymphocytes and NK cells, suggesting that these antigens could be used to increase the immune system’s responsiveness to vaccination with BCG.

5.2 TASK B: investigation of PLGA and pSi NPs as nano-delivery system for human DCs targeting.

RESULTS

Evaluation of PLGA and pSi nanoparticle toxicity on human DCs

It is well accepted that to be suitable candidates for various biomedical purposes, nanoparticles (NPs) should not induce toxic effect and/or adverse immune reactions. Therefore, preventive to the use of PLGA (poly (lactic-co-glycolic acid) and pSi (porous silicon) NPs as BCG/ESAT-6/HspX carriers into DCs, we determined whether the NPs exerted *per se* toxic effects on these cells. For this purpose, iDCs prepared as previously described (see TASK A and methods) were challenged with different doses of PLGA or pSi NPs (kindly provided by Dr H. Monaco and Dr. N. Daldosso respectively). After a 24 hr treatment the cell viability was assessed by Annexin V staining and flow cytometric analysis.

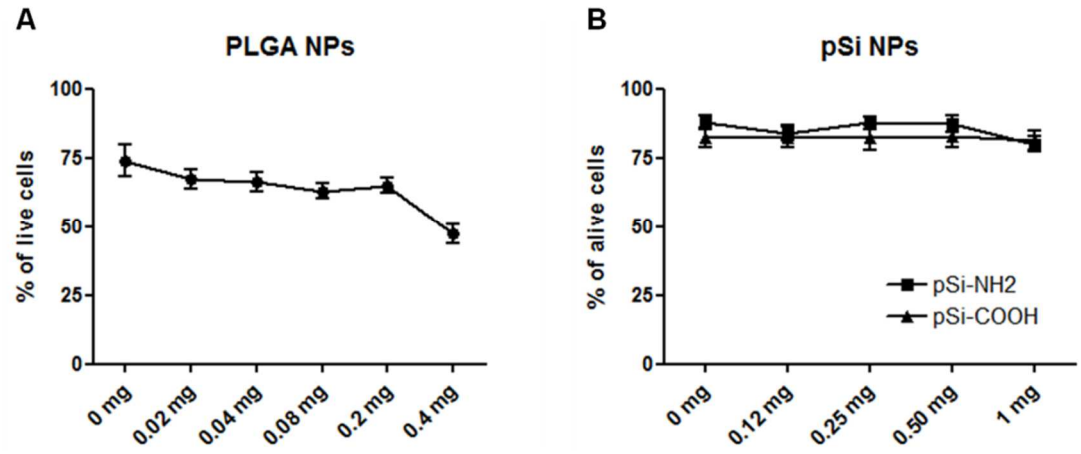

Figure 11. Effects of PLGA and pSi NPs on DCs viability
DCs were treated with the indicated doses of PLGA (A) or pSi (B) NPs. After a 24 hour incubation, cells were stained with annexin V for the identification of apoptotic cells, and analyzed by flow cytometry. The results are expressed as the mean value + SD of three independent experiments.

As shown in figure 11A, the incubation with PLGA NPs at doses of 0.02, 0.08 and 0.2 mg did not affect DC viability. However, at the highest dose used (0.4 mg) the percentage of alive DCs was about 50%, whereas that of control untreated cells was 80%, indicating that this amount of PLGA NPs was quite toxic (Fig 11A). On the bases of this evidence, the subsequent experiments involving PLGA NPs were performed using particle doses lower than 0.4 mg. Conversely, as depicted in figure 11B the incubation of DCs with doses of pSi NPs ranging between 0.25 and 1 mg did not lead to any DC apoptosis event. This is a very important point considering that 1 mg represents an extremely high dose for the *in vitro* stimulation of DCs. Moreover, a different pSi functionalization, consisting in the presence of NH_2 or COOH groups at the NP surface, did not cause relevant changes of NP toxicity on DCs. This result underlines the possibility to expose various chemical groups at the NP surface in order to increase and/or maintain NP stability without enhancing their toxicity.

Evaluation of PLGA and pSi nanoparticle effects on human DC activation

The activation of DCs results in the production of various cytokines that stimulate the inflammatory process and the immune response [71]. Therefore it is important to assess whether nanostructures that have to be used for medical applications stimulate these cells causing inflammation and/or adverse immune reactions once injected in patients [103]. We then examined whether our NPs induce pro-inflammatory cytokine secretion by DCs. For these reasons iDCs were challenged with various doses of PLGA or pSi NPs for 24 hrs in absence or presence of LPS. Subsequently, the supernatants were collected and checked for the presence of the pro-inflammatory cytokines IL-12, TNF α, IL-6 and IL-23. As clearly shown in figure 12, the incubation of resting DCs with various doses of PLGA NPs (ranging between 0.02 and 0.2 mg) did not elicit any pro-inflammatory cytokine release by DCs and did not significantly affect the LPS-induced cytokine production. This finding indicates that the PLGA NPs synthesized following the protocol of Dr H. Monaco are unable to interfere *per se* with human DC activation mechanisms and therefore, they represent suitable tools to investigate the effects of NP-associated molecules on these cells. Similar results were obtained when DCs were treated with doses of pSi NPs ranging between 0.25 and 1 mg (results not shown). Hence, these NPs represent a promising tool for antigens delivery into human DCs.

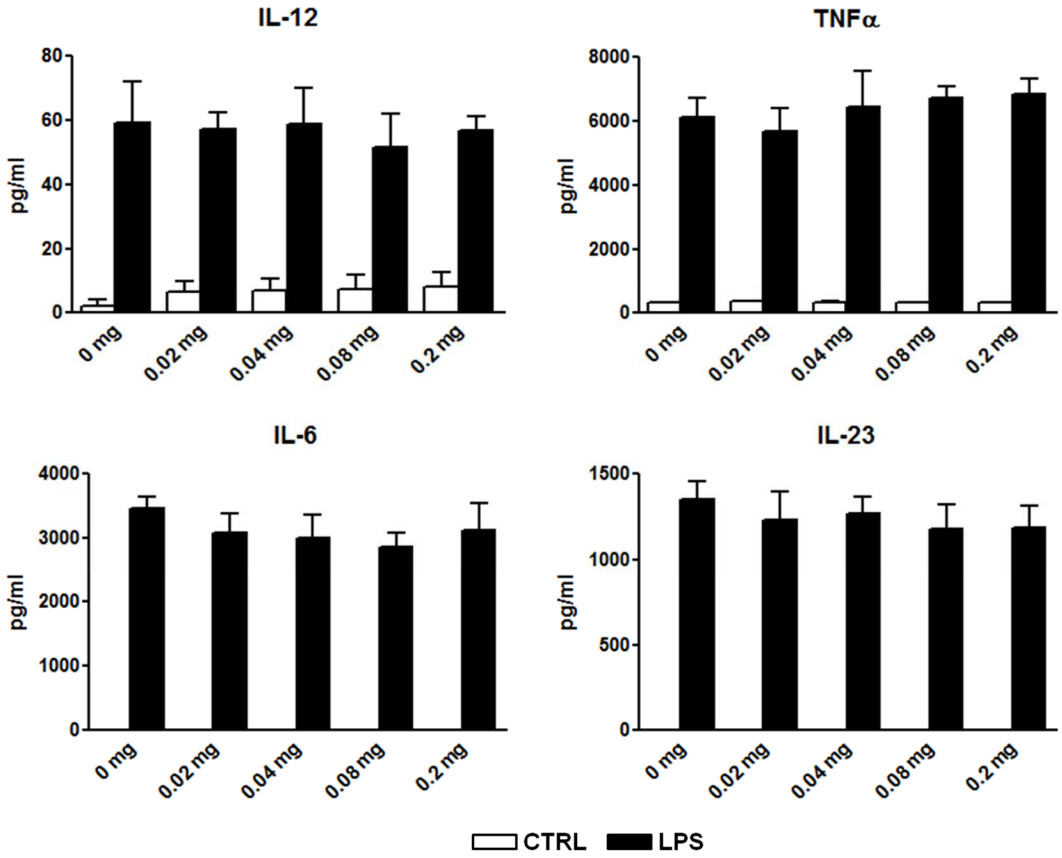

Figure 12. Effects of PLGA NPs on DCs cytokine secretion
iDCs were treated for 24 hours with the indicated concentrations of PLGA NPs in the absence (open bars) or presence (filled bars) of 100 ng of LPS. The release of the indicated cytokines in culture supernatants was evaluated by ELISA assay. The results are expressed as the mean value + SD of three independent experiments.

Evaluation of PLGA and pSi nanoparticle internalization by human DCs

In order to transport molecules into cells, it is important to choose a carrier that could be efficiently ingested by the cells. Hence, we investigated whether human DCs were able to internalize PLGA and pSi NPs. For this purpose human DCs were incubated with rhodamine B-coniugated PLGA NPs and after 24 hrs the NPs uptake by DCs was checked by confocal microscopy. This analysis revealed that NPs were efficiently internalized by human DCs (Figure 13). For example, Figure 13 panel B illustrates a single DC that ingested more than ten PLGA NPs. Moreover, the different z-stacks of the same

field shown in panels C-E demonstrate that the particles are indeed localized inside the cells.

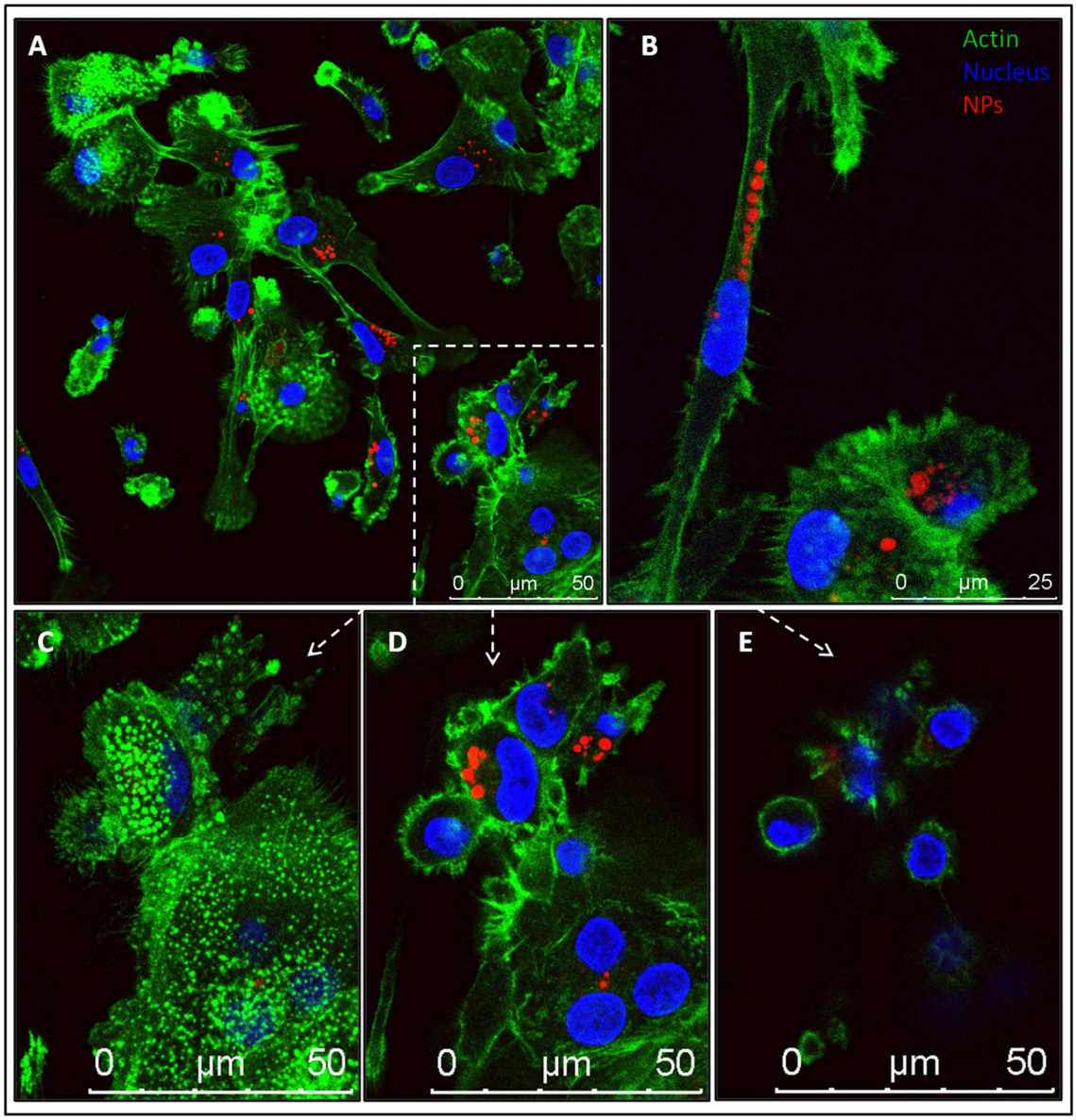

Figure 13. PLGA NPs internalization by human DCs
Confocal microscopy images of DCs treated with 500 µg of rhodamine B-coniugated PLGA NPs (red). After a 24 hour treatment the cells were fixed and stained with Phalloidin to label filamentous F-actin (green), and with DAPI to visualize nuclei (blue). One representative experiment of three is shown. (A) zoom-out and (B) zoom-out represent two different fields of individual experiments; (C-E) zoom-in of (A) represent 3 different z-stacks of the same field: upper (C), middle (D) and lower (E).

Notably, we did not observe any extracellular particle in the examined fields, indicating that the NPs were effectively swallowed by DCs. These results demonstrate that

human DCs are able to internalize large amounts of PLGA particles and therefore these nanostructures represent good vehicles to carry molecules into the cells. Similar experiments have been done incubating DCs with pSi NPs for 24 hrs and analyzing the NP uptake by confocal microscopy. However in this case we exploited the intrinsic fluorescence of these NPs without the need of any fluorochrome conjugation. This analysis revealed that the pSi NPs were internalized by human DCs less efficiently than PLGA NPs (about 1 pSi/cell *vs* 10 PLGA/cell). For example, the figure 14A shows a confocal microscopy field in which yellow arrows indicate the pSi NPs-NH_2 that have not been internalized by DCs. Conversely, in panel B is shown a zoom-in of the above field highlighting the only cell showing an ingested particle. These data have been confirmed by other experiments performed in the same experimental conditions. Moreover, similar results have been obtained using pSi NPs-COOH (results not shown), indicating that the presence of NH_2 or COOH on pSi surface did not change the particle uptake by DCs.

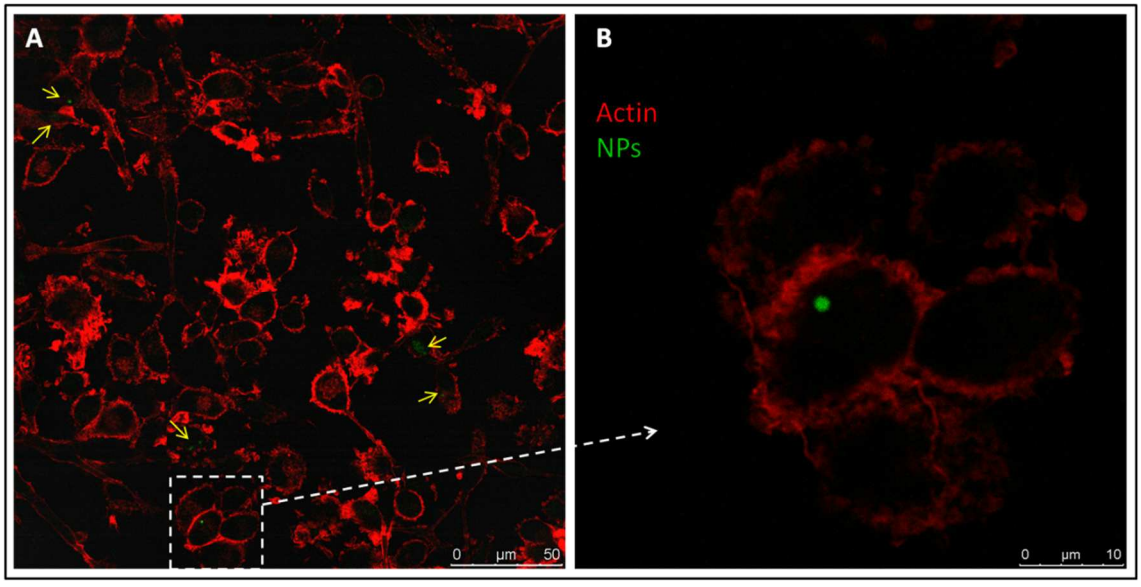

Figure 14. pSi-NH2 NPs internalization by human DCs
Confocal microscopy images of DCs treated with 500 µg of pSi-NH2 NPs (green). After a 24 hour treatment the cells were fixed and stained with Phalloidin to label filamentous F-actin (red). One representative experiment of three is shown. (A) Internal z-stack of a general field; (B) a zoom-in of (A).

DISCUSSION

In the second part of this work we tested the effects of PLGA and pSi NPs on human DCs. Our interest on these NPs is due to their characteristic biodegradability and their wide use for biomedical applications in humans [104, 105]. Moreover, the fact that PLGA is a FDA approved polymer and that pSi have intrinsic stable fluorescence [105] renders this NPs suitable tools as carriers of BCG/HspX/ESAT-6 into human DCs. First of all we decided to explore the consequences of the interaction of PLGA and pSi NPs with DCs. In order to perform these investigations, we started a collaboration with two research groups of Verona University, that produced and characterized NPs. The PLGA NPs used in this work, characterized by an uniform size (diameter about 100-150 nm) and a negative charge, have been kindly provided by Dr H. Monaco. The pSi NPs used in this work have been provided by Dr. N. Daldosso. The group of Dr. Daldosso produced pSi NPs with NH_2 or COOH groups on their surface, leading to a different electric charge. Moreover, this group characterized the optical properties of these NPs. The final size of these particles is highly heterogeneous as it is due to the random aggregation of various small pSi NPs. Importantly, all the results obtained by treating DCs with pSi-NH_2 or pSi-COOH are completely comparable demonstrating that the presence of NH_2 or COOH is irrelevant for pSi NPs-DCs interaction.

A major problem holding back the use of particles in targeted drug delivery is the ability of some of them to activate immune cells, thus evoking pro-inflammatory effects that hinder their use for *in vitro* experiments, and *in vivo* can lead to adverse reactions such as fever, allergy or autoimmunity. These effects can be ascribed to interactions between particle components and immune cell structures, as well as to microorganisms or their derivatives contaminating the particles and engaging pattern recognition receptors (PRRs) on the cell surface. The results of this second part of the work clearly show that both PLGA and pSi NPs do not affect the DC viability. However while the treatment of DCs with extremely high doses of pSi NPs does not affect cell viability, high doses of PLGA NPs result to be quite toxic. Nevertheless, it is important to note that the toxic effect of PLGA NPs on DCs has been observed upon cell treatment with amounts of particles which are unusually high for *in vitro* DC stimulation.

The data obtained herein show that both PLGA and pSi NPs are unable to activate a substantial cytokine production by resting DCs, thus ruling out the possibility of particle-mediated stimulatory effects as well as an eventual their contamination by microorganisms. Moreover, the PLGA and pSi NPs do not affect the cytokine production by LPS-stimulated DCs, indicating that also in the presence of a pro-inflammatory agent, these NPs are immunologically inert and harmless.

Several studies on PLGA NPs have shown that these structures can entrap a wide range of biologically active compounds and are able to enter inside the cells where they release the loaded molecules [106]. Conversely, little is known about the ability of pSi NPs to be internalized by human primary cells. Indeed, the few data published on this matter have been obtained using pSi particles having sizes and functionalizations different from those presented in this work [105, 106]. The confocal microscopy analyses performed herein demonstrate that both PLGA and pSi NPs are taken up by human DCs. However, our results demonstrate that PLGA NPs are more efficiently internalized by DCs than pSi NPs. Our findings are in line with previous reports showing that PLGA particles could be easily ingested by human moDCs [107], human cord -blood $CD34^+$ stem cells-derived DCs [108] and mouse bone marrow-derived DCs [109].

The discrepancy between pSi and PLGA NPs internalization by human DCs could be ascribed to their different chemical nature and/or to their size. It's important to underline that PLGA NP size is well defined, whereas small pSi NPs can easily aggregate producing particles with heterogeneous sizes, becoming too big to be taken up by DCs or being too small to remain inside the cells.

Some authors suggest the PLGA NPs are preferentially taken up by both human and mouse DCs rather than by other cell types [107-109]. However, we performed control experiments in which HUVEC cells have been treated with PLGA NPs, and we found that these endothelial cells efficiently internalized PLGA NPs (data not shown). These results suggest that in our experimental conditions the uptake of PLGA NPs by human moDCs is not selective, pointing out the necessity to attach a specific DC-recognizing antibody on NP surface. This point will be the subject of future investigations.

In conclusion, considering their scarce toxicity, their low ability to modulate the immune response and their good internalization by human moDCs, we believe that PLGA NPs could be a good tool to be used for targeting molecules into human DCs.

4. CONCLUSION

The results reported in this study demonstrate that HspX and ESAT-6 cooperate to enhance the capacity of human BCG-primed DCs to produce IL-12 which, in turn, induces an effective Th1 and NK cell response. These findings suggest that the combination of antigens and bacteria investigated by us could be used to increase the immune system's responsiveness to vaccination with BCG. Moreover in this work we identified and tested a nano delivery system based on PLGA NPs that could improve the capture of BCG/HspX/ESAT-6 by human moDCs. Collectively these results suggest a new vaccination strategy against TB based on i) the combination of BCG/HspX/ESAT-6 in order to stimulate the immune system, and ii) the use of PLGA NPs to target antigens into human DCs.

Of course, our *in vitro* model needs to be tested also *in vivo*. Moreover, further experiments are ongoing in order to develop a suitable protocol to encapsulate BCG/HspX/ESAT-6 into PLGA NPs.

In conclusion, the results of the investigations reported herein on DC-mediated immune response could improve our knowledge in the field of TB vaccine and could lead to the development of vaccine formulations suitable to efficiently prevent this re-emerging disease. Furthermore, the strategy presented herein could be easily extended to other experimental approaches based on DC targeting, i.g. cancer immunotherapy *via* DCs.

5. Appendix 1: ABBREVIATIONS

Ab: antibody

APCs: antigen presenting cells

BCG: Bacillus Calmette-Guerin

BFA: brefeldin A

CD: cluster of D*ifferentiation* m*arker*

CFP-10: culture filtrate protein 10 KDa

CLRs: C-type lectin receptors

CRDs: carbohydrate recognition domains

DCs: dendritic cells

DC-SIGN: dendritic cell-specific intercellular cell adhesion molecule grabbing non-integrin

Dectin-1: dendritic-cell-associated C-type lectin-1

ELISA: enzyme linked immuno sorbent assay

ESAT-6: early secreted antigen target 6 KDa

FACS: fluorescence-activated cell sorter

FC: fold Change

FDA: food and drug administration

GATA3: GATA binding protein 3

GM-CSF: granulocyte-macrophages colony-stimulating factor

HLA: human leukocyte antigen

HspX: heat shock protein X

HUVEC: human umbilical vein endothelial cell

iDCs: immature dendritic cells

IFNs: interferons

Ig: immunoglobulin

IL: interleukin

LPS: lipopolysaccharide

mDCs: mature dendritic cells

MHC: major histocompatibility complex

moDCs: monocyte-derived Dendritic Cells

Mtb: *Mycobacterium tuberculosis* H37 Rv

NF-kB: nuclear factor κappa B

NPs: nanoparticles

Pam_3CSK_4 : palmitoyl-3-Cysteine-Serine-Lysine-4

PAMPs : pathogen-associated molecular pattern

PBMC: peripheral blood mononuclear cells

PLGA: poly lactic-co-glycolic acid

PMN: polymorphonuclear neutrophils

pSi: porous Silicon

PRRs: pattern recognition receptors

RD1: region of difference-1

RNA: ribonucleic acid

pSi: porous silicon

SD: standard deviation

Th: T helper lymphocytes

TB: tuberculosis

T-bet: T-box expressed in T cells

TGF: transforming growth factor

TLRs: Toll-Like Receptor

TNF-α: tumor necrosis factor α

6. APPENDIX 2: Pubblications

The results of the studies conducted during my PhD, including those presented in this thesis, have been published in international scientific journals. In this appendix the list of my publications and of the manuscripts submitted or in preparations is reported.

1) **Marongiu L**, Donini M, Toffali L, Zenaro E and Dusi S. "ESAT-6 and HspX improve the effectiveness of BCG to induce human Dendritic Cells dependent Th1 and NK cells activation". PlosOne 2013 Oct; e75684doi :10.1371 / journal.pone.0075684. IF 3.730

2) Donini M, **Marongiu L**, Fontana E and Dusi S. "Prostate Carcinoma Cells LNCaP and Glucan Cooperate in Induction of Cytokine Synthesis by Dendritic Cells : Effect on Natural Killer Cells and CD4+ Lymphocytes Activation". Prostate. 2012 Apr; 72(5):566-76. Epub 2011 Jul 27. IF 3.485

3) Cantarelli IX, Pedroni M, Piccinelli F, Marzola P, Boschi F, Conti G, Mosconi E, Sbarbati A, Bernardi P, Perbellini L, Sorace L, **Marongiu L,** Donini M, Dusi S, Innocenti C, Fantechi E, Sangregorio C and Speghini A. "Lanthanide doped CaF2 nanopraticles as multimodal bioprobes". Submitted to ACSnano.

4) **Marongiu L**, Donini M, Bovi M, Perduca M, Vivian F, Romeo A, Mariotto S, Monaco HL, and Dusi S. "The inclusion into PLGA nanoparticles enables α-bisabolol to efficiently inhibit the human Dendritic Cell pro-inflammatory activity". Submitted to Int. J. Nanomedicine.

5) Ghafarinazari A , Cortelletti P, **Marongiu L,** Donini M, Paterlini V, Bettotti P, Froner E, Daldosso N, Dusi S and Scarpa M. "Luminescent porous silicon micro-particles as biocompatible and traceable drug delivery system". Manuscript in preparation

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